

## Interaction Between PEVK-Titin and Actin Filaments Origin of a Viscous Force Component in Cardiac Myofibrils

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**Abstract**—The giant muscle protein titin contains a unique sequence, the PEVK domain, the elastic properties of which contribute to the mechanical behavior of relaxed cardiomyocytes. Here, human N2-B–cardiac PEVK was expressed in *Escherichia coli* and tested—along with recombinant cardiac titin constructs containing immunoglobulin-like or fibronectin-like domains—for a possible interaction with actin filaments. In the actomyosin in vitro motility assay, only the PEVK construct inhibited actin filament sliding over myosin. The slowdown occurred in a concentration-dependent manner and was accompanied by an increase in the number of stationary actin filaments. High  $[Ca^{2+}]$  reversed the PEVK effect. PEVK concentrations  $\geq 10 \mu\text{g/mL}$  caused actin bundling. Actin-PEVK association was found also in actin fluorescence binding assays without myosin at physiological ionic strength. In cosedimentation assays, PEVK-titin interacted weakly with actin at  $0^\circ\text{C}$ , but more strongly at  $30^\circ\text{C}$ , suggesting involvement of hydrophobic interactions. To probe the interaction in a more physiological environment, nonactivated cardiac myofibrils were stretched quickly, and force was measured during the subsequent hold period. The observed force decline could be fit with a three-order exponential-decay function, which revealed an initial rapid-decay component (time constant, 4 to 5 ms) making up 30% to 50% of the whole decay amplitude. The rapid, viscous decay component, but not the slower decay components, decreased greatly and immediately on actin extraction with  $Ca^{2+}$ -independent gelsolin fragment, both at physiological sarcomere lengths and beyond actin-myosin overlap. Steady-state passive force dropped only after longer exposure to gelsolin. We conclude that interaction between PEVK-titin and actin occurs in the sarcomere and may cause viscous drag during diastolic stretch of cardiac myofibrils. The interaction could also oppose shortening during contraction. (*Circ Res.* 2001;89:874-881.)

**Key Words:** connectin ■ passive tension ■ myofibril mechanics ■ myocardial viscosity ■ actin binding protein

Viscosity has long been known to be part of the mechanical characteristics of cardiac muscle.<sup>1</sup> Some 25 years ago, Noble<sup>2</sup> measured the force of cat papillary muscle during diastole at different stretch velocities. A viscous force component appearing above intermediate velocities was hypothesized to cause resistance to stretch during rapid filling of the left ventricle. Chiu et al<sup>3</sup> reported viscous resistance to both stretch and active shortening of cat papillary muscle. They concluded that, at low levels of contractile activation, an internal viscous load may limit the “unloaded” velocity of sarcomere shortening. In a later study, rapid stretch protocols were applied to rat heart trabeculae during the pause between active contractions to investigate a possible origin of the internal viscous force.<sup>4</sup> It was suggested that a main contributor to the viscous effects may be the sarcomere protein titin (also known as connectin). Related studies then raised the possibility that the viscosity may be modulated by  $[Ca^{2+}]$  in a concentration range in which structures responsible for the generation of contractile force are not yet active; again, titin

was considered the main factor involved.<sup>5,6</sup> However, titin may not be the only element important for the viscosity of cardiac muscle; chronic changes in diastolic viscous properties have been ascribed to increased microtubule density, at least in pressure-overload cardiac hypertrophy.<sup>7,8</sup> On the other hand, titin expression also appears to be altered in cardiac hypertrophy and failure.<sup>9</sup> Establishing the causes of myocardial viscosity will be important to improve understanding of overall cardiac function in both normal and pathological states.

In contrast to the origin of viscosity, the source of the elasticity of cardiac muscle is well known. Up to modest physiological sarcomere lengths (SLs), the main determinant of elasticity is titin, whereas collagen stiffness dominates at longer lengths.<sup>10,11</sup> The titin molecules span the I-band of the sarcomere from the Z-line to the A-band edge and continue to run along the length of the half-thick filament, where they are bound to myosin.<sup>12</sup> After the determination of the human titin sequence,<sup>13</sup> the molecular basis of cardiac-titin elasticity was

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investigated in a series of studies.<sup>14–19</sup> It was shown that structurally distinct regions of cardiac titin are recruited in a sequential manner during sarcomere stretch.<sup>19</sup> At low stretch forces, chains of serially linked immunoglobulin (Ig)-like domains, which adopt a fairly stable  $\beta$ -sheet fold,<sup>20</sup> extend mainly by straightening. At higher forces, two longer sequence insertions of less obvious secondary/tertiary structure unravel, the so-called PEVK domain and a cardiac-specific “N2-B–unique sequence.” Cardiac titin exists in two main isoforms termed N2-B and N2-BA; these isoforms express different-length variants of Ig-like regions and PEVK segment.<sup>21</sup> Interestingly, the N2-B:N2-BA isoform expression pattern is altered in dog hearts exposed to prolonged pacing tachycardia, which impairs determinants of left ventricular diastolic suction.<sup>22</sup>

Preliminary evidence suggested that the extensible region of cardiac titin might interact with other I-band proteins.<sup>23</sup> Interaction of whole titin with actin earlier was demonstrated in vitro.<sup>24–26</sup> However, it remained unclear which sites along the titin molecule are involved in the interaction. A couple of expressed globular titin domains derived from the A-band edge were found to associate with actin in vitro,<sup>27,28</sup> and the interaction was proposed to be important for the viscosity of the sarcomere.<sup>6</sup> However, a study from our laboratory showed that actin binding is no general feature of the globular domains in cardiac titin.<sup>17</sup> In the present work, we confirmed the lack of interaction between actin and  $\beta$ -sheet titin domains and probed a possible actin association of the cardiac PEVK domain. Results show that recombinant cardiac PEVK constructs do interact with actin and—in contrast to other expressed titin fragments—slow down actin filament sliding over myosin in the in vitro motility assay. Hydrophobic and electrostatic interactions are likely to participate in these processes. The relatively weak actin-PEVK association, confirmed in actin fluorescence binding assays without myosin and in cosedimentation assays, results in viscous drag appearing during SL changes of passive cardiac myofibrils. The involvement of actin-titin interactions in determining the viscous force of the sarcomere is further demonstrated in selective actin-extraction and titin-proteolysis protocols. The viscous force component will impose a significant internal load onto cardiac myocytes during diastolic stretch but may also oppose shortening during active contraction.

## Materials and Methods

### Expression of Titin Constructs

cDNA fragments corresponding to different regions of the human cardiac N2-B–titin nucleotide sequence (EMBL accession No. X90568)<sup>13</sup> were expressed solubly in *Escherichia coli* BL21[DE3]pLysS cells (Stratagene) as described.<sup>17,29</sup> The following titin constructs were obtained (see Figure 1A): I24/I25 (two N2-B–specific Ig-like domains flanking a short unique sequence), I26/I27–I84 (cardiac-PEVK flanked by two N-terminal Ig-like domains and a C-terminal Ig-module), I26/I27 (two Ig-like domains); I84 (a single Ig-like domain), and (FN3)<sub>6</sub> (six fibronectin type 3 domains located at the A-band edge). Nomenclature is according to Reference 21.

### In Vitro Motility Assay

An actin-myosin in vitro motility assay was performed with heavy meromyosin (HMM), sometimes also with whole myosin, from chicken breast muscle.<sup>30</sup> Actin was prepared from rabbit back

muscle. Measurements were done at 30°C using pure F-actin or tropomyosin-reconstituted F-actin. Tropomyosin was a kind gift of Dr B. Bullard (EMBL Heidelberg, Germany). For buffer composition, see Reference 30. Sliding of rhodamine/phalloidin-labeled actin filaments was monitored in the absence and presence of recombinant titin constructs (0.001 to 100  $\mu\text{g}/\text{mL}$ ). Videotaped movies were analyzed with tracking software (available at <http://mc11.mcri.ac.uk/Retrac>) to determine the sliding speed of randomly selected actin filaments within the field of view. The number of stationary filaments was counted within a 50 $\times$ 50- $\mu\text{m}$  region of interest.

### Fluorescence Actin Binding Assay

This assay was performed in motility-assay flow cells by adding PEVK-titin construct to rhodamine/phalloidin-labeled actin filaments at high (170 mmol/L) and low (50 mmol/L) ionic strength (IS) in the absence of myosin.<sup>26</sup> Bound actin filaments were counted in a 50 $\times$ 50- $\mu\text{m}$  region of interest.

### Actin-Titin Cosedimentation Assay

Actin-titin binding assays were essentially performed as described.<sup>17</sup> G-Actin and recombinant cardiac-titin constructs were incubated with  $\text{CaCl}_2$  or EGTA at 0°C or 30°C. Prepared samples were separated by SDS-PAGE and Coomassie stained. PEVK-titin was visualized also by Western blot using 9D10 monoclonal antibody (Hybridoma Bank, University of Iowa). Scanned images were analyzed with Intelligent Quantifier software (Bioimage).

### Cardiac Myofibril Mechanics

Myofibrils were isolated from freshly excised rabbit (male New Zealand White) heart.<sup>17</sup> Small bundles of three to four myofibrils were suspended between a piezomotor (Physik Instrumente) and a force sensor (home-built) with nanonewton resolution.<sup>10,17</sup> Specimens were held at room temperature in relaxing buffer (IS, 200 mmol/L, pH 7.1) containing 40  $\mu\text{g}/\text{mL}$  leupeptin and 20 mmol/L active-force suppressing agent, 2,3-butanedione monoxime. Data acquisition/analysis was done using a personal computer, data acquisition board, and LabView software (National Instruments). Myofibril images were videotaped during experiments for later analysis of SL.

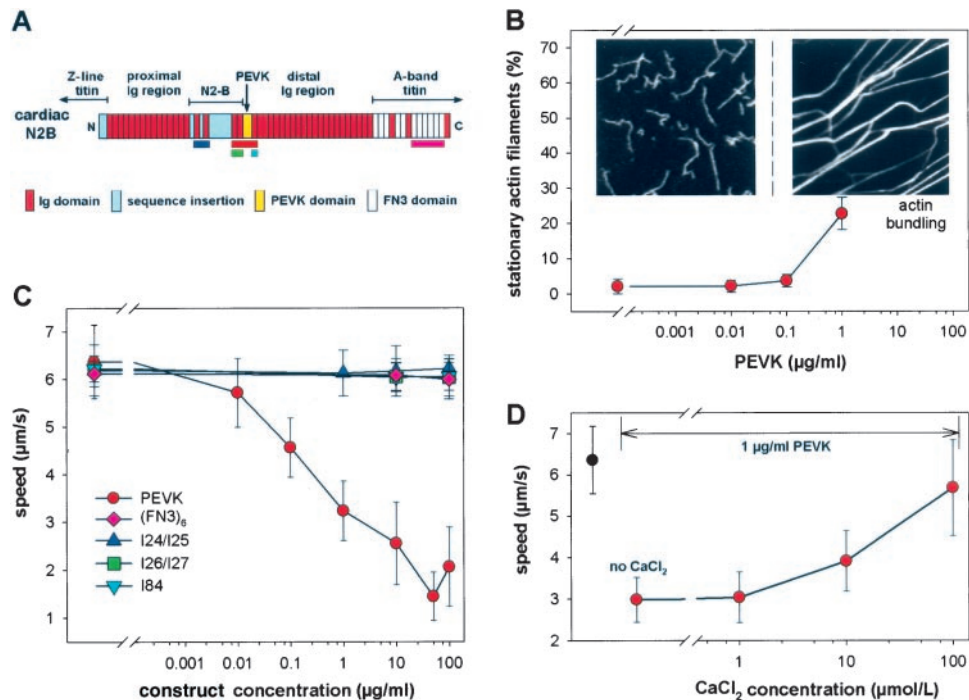
In a typical experimental protocol (compare Reference 4), relaxed myofibrils were extended to 2.1 or 3.6  $\mu\text{m}$  SL, subjected to a  $\approx 0.2$   $\mu\text{m}/\text{sarcomere}$  ramp stretch completed within 4 or 20 ms, held isometrically for 10 to 20 seconds, and released quickly to initial SL. Force sampling rate was 5 kHz. Three identical stretch protocols were carried out (rest interval, 1 minute), and the stress-relaxation response in each recording was fit with a three-order exponential-decay function. A  $\text{Ca}^{2+}$ -independent gelsolin fragment (kindly provided by Dr H. Hinssen, University of Bielefeld, Germany) was added to the relaxing buffer (final concentration, 0.2 mg/mL) to extract actin.<sup>17</sup> The ramp-stretch protocols were then repeated every minute, and stress relaxation was analyzed. Actin extraction was routinely tested by rhodamine/phalloidin fluorescence.<sup>17</sup> In some experiments, stress relaxation was measured during selective proteolysis of titin with 0.03  $\mu\text{g}/\text{mL}$  trypsin<sup>11</sup> and confirmed by 2.8% SDS-polyacrylamide gel electrophoresis.<sup>17</sup>

An expanded Materials and Methods section can be found in the online data supplement available at <http://www.circresaha.org>.

## Results

### PEVK-Titin Slows Down Actin Filament Sliding in the In Vitro Motility Assay

A possible interaction between recombinant cardiac-titin constructs and actin was tested in the actomyosin in vitro motility assay. Actin movement should be impaired, if a titin construct tethered actin filaments to the surface of the motility chamber.<sup>31</sup> Altogether five different construct types were used, derived from structurally distinct regions along the N2-B cardiac-titin sequence (Figure 1A). A titin segment



**Figure 1.** Effect of recombinant cardiac-titin constructs on actomyosin in vitro motility. **A**, Domain architecture of I-band titin in cardiac muscle (N2-B isoform)<sup>13</sup> and construct types used in in vitro motility assay (horizontal bars; color coding as in panel C). **B**, Effect of PEVK-titin (flanked by Ig-like domains I26/I27 and I84) on number of stationary actin filaments, in fields of view of same size. Images show rhodamine/phalloidin fluorescence of actin filaments at low (left) and high (right) PEVK concentrations. Number of individual immobile filaments at  $\geq 10$   $\mu\text{g/ml}$  PEVK could not be counted because of actin bundling. **C**, Dependence of actin sliding speed on titin construct concentration. Only the construct containing cardiac PEVK domain inhibited actin sliding in a concentration-dependent manner.  $n=20$  filaments per construct. Domain nomenclature was according to Reference 21. **D**,  $\text{Ca}^{2+}$  effect on PEVK-induced slowdown of actin sliding.  $n=20$ . Panels B through D, data are mean  $\pm$  SD.

encompassing Ig-like domains I24 and I25 recently was shown to disrupt sarcomeric actin on overexpression in cardiac myocytes.<sup>19</sup> However, the recombinant I24/I25 construct, which contains a 23-residue unique sequence, did not affect actin filament sliding over myosin (Figure 1C). Similarly, the sliding speed was not altered by a titin fragment made up of six FN3 domains at the A-band edge. The only construct consistently showing an effect in the in vitro motility assay was recombinant PEVK-titin, including the flanking Ig-like domains I26/I27 and I84 (Figure 1C). As a control, I26/I27 and I84 alone were tested for their impact on actin sliding speed, but no effect was found (Figure 1C). As for the PEVK construct, three different batches of expressed protein were studied, all with comparable results. Cardiac PEVK inhibited actin sliding in a concentration-dependent manner; little effect was seen at 10  $\mu\text{g/ml}$ , whereas a  $\approx 75\%$  speed reduction occurred at 50  $\mu\text{g/ml}$  PEVK (Figure 1C). The range of PEVK concentrations used (0.01 to 100  $\mu\text{g/ml}$ ) corresponds to a PEVK:actin molar ratio between 0.032 and 320. At PEVK concentrations mimicking the physiological situation—near 1  $\mu\text{g/ml}$ —actin sliding speed was decreased by  $\approx 50\%$  (Figure 1C). This slowdown was accompanied by an increase in the number of stationary actin filaments (Figure 1B; these filaments were not included in the analysis of Figure 1C). Concentrations  $\geq 10$   $\mu\text{g/ml}$  PEVK usually resulted in actin bundling (upper right image, Figure 1B). Large actin bundles developing in the presence of 50 to 100  $\mu\text{g/ml}$  PEVK were seen also by electron microscopy (not

shown). The observed effects of cardiac PEVK construct on both actin bundling propensity and actin sliding speed were similar when pure actin filaments, tropomyosin-reconstituted F-actin, HMM, or whole myosin were used. Data presented in Figure 1 are from experiments with HMM and pure F-actin.

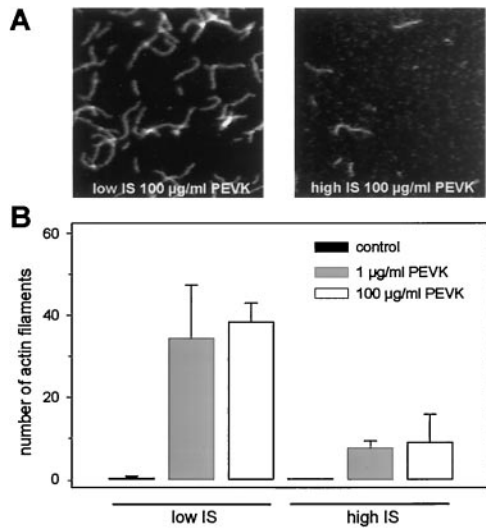
Because the concentration of intracellular free  $\text{Ca}^{2+}$  changes during muscle activation, we investigated whether  $\text{Ca}^{2+}$  affects the PEVK-induced slowdown of actin sliding. Figure 1D shows that high  $[\text{Ca}^{2+}]$  reversed the PEVK effect. The calcium concentration needed for near-complete reversal was higher (100  $\mu\text{mol/L}$ ) than what is considered physiological (up to  $\approx 10$   $\mu\text{mol/L}$ ). We then tested whether  $\text{Ca}^{2+}$ -calmodulin and/or  $\text{Ca}^{2+}$ -S-100 protein could modulate the effect observed with  $\text{Ca}^{2+}$  (for rationale, see Gutierrez-Cruz et al<sup>32</sup>). However, neither substance (at concentrations of 10 or 100  $\mu\text{g/ml}$ ;  $\text{Ca}^{2+}$ , 10 or 100  $\mu\text{mol/L}$ ) altered the actin sliding speed in the presence of cardiac PEVK construct in a statistically significant manner (data not shown).

## Interaction Between Cardiac PEVK-Titin and Actin

### Impact of IS

Because the PEVK region is highly charged, it was desirable to study whether IS could affect the interaction between cardiac PEVK-titin and actin. However, in vitro motility assays are generally carried out at low IS (maximum, 50 mmol/L). To test for actin-PEVK association under ionic conditions resembling physiological ( $\approx 170$  mmol/L), an





**Figure 2.** IS dependency of actin binding to cardiac PEVK construct, in the absence of myosin. A, Images of fluorescent actin filaments tethered by PEVK-titin to the cover-glass surface. B, Summary of results of in vitro actin fluorescence binding assay (mean±SD; n=15 regions of interest of 50×50 µm). Low IS=50 mmol/L; high IS=170 mmol/L.

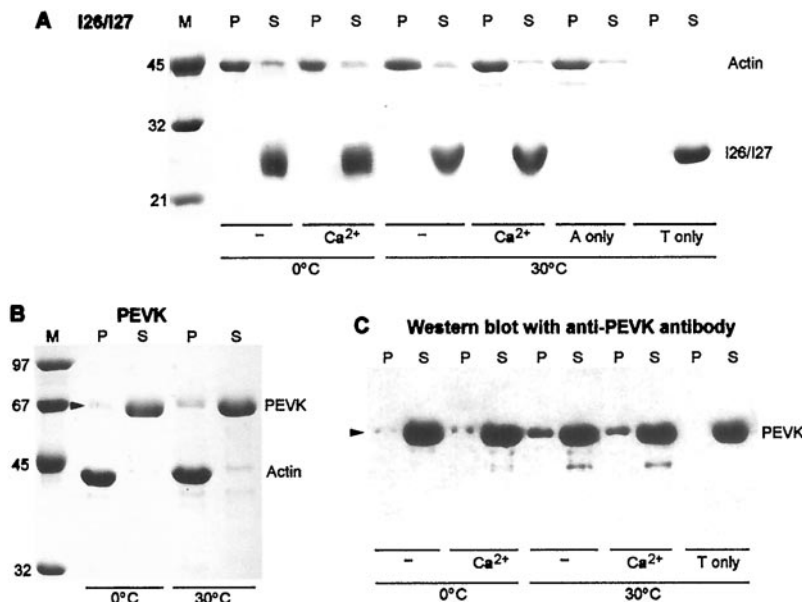
actin fluorescence binding assay<sup>26</sup> was used. Rhodamine/phalloidin-labeled actin filaments were incubated with PEVK-titin in the absence of myosin, and the number of fluorescent actin filaments still attached to the coverslip surface after repeated washes was counted (Figure 2A). Actin was tethered by PEVK-titin (concentration, 1 or 100 µg/mL) to both nitrocellulose-coated coverslips and untreated glass surfaces. Figure 2B shows that many actin filaments were still seen to be bound at high IS, although the number of filaments per field of view was ≈5 times lower than at 50 mmol/L IS. In the absence of PEVK construct, almost no actin filaments remained attached (Figure 2B, control). Thus, actin and PEVK-titin may associate at physiological IS.

**Temperature Dependency**

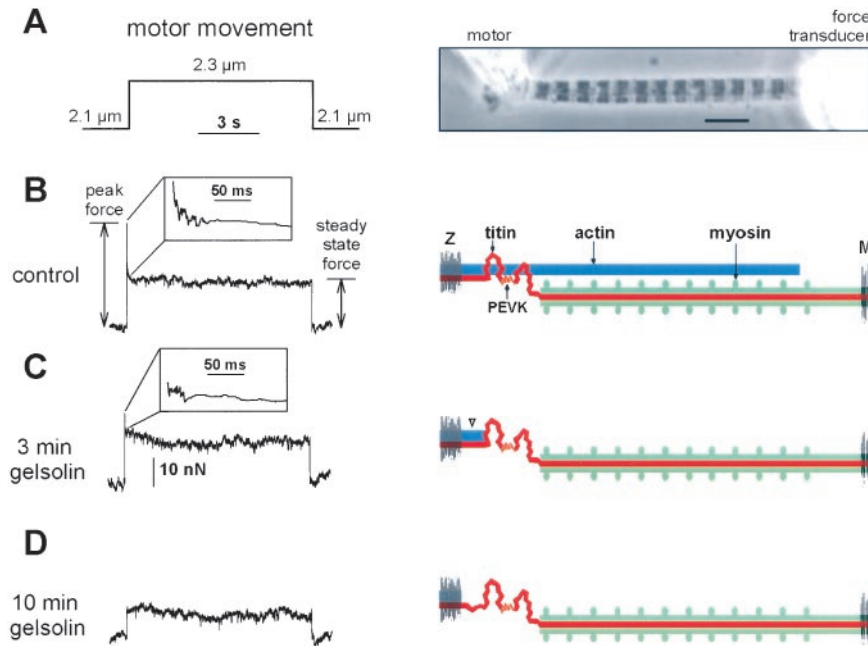
Earlier, we studied cloned fragments derived from different regions of cardiac titin for a possible interaction with actin in cosedimentation assays.<sup>17</sup> At that time, no actin binding to FN3 modules or Ig-like domains was detected, except to a two-Ig fragment from the functionally stiff Z-disc region of titin. Extending the earlier findings, we show here that also the cardiac N2-B-specific Ig-like domains I26/I27 do not interact with actin in the cosedimentation assay (Figure 3A). In contrast, cardiac PEVK (including the flanking Ig-like domains I26/I27 and I84, respectively) does exhibit actin-binding propensity. The actin-PEVK association is weak at 0°C, but stronger at 30°C (examples in Figures 3B and 3C). On Coomassie-stained SDS gels, the interaction was barely detectable (Figure 3B), but Western blots with α-PEVK antibody 9D10 established the temperature-dependent binding beyond doubt (Figure 3C). On average, the actin-PEVK binding propensity was three to four times higher at 30°C than at 0°C, both in the absence and presence of Ca<sup>2+</sup> (Figure 3C). Ca<sup>2+</sup> (concentration, 50 µmol/L) appeared to decrease the actin-PEVK affinity somewhat, by a factor of about two at 30°C. At 0°C, the differences were too small to allow reliable conclusions to be drawn.

**Actin-Titin Interaction Is the Source of a Viscous Force in Cardiac Myofibrils**

To probe a physiological relevance of the actin-PEVK association, force measurements were carried out on rabbit cardiac myofibrils. Nonactivated specimens containing no more than four parallel myofibrils were extended within 4 ms from 2.1 to 2.3 µm SL and held in the stretched state, and stress relaxation was measured (Figure 4A). Passive force during the hold period initially decayed rapidly, but then more slowly, to a quasi-steady-state level (Figure 4B). The force decline could be fit with a three-order exponential-decay function; the Table shows that the decay time constants were 4 to 5 ms, 50 to 60 ms, and ≈30 seconds, respectively. The amplitude of the quickest decay component was strongly



**Figure 3.** Examples of results of actin-titin cosedimentation assays. A, SDS gel demonstrates lack of interaction (see pellet) between actin and the titin construct with two Ig-like domains, I26/I27, under various experimental conditions. B, Association between cardiac PEVK-titin (including flanking Ig-like domains I26/I27-I84) and actin probed at two different temperatures. Bound actin-PEVK complex is found in the pellet (arrowhead). C, Western blot to study actin-PEVK association, using monoclonal PEVK antibody 9D10. Arrowhead indicates actin-PEVK binding (see pellet) in the presence of 1 mmol/L EGTA (-) or 50 µmol/L CaCl<sub>2</sub> (Ca<sup>2+</sup>), at either 0°C or 30°C. M indicates molecular weight markers; P, pellet; S, supernatant; A, actin; and T, titin construct. Concentrations are as follows: titin constructs, 20 µmol/L; actin, 40 µmol/L.



**Figure 4.** Force measurements on relaxed cardiac myofibrils before and during actin extraction. A, Motor ramp (stretch/release completed within 4 ms) and phase image of a representative bundle of three myofibrils (right scale bar, 5 μm). B, Stress-relaxation before actin extraction (left) and scheme of filament arrangement in a half-sarcomere (right). Z indicates Z-disc, and M, M-line. C, Peak force is decreased after 3-minute treatment with a Ca<sup>2+</sup>-independent gelsolin fragment (left), which extracts actin, except the portion adjoining the Z-line (arrowhead in scheme). (Force calibration bar also applies to panels B and D.) D, Both peak force and steady-state force are decreased 10 minutes after gelsolin application (left); the Z-disc-adjacent actin is removed, but not the central Z-disc actin (right).

dependent on stretch speed (data not shown, but compare Reference 33) and made up a large portion of the whole decay amplitude after 4-ms stretches (Table). This quick force decay was shown elsewhere to be viscous in nature, whereas the slower decay components are viscoelastic and may result from unfolding or structural rearrangement of titin domains.<sup>33</sup>

The force decay after stretch then was studied in relaxed cardiac myofibrils exposed to a Ca<sup>2+</sup>-independent gelsolin fragment to extract actin. Gelsolin treatment removes actin filaments rapidly,<sup>17</sup> except for the portion attached to titin in a ≈100-nm-wide region adjoining the Z-line.<sup>16</sup> Severing of the Z-line-adjacent actin takes several minutes, whereas the central Z-disc actin cannot be extracted by gelsolin.<sup>16,17</sup> This scenario is depicted in Figure 4B through 4D (right panels). Analysis of stress relaxation during gelsolin treatment showed that the whole decay amplitude after 4-ms stretches dropped by ≈30% within 3 minutes (Figure 4C; Table).

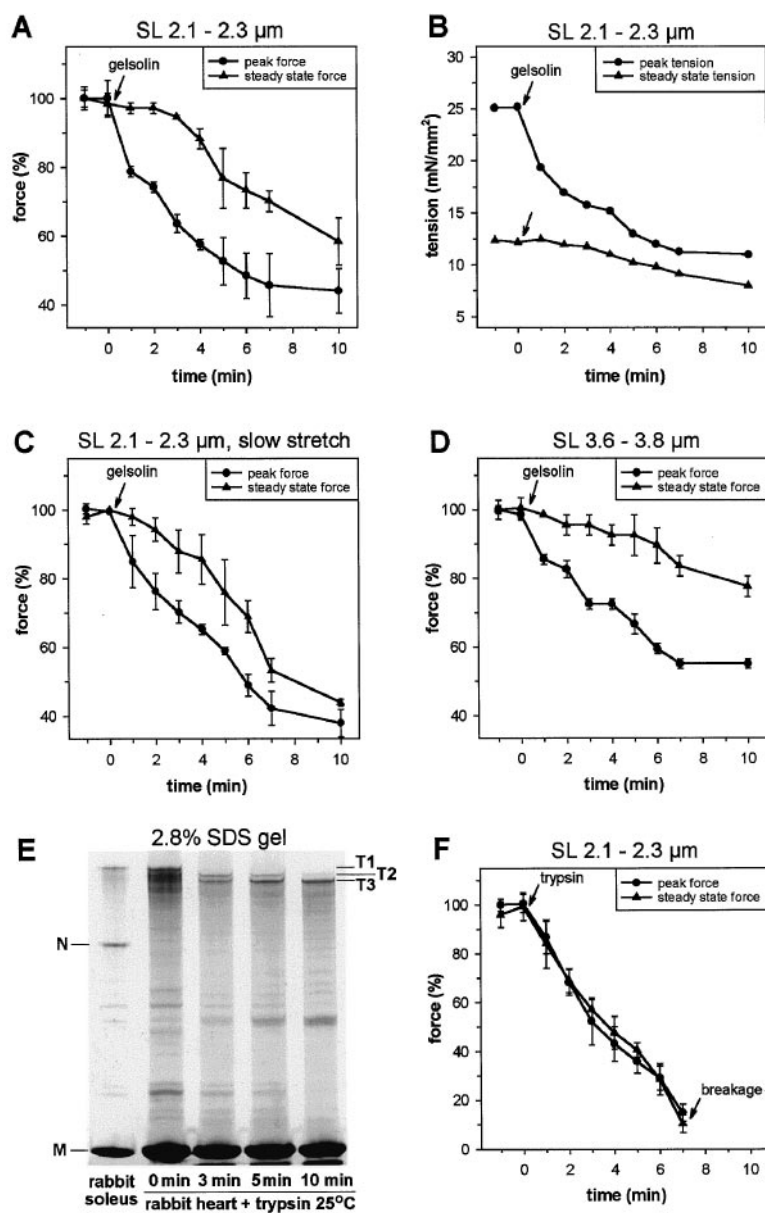
**Summary of Results of Stress-Relaxation Measurements on Cardiac Myofibrils (n=6) Stretched From 2.1 to 2.3 μm SL Within 4 ms and Held at the Stretched Length**

	Control		Gelsolin (3 Minutes)	
	Mean	±SD	Mean	±SD
A, mN/mm <sup>2</sup>	11.98	0.54	6.71	0.24
A <sub>1</sub> , mN/mm <sup>2</sup>	6.92	0.68	0.91	0.43
t <sub>1</sub> , ms	4.60	0.75	4.91	0.47
A <sub>2</sub> , mN/mm <sup>2</sup>	3.68	0.27	4.44	0.42
t <sub>2</sub> , ms	56.70	4.70	60.80	6.80
A <sub>3</sub> , mN/mm <sup>2</sup>	1.19	0.02	1.11	0.10
t <sub>3</sub> , ms	30 945	515	30 513	2178

A is the whole amplitude of tension decay measured from original force data (peak force minus steady-state force). The force decline also was fit with a 3-order exponential decay function of the type  $f(t) = f_0 + A_1 \times \exp(-t/t_1) + A_2 \times \exp(-t/t_2) + A_3 \times \exp(-t/t_3)$ , where  $f_0$  is offset,  $A_1$ - $A_3$  are decay amplitudes, and  $t_1$ - $t_3$  are decay time constants.

Exponential-decay fits revealed that this relatively fast drop is due to a decrease of the quickly decaying force component exclusively; the slower-decay components initially are not affected (Table). More than 3 minutes after gelsolin application, steady-state tension also dropped and stress relaxation became very small (Figure 4D). Results of force measurements over a 10-minute period of gelsolin treatment are summarized in Figure 5A; peak force just after completion of the 4-ms stretch is compared with steady-state force at the end of a 10-second hold period. Figure 5B shows the results of a single experiment to demonstrate the absolute magnitude of tension changes observed. Because 4-ms stretches of 2.1-μm-long sarcomeres by 0.2 μm correspond to high stretch speeds of nearly 25 muscle-lengths per second, stress relaxation was also studied after 20-ms stretches corresponding to more physiological stretch speeds (Figure 5C). Although the rapidly decaying viscous component was lower than in the 4-ms stretch protocols (data not shown), the differences between the time courses of peak force decrease and steady-state force decrease during gelsolin treatment persisted. To conclude, gelsolin application almost immediately decreased the viscous force component during filament sliding, suggesting that interactions involving actin play a main role in the viscosity.

The results described above were obtained at physiological SL, at which weak interactions between actin and myosin in the relaxed state could contribute to viscous drag during SL changes. Therefore, isolated cardiac myofibrils were stretched to beyond overlap of actin and myosin filaments (3.6 to 3.8 μm SL), and the stretch-hold protocol again was applied before and during actin extraction. Figure 5D shows that results were qualitatively similar to those obtained at shorter SLs. Ten minutes of gelsolin treatment decreased peak force by ≈45%, only slightly less than the ≈55% seen at short SLs. Thus, actin-myosin interactions may contribute to some degree to the viscous force decay after quick stretch. However, the majority of this decay must come from other sources, most likely actin-titin interactions.



**Figure 5.** Summary of results of stress-relaxation measurements on cardiac myofibrils. A, Time course of decrease in peak force and steady-state force during gelsolin treatment, for 4-ms stretches followed by a hold period, at physiological SLs. B, Example showing absolute force change related to myofibrillar cross-sectional area. C, Results of 20-ms stretch protocols at physiological SLs. D, Results of 4-ms stretch protocols at zero actin-myosin overlap. Data in panels A, C, and D are mean  $\pm$  SD ( $n=6$ ). E, 2.8% SDS-PAGE shows titin proteolysis in rabbit cardiac myofibrils at different time points during treatment with 0.03  $\mu\text{g}/\text{mL}$  trypsin. Rabbit soleus proteins are shown for comparison. T1 indicates native titin; T2–T3, titin degradation products; N, nebulin; and M, myosin heavy chain. F, Time course of decrease in peak force and steady-state force during trypsin treatment for 4-ms stretch protocols at physiological SLs (mean  $\pm$  SD,  $n=5$ ).

To exclude the possibility that merely actin filament sliding in the medium is responsible for the viscous drag, titin filaments were selectively degraded by low doses of trypsin (0.03  $\mu\text{g}/\text{mL}$ ), which affects preferentially the PEVK region.<sup>34</sup> 2.8% SDS-PAGE confirmed effective proteolysis (Figure 5E), which was characterized by a quick loss of the native T1-titin band and appearance of titin degradation bands (T2 and T3). Stress-relaxation measurements showed that peak force and steady-state force decreased in parallel during trypsin treatment to near-zero levels after 7 to 8 minutes, when myofibrils usually broke apart (Figure 5F). Thus, large viscous forces resulting from actin filament sliding could be excluded. Instead, the combined results of this study suggest that it is the titin filaments, most likely the PEVK domain of titin, which may associate with actin in the sarcomere, thereby contributing to the viscous force during SL changes.

## Discussion

Analysis of the diastolic mechanical properties has long been part of the assessment of the performance characteristics of the heart. Important contributors to diastolic wall stiffness are extracellular elements, such as collagen and elastin, albeit mainly toward the high end of the physiological SL range.<sup>10,11</sup> The principal determinants of passive-tension development at physiological SLs lie inside the cell and were shown to be the titin filaments<sup>10,11</sup>; intermediate filaments play only a minor role.<sup>11</sup> Furthermore, titin was recently suggested to determine, to a large part, restoring forces in cardiomyocytes<sup>34</sup> and left ventricular diastolic suction.<sup>22</sup> Whereas titin filaments are the source of much of the elasticity of cardiac muscle, there are also viscous forces appearing during ventricular filling and contraction.<sup>1–3</sup> The origin of the viscosity is less well understood, although titin has been suggested to contribute to the phenomenon.<sup>4–6</sup> A viscous load on the contractile apparatus may also



arise from an increased microtubule content of cardiac cells, at least in pressure-overload hypertrophy.<sup>7,8</sup> Here, we propose that viscous effects in normal cardiac muscle may be due to an interaction between actin and PEVK-titin in the sarcomeres.

A previous report suggested, from the study of electron micrographs of rabbit papillary muscle, that actin filaments may laterally associate with titin in the elastic I-band region.<sup>23</sup> Because it was difficult to imagine, at the same time, both actin-titin association along major portions of the sarcomere and relative sliding of thin and thick filaments past one another, the interaction was proposed to be transient or weak. *In vitro* studies confirmed that actin may interact with whole titin,<sup>24,25</sup> and native titin was shown to slow down actin filament sliding over myosin in the *in vitro* motility assay.<sup>26</sup> One report also found binding of actin to an expressed titin-FN3 module or to such a module combined with an Ig-like domain (derived from the A-band edge),<sup>27</sup> leading to the inhibition of actomyosin *in vitro* motility.<sup>28</sup> However, Ig/FN3-like domains of titin do not interact with actin generally; we confirmed actin binding propensity only for a recombinant two-Ig-like-domain construct from the Z-line-adjacent (functionally stiff) titin region.<sup>17</sup>

These issues were addressed in the present work by testing structurally distinct regions of N2-B-cardiac titin for a possible interaction with actin in the *in vitro* motility assay. This assay was chosen because an effective slowdown of actomyosin motility is characteristic of actin-binding protein.<sup>31</sup> None of the recombinant titin constructs containing exclusively Ig-like or FN3 domains slowed down the sliding of actin filaments (or tropomyosin-reconstituted F-actin) over HMM or whole myosin. Similarly, an expressed construct containing a unique sequence flanked by Ig-like domains I24/I25 had no effect on actin sliding speed. The I24/I25 segment was of particular interest to us, because its overexpression in cardiomyocytes causes disruption of sarcomeric actin filaments.<sup>19</sup> The present findings make it unlikely that the disruption results from direct actin binding to I24/I25. What became clear is that the sliding of actin filaments is inhibited in a concentration-dependent manner specifically by PEVK-titin (Figure 1). Relatively weak actin-PEVK interaction was confirmed in cosedimentation assays, consistent with a recent report on skeletal (fetal) PEVK-titin.<sup>32</sup> In contrast, Ig-like domains from the N2-B-titin region completely lacked actin binding propensity (Figure 3). Taken together with earlier results,<sup>17</sup> we conclude that the actin-titin association most likely involves the PEVK region, but not the vast majority of titin domains outside the Z-disc ( $\approx 90\%$  of titin consists of Ig-like domains and FN3 modules).<sup>12</sup> At this point we cannot exclude that unique sequences other than the PEVK domain also interact with actin. For example, the N2-B-unique sequence of cardiac titin contains 572 residues, much more than the 163 residues of N2-B-PEVK.<sup>13</sup> However, the fact that a 23-residue insertion contained within the I24/I25 construct did not reduce actin-filament motility suggests that the unique sequences of titin do not bind to actin generally.

An intriguing result is that the interaction between actin and cardiac PEVK titin is modified by temperature (Figures 3B and 3C) and ionic conditions (Figure 2). At physiological IS the binding was weaker than at low IS, which is not

unrealistic, considering that charged residues along the PEVK domain<sup>35</sup> may become electrostatically shielded. We propose that electrostatic interactions may be important for both the elastic properties of PEVK titin<sup>36</sup> and the actin-PEVK association. Moreover, the  $\approx 4$ -fold increase in actin-PEVK binding strength on raising temperature from 0°C to 30°C (Figures 3B and 3C) hints at the possibility that the actin-PEVK association involves hydrophobic interactions.

High  $\text{Ca}^{2+}$  concentrations reversed the slowdown of actin sliding by cardiac PEVK-titin and slightly inhibited actin-PEVK binding at 30°C in the cosedimentation assay. This is just the opposite of what was found earlier in the case of actin and whole titin.<sup>26</sup> The reason for this discrepancy is unclear at this point. In our hands,  $\text{Ca}^{2+}$  alone did not appear to be sufficient for effective regulation of actin-PEVK binding, as the ion concentration producing an effect was an order of magnitude higher than that reached under physiological conditions. Attempts to modify the  $\text{Ca}^{2+}$  effect by calmodulin or S-100 protein were unsuccessful. It will be interesting to identify possible mechanisms of  $\text{Ca}^{2+}$ -dependent regulation of actin-PEVK binding. Our result that  $\text{Ca}^{2+}$  counteracts the actin-PEVK binding is consistent with a finding by Stuyvers et al,<sup>6</sup> who reported that length and stiffness of the sarcomeres increased in the diastolic interval of cardiac muscle, whereas cytosolic free  $[\text{Ca}^{2+}]$  gradually declined in the submicromolar range. The authors hypothesized that the  $\text{Ca}^{2+}$  sensitivity of sarcomere stiffness observed at low  $[\text{Ca}^{2+}]$  could result from a  $\text{Ca}^{2+}$ -dependent alteration of interaction between titin and thin filaments. Moreover, a cloned fragment consisting of two Ig/FN3-titin domains eliminated the  $\text{Ca}^{2+}$  dependence of stiffness, perhaps by competing with endogenous titin for the same binding site on actin.<sup>6</sup> In contrast, findings from our laboratory showing no actin binding propensity of the  $\beta$ -sheet domains of titin (Figure 3A and Reference 17) argue against the possibility that such domains are involved in  $\text{Ca}^{2+}$ -regulated actin binding. Given that the two domains used by Stuyvers et al<sup>6</sup> were derived from the A-band edge, where six individual titin filaments may associate into a supramolecular complex that binds to the thick filament,<sup>37</sup> it is possible that as-yet-unrecognized factors modulate actin-titin interactions. However, the results of this study rather suggest that the most likely site on titin to associate with actin is the PEVK segment.

If binding between actin and titin increases sarcomere stiffness, removal of one of these proteins should decrease stiffness. Indeed, selective actin extraction by gelsolin fragment lowered the stiffness of nonactivated cardiac myofibrils by  $\approx 60\%$ .<sup>16,17</sup> In the present study, the gelsolin effect on the mechanical properties of relaxed cardiac myofibrils was studied in more detail. The investigation was triggered by the finding that skeletal myofibrils exhibit, during a hold period after rapid stretch, a quick viscous force decline that cannot be explained by the viscoelastic properties of titin.<sup>33</sup> Here we showed, on cardiac myofibrils, that actin extraction removes the viscous force component—an effect depending only little on actin-myosin overlap (Figures 5A through 5D). Further, selective proteolysis of titin drastically lowered the viscous force as well, demonstrating that viscous drag possibly brought about by thin-filament sliding in the surrounding medium is unimportant. These results unequivocally establish

actin-titin interaction as a source of viscous force in the sarcomere. Although a main contributor to viscosity most likely is actin-PEVK association, it remains to be seen whether or not some titin domains not studied by us (other Ig-like domains and/or the N2-B–unique sequence) contribute to the phenomenon. In any case, a viscous force component due to actin-titin interaction may not only show up during rapid ventricular filling, but to some degree also during contraction, thereby opposing active shortening.<sup>3,4</sup> Finally, because the length of the PEVK domain of titin differs in different cardiac-titin isoforms,<sup>21</sup> it is not unreasonable to expect changes in viscous loading when the isoform expression pattern is altered under certain pathological conditions. Increased expression of N2-BA–titin containing a longer PEVK region has been found after 14 days of pacing tachycardia in dog hearts.<sup>22</sup>

In summary, titin may interact with actin filaments in the cardiac-muscle sarcomere. During sarcomere-length changes, this interaction may cause viscous drag, which manifests itself in quick stress relaxation after rapid stretch and probably in a force that opposes shortening. Several lines of evidence suggest that actin binding is a property of the PEVK region of titin, as follows: (1) recombinant PEVK constructs (but not Ig/FN3 domains) inhibit actomyosin motility, (2) PEVK-titin binds to actin in *in vitro* fluorescence binding assays; and (3) PEVK-titin and actin interact weakly in cosedimentation assays. Both hydrophobic and electrostatic interactions are likely to contribute to the actin-PEVK association.

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### **INTERACTION BETWEEN PEVK-TITIN AND ACTIN FILAMENTS: ORIGIN OF A VISCOUS FORCE COMPONENT IN CARDIAC MYOFIBRILS**

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### **Expanded Materials and Methods**

## Expression of recombinant titin constructs

Titin fragments were expressed essentially as described.<sup>1</sup> cDNA fragments corresponding to different regions of the human cardiac N2B-titin sequence were obtained by PCR and subcloned into a modified pET vector.<sup>2</sup> The sequence of the inserts was confirmed and the expression vectors transformed into *E. coli* BL21[DE3]pLysS cells (Stratagene). His<sub>6</sub>-tagged protein was purified on Ni<sup>2+</sup>-NTA columns following manufacturer's instructions (Qiagen, Germany). Further purification was achieved by using anion exchange chromatography on a Mono Q column (Pharmacia, Sweden). In some experiments, the His<sub>6</sub> tag was cleaved off by recombinant TEV protease, without affecting the binding properties. Concentration determination was carried out by UV spectroscopy.

Nomenclature of titin constructs is according to Ref. 3; residue numbers of the human cardiac titin sequence are found in Ref. 4 (EMBL accession number X90568). The following titin constructs were obtained: I24/I25 (two N2-B-specific Ig-domains flanking a short unique sequence); I26/I27-I84 (cardiac PEVK flanked by two N-terminal Ig-domains and a C-terminal Ig-module); I26/I27 (two Ig-domains); I84 (single Ig-domain); and (FN3)<sub>6</sub> (six fibronectin-type-3 domains located at the A-band edge).

## In-vitro motility assay

The actin-myosin *in-vitro* motility assay was performed according to the method of Anson et al.,<sup>5</sup> with some modifications. Briefly, motility chambers were constructed from glass slides coated with bovine serum albumine (BSA) and 18 mm square coverslips coated with a thin nitrocellulose film. Experiments were carried out mainly with heavy meromyosin (HMM), sometimes also with whole myosin, obtained from chicken breast muscle. Actin was prepared from rabbit back muscle. For solution component conditions, see Ref. 5. Measurements were done with pure actin filaments or tropomyosin-reconstituted F-actin. Tropomyosin was a kind gift of Dr. B. Bullard. To test the impact of several recombinant titin construct on actin-sliding speed, a given construct usually was added to the ATP-containing buffer infused into the motility chamber to initiate movement of actin filaments. Filament-sliding velocity was monitored in the absence and presence, respectively, of different concentrations of titin construct (10 ng/ml - 100 µg/ml). As for the cardiac PEVK-domain, a concentration of 10 ng/ml corresponds to 0.18 pM PEVK, which compares to 5.7 pM F-actin used (assuming an average actin-filament length of 10 µm). Thus, the range of PEVK:actin molar ratios used was 0.032 to 320 (0.01 to 100 µg/ml).



PEVK). With presumably three titin filaments per actin filament present in the half-sarcomere,<sup>6</sup> a PEVK concentration of 1 µg/ml comes close to physiological.

The assay was carried out on the stage of an inverted microscope (Zeiss Axiovert 135, Göttingen, Germany) equipped with a 40x objective and 1.6x lens slider. The objective was heated to 30°C by using a peltier device. Images were recorded with an intensified video camera (Photonic Science) and S-VHS video recorder (JVC) via an image-processing system (Hamamatsu, Argus 20). Images were digitized with a PC and frame-grabber board (CG7, Scion Corp.). Actin filament-sliding speed was determined by tracking filaments using the PC-based tracking software Retrac v1.80 (<http://mc11.mcri.ac.uk/Retrac>). A region of interest (ROI, 50 x 50 µm) was defined within which the number of stationary filaments was determined.

Different concentrations of Ca<sup>2+</sup>, Ca<sup>2+</sup>-Calmodulin (P 2277, Sigma) and Ca<sup>2+</sup>-S-100 (S-100A (α,β), Sigma) were added directly to the motility buffer (containing 0.5% methylcellulose and 2 mmol/L ATP), and the effect on actin-sliding speed was monitored in the presence of cardiac PEVK-construct.

### **Actin fluorescence binding assay**

Actin fluorescence-binding assay was performed by flushing 50 µl flow cells with 100 µl assay buffer (in mmol/L, imidazol 25, KCl 25, MgCl<sub>2</sub> 4, EGTA 1, DTT 10; 0.5 mg/ml BSA) containing various amounts of PEVK-construct. After addition of 100 µl rhodamine/phalloidin-labeled F-actin, the flow cell was rinsed with assay buffer. To prevent photobleaching, flow cells were infused with assay buffer containing 5 mg/ml glucose, 0.1 mg/ml glucose oxidase and 0.02 mg/ml catalase. The assay was carried out at high (170 mmol/L) and low (50 mmol/L) ionic strength (IS) in the absence of myosin (cf., Ref. 7).

### **Cosedimentation assay**

All proteins were centrifuged prior to use at 4°C for 30 min at 400,000 g using a Beckman TLA 100.1 rotor and Optima™ TL ultracentrifuge. Appropriate amounts were mixed in Beckman polycarbonate centrifuge tubes (No. 343776), and the volume was made up to 100 µl with F-actin buffer (in mmol/L, Tris/Cl 50, pH 8, NaCl 130, DTT 2, ATP 0.2, MgCl<sub>2</sub> 2). Actin-binding assays were essentially performed as described.<sup>8</sup> G-actin and the recombinant titin fragments were incubated in 100 µl F-actin buffer, with 0.05 mmol/L CaCl<sub>2</sub> or 1 mmol/L EGTA at either 0°C or

30°C, and with 40 µmol/L actin and 20 µmol/L titin construct. Following 30 min incubation, the F-actin was pelleted at 400,000 g for 30 min, and the supernatant was separated. The pellet was resuspended in 100 µl urea (4 mol/L). After addition of 100 µl sample buffer,<sup>9</sup> samples were heated to 95°C for 3 min. For analysis, 5 to 10 µl of the sample were separated on 15% sodium dodecyl sulphate polyacrylamide gels. Correction of the quantitation was done with the volume factor of the amount of pellet loaded. Gels were stained for 24 h in staining solution (1.5% w/v Coomassie brilliant blue R250, 40% ethanol, 10% acetic acid) and subsequently destained for 48 h. For analysis, the wet gel was scanned using a Mustek SP1200 scanner. PEVK-titin was visualized also by Western blot using 9D10 monoclonal IgM antibody (Developmental Studies Hybridoma Bank, University of Iowa).<sup>10</sup> Bound antibody was detected with ECL kit (Amersham). Quantitation of the bands was performed with software Intelligent Quantifier (Bioimage). The concentration of bound protein fragment was calculated from the relative intensities of supernatant and pellet and from the known total concentration.

### **Myofibril mechanics**

*Myofibril preparation:* Isolated myofibrils were prepared as described<sup>8</sup> from freshly excised rabbit ventricular tissue. Tissue samples were tied to thin glass rods and incubated overnight in rigor solution (in mmol/L; KCl 75, Tris 10, MgCl<sub>2</sub> 2, EGTA 2, 40 µg/ml leupeptin, pH 7.1) containing 0.5% Triton X-100. After repeated washes in rigor buffer without Triton, muscle strips were homogenized in a blender, and myofibrils were used within 5 hours. All steps were performed at 4°C. Relaxing buffer was prepared according to Ref. 11 and generally contained 20 mmol/L 2,3-butanedione monoxime (BDM), an active-force suppressing agent.

*Stress relaxation measurements:* The setup used for force measurements is centered around a Zeiss Axiovert 135 inverted microscope equipped with phase-contrast optics.<sup>8</sup> Glass microneedles attached to a piezoelectric micromotor (Physik Instrumente, Waldbronn, Germany) and a sensitive force transducer (home-built), respectively, were used to pick up a myofibril. To firmly anchor the myofibril ends, the motor/force-transducer needle tips were coated with a water-curing silicone adhesive in a 2:1 (v/v) mixture of Dow Corning 3145 RTV and 3140 RTV. Water-hydraulic micromanipulators (Narishige, Japan) were used to control the position of the needles. At the beginning of an experiment, the myofibril was transferred to relaxing solution and stretched to either ~2.1 or ~3.6 µm sarcomere length (SL). To measure stress relaxation, the myofibril was exposed to a motor step consisting of a quick 0.2 µm/sarcomere stretch completed within 4 ms. Sometimes, the stretch time was 20 ms, corresponding to a more physiological stretch speed of ~5 muscle lengths per second. Following the stretch, the specimen was held at

constant SL for 10-20 s, before being released to the initial SL. Three identical stretch protocols were carried out (rest interval, 1 min). Force was recorded during the entire experiment (sampling rate: 5 kHz). Data acquisition and data analysis were done with a Pentium-II PC, National Instruments data acquisition board (PCI-MIO-16E-1) and custom-written LABVIEW software (National Instruments). Myofibril images were recorded with a 3CCD color video camera (Sony, AVT Horn, Germany) and VCR (Panasonic NV-SD45) during experiments, digitized with a PC and frame grabber (CG7, Scion Corp.), and used to measure SL.

The force decline during stress relaxation (force,  $f$ , as a function of time,  $t$ ) was fit with an exponential-decay function of the type:

$$f(t) = f_0 + A_1 \times \exp(-t/t_1) + A_2 \times \exp(-t/t_2) + A_3 \times \exp(-t/t_3) \quad , \quad (1)$$

where  $f_0$  is offset,  $A_1$  through  $A_3$  are decay amplitudes, and  $t_1$  through  $t_3$  are decay time constants. Curve fitting was done by using a nonlinear least-squares method (Levenberg-Marquardt algorithm, Origin 5.0 software by Microcal, Amherst, MA).

Force-measurement protocols were repeated during exposure of the specimens to a  $\text{Ca}^{2+}$ -independent gelsolin fragment obtained from pig stomach smooth-muscle gelsolin.<sup>12</sup> Small units of gelsolin (concentration, 0.6-0.9 mg/ml) stored at  $-80^\circ\text{C}$  in a buffer containing (in mmol/L) KCl 50,  $\text{MgCl}_2$  1, imidazol 1, pH 7.2, were thawed shortly before usage. Final concentration of gelsolin fragment in relaxing buffer was 0.2 mg/ml. At the end of an experiment, actin extraction was routinely tested by rhodamine/phalloidin fluorescence. The gelsolin fragment removed all but the central Z-disc actin (cf., Ref. 8). In another series of experiments, stress relaxation after quick stretches was measured before and during treatment of cardiac myofibrils with low doses of trypsin (0.03  $\mu\text{g/ml}$ ) in relaxing buffer to selectively degrade the titin filaments.<sup>13</sup>

### **SDS-polyacrylamide gel electrophoresis**

Low-percentage SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was employed to investigate the time course of titin degradation upon treatment with low concentrations of trypsin (0.03  $\mu\text{g/ml}$ ). Procedures were carried out as described previously.<sup>8</sup> To detect both titin and myosin heavy chain of rabbit cardiac muscle on the same gels, agarose-strengthened 2.8% acrylamide gels were prepared.<sup>14</sup> Protein bands were visualized with Coomassie brilliant blue R.



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