Molecular Biology of the Cell
Vol. 8, 705–717, April 1997

An N-Terminal Fragment of Titin Coupled to Green Fluorescent Protein Localizes to the Z-Bands in Living Muscle Cells: Overexpression Leads to Myofibril Disassembly

Kenan K. Turnacioglu,* Balraj Mittal,† Guissou A. Dabiri,* Jean M. Sanger,* and Joseph W. Sanger*‡

*Department of Cell and Developmental Biology and the Pennsylvania Muscle Institute, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19104-6058; and †Department of Genetics, Sanjay Gandhi Institute of Medical Sciences, Lucknow, India

Submitted September 19, 1996; Accepted December 23, 1996
Monitoring Editor: J. Richard McIntosh

Cultures of nonmuscle cells, skeletal myotubes, and cardiomyocytes were transfected with a fusion construct (Z1.1GFP) consisting of a 1.1-kb cDNA (Z1.1) fragment from the Z-band region of titin linked to the cDNA for green fluorescent protein (GFP). The Z1.1 cDNA encodes only 362 amino acids of the approximately 2000 amino acids that make up the Z-band region of titin; nevertheless, the Z1.1GFP fusion protein targets the α-actinin–rich Z-bands of contracting myofibrils in vivo. This fluorescent fusion protein also localizes in the nascent and premyofibrils at the edges of spreading cardiomyocytes. Similarly, in transfected nonmuscle cells, the Z1.1GFP fusion protein localizes to the α-actinin–containing dense bodies of the stress fibers in vivo. A dominant negative phenotype was also observed in living cells expressing high levels of this Z1.1GFP fusion protein, with myofibril disassembly occurring as titin-GFP fragments accumulated. These data indicate that the Z-band region of titin plays an important role in maintaining and organizing the structure of the myofibril. The Z1.1 cDNA was derived from a chicken cardiac λgt11 expression library, screened with a zeugmatin antibody. Recent work has suggested that zeugmatin is actually part of the N-terminal region of the 81-kb titin cDNA. A reverse transcriptase polymerase chain reaction using a primer from the distal end (5′ end) of the Z1.1 zeugmatin cDNA and a primer from the nearest known proximal (3′ end) chicken titin (also called connectin) cDNA resulted in a predicted 0.3-kb polymerase chain reaction product linking the two known chicken titin cDNAs to each other. The linking region had a 79% identity at the amino acid level to human cardiac titin. This result and a Southern blot analysis of chicken genomic DNA hybridized with Z1.1 add further support to our original suggestion that zeugmatin is a proteolytic fragment from the N-terminal region of titin.

INTRODUCTION

The Z-bands of striated muscles anchor the thin filaments of the sarcomere and are also sites of insertion for the two largest muscle proteins: nebulin and titin. These proteins exist as single polypeptides that bind along either the actin filaments (nebulin) or the myosin filaments (titin). Nebulin, a 775-kDa protein with its C-terminal end embedded in the Z-band, binds along the entire 1-μm length of the thin filament (Labeit and Kolmerer, 1995b). Titin, also known as connectin, is the largest polypeptide known with a molecular mass of 3–3.7 megadaltons. Titin is up to 10% of the total protein in skeletal and cardiac muscles,

‡ Corresponding author.

behind only myosin and actin in abundance (for review, see Wang *et al.*, 1979; Fulton and Isaacs, 1991; Maruyama, 1994; Trinick, 1994). A single titin polypeptide with its N-terminal region embedded in the Z-band extends to the M-line of the thick filaments. It is thought that six to eight titin filaments can associate with each half of a thick filament (Granzier and Irving, 1995), centering it in the sarcomere and providing the passive tension of vertebrate muscle (Horowits and Podolsky, 1987). In comparison, there are only two actin filaments per half thick filament in the sarcomere (Huxley, 1972).

The recently sequenced 81-kb cDNA of human cardiac titin (Labeit and Kolmerer, 1995a) codes for a polypeptide of 26,926 amino acid residues with a protein kinase domain, 132 fibronectin type III (FN3)[1] domains (class I motif), and 112 immunoglobulin-like domains (Igs) of the I-set (Harpaz and Chothia, 1994). These three domains are also found in the myosin-binding superfamily, which includes such proteins as projectin (Ayme-Southgate *et al.*, 1991), twitchin (Benian *et al.*, 1989), kettin (Lakey *et al.*, 1993), myosin light chain kinase (Olson *et al.*, 1990), skelemin (Price and Gomer, 1993), and C-protein (Einheber and Fischman, 1990). In titin, the Ig and FN3 domains are each approximately 100 amino acid long and make up 90% of the titin molecule. Each motif forms similar seven-stranded antiparallel β-sheets that fold upon themselves to form a globular 4.0-nm motif in unstrained titin. Unfolding of the parallel β-sheets of these domains (Soteriou *et al.*, 1993; Erickson, 1994) is thought to act together with the proline-glutamine-valine-lysine-rich domain (Labeit and Kolmerer, 1995a) in producing the elasticity of the I-band region of the molecule.

Correlations of the sequence data with antibody localization data have produced a model of the titin molecule in which approximately 2000 amino acids of the N-terminal part of the molecule reside in the Z-band. This segment of titin contains only 10 Ig repeats interspersed with nonrepeating sequences in a pattern similar to the M-band region of titin (Nave *et al.*, 1989; Labeit and Kolmerer, 1995a). The Z- and M-band segments of titin contain the fewest Ig and FN3 domains in the entire molecule.

In a recent study, we isolated a 1.8-kb cDNA from a chicken cardiac expression library (Turnacioglu *et al.*, 1996) by using an anti-zeugmatin antibody (Maher *et al.*, 1985). This chicken cDNA was found to be 60% identical at the amino acid level to a segment of the Z-band region of human cardiac titin. This homology along with Western blot analysis with purified titin suggested that zeugmatin is in fact part of the N-terminal region of chicken titin. In addition, this fragment was also shown to interact with α-actinin (Turnacioglu *et al.*, 1996). A purified bacterially expressed zeugmatin fragment (Z1.1) bound to α-actinin as shown by immunoprecipitation. When expressed in nonmuscle cells, Z1.1 colocalized with the α-actinin in dense bodies of the stress fibers. Furthermore, prolonged expression of the Z1.1 protein in transfected nonmuscle cells resulted in stress fiber disassembly, supporting the evidence of Eilersten *et al.* (1994) that a cellular form of titin is important in assembling and maintaining stress fibers.

In this study, we have further characterized this part of the N-terminal region of titin by examining its expression in cardiac and skeletal muscle cells. Although Z1.1 contains only 19% of the 2000 amino acids in the Z-band region of titin, it was targeted in vivo to the Z-bands of muscle cells that were transfected with a fusion construct of Z1.1 and a sequence encoding green fluorescent protein (GFP). We also show by time-lapse observations of live cells that as high levels of the titin fragment accumulated in a cell, myofibril disassembly occurred. Z-bands were disrupted and A-bands that are bound to the C-terminal region of titin were also disrupted.

MATERIALS AND METHODS

Chicken Cardiac Muscle RNA Extraction and Reverse Transcription- Polymerase Chain Reaction (RT-PCR)

The heart of an adult Leghorn chicken was dissected, flash frozen in liquid nitrogen, and pulverized with a mortar and pestle that was bathed in liquid nitrogen. Total RNA was then extracted with a guanidinium thiocyanate/phenol-chloroform extraction procedure (Chomczynski and Sacchi, 1987). RT-PCRs were done in a single tube as follows. One microgram of total RNA was reverse transcribed at 42°C for 1 h followed by heating at 94°C for 5 min with the antisense primer connI, 5′-TCTGGTGGAATCTAGTTGAG-3′ (Maruyama *et al.*, 1994). Reverse transcription was carried out in a total reaction volume of 20 μl containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 5 mM $MgCl_2$, 40 U of RNasin (Promega, Madison, WI), all four deoxynucleotide triphosphates (each at 50 μM), and 5 units of avian myeloblastosis virus (AMV) RT (Promega). A PCR was then carried out with the sense primer zeug3 (5′-GAGTTCCAAGTAGGGGGCAACCC-3′) and the antisense primer connI. PCRs were carried out in a total volume of 100 μl in 10 mM Tris-HCl (pH 8.3), 50 mM KCl, all four deoxynucleotide triphosphates (each at 0.5 mM), 1.5 mM $MgCl_2$, each primer at 15 μM, and 2.5 U of *Taq* DNA polymerase (Perkin-Elmer Cetus, Norwalk, CT). The first round of PCR was at 94°C for 5 min followed by 35 cycles at 94°C for 30 s, 45°C for 45 s, and 65°C for 2 min. The PCR product was gel purified, subcloned into pcDNA3 (Invitrogen, San Diego, CA), and sequenced on both strands by an automated sequencer (Applied Biosystems, Foster City, CA).

Southern Blot Analysis of Chicken Genomic DNA

High molecular weight genomic DNA from an adult Leghorn chicken heart was extracted using standard methods (Sambrook *et al.*, 1986). Twenty micrograms of DNA were digested separately with *Eco*RI, *Bam*HI, *Hind*III, and *Pst*I overnight at 37°C and sub-

[1] Abbreviations used: cCCD, cooled charge-coupled device; FN3, fibronectin-like type III domain; GFP, green fluorescent protein, Ig, Ig-like domain.

jected to electrophoresis on a 1.0% agarose gel in Tris-acetate-EDTA and transferred to HyBond TM-N nylon membrane (Amersham, Arlington Heights, IL) in 10× standard saline citrate (SSC). A 1.1-kb zeugmatin cDNA fragment, Z1.1 (Turnacioglu *et al.*, 1996), was random prime labeled with ^{32}P and hybridized at 65°C in 5× SSC, 5× Denhardt's, 0.1% SDS, and 100 μg/ml salmon sperm DNA. After a quick rinse of the filters in a 2× SSC and 0.1% SDS at room temperature, the filters were washed in 2× SSC and 0.1% SDS at room temperature for 30 min, followed by a wash in 0.2× SSC and 0.1% SDS at 65°C for 30 min, and autoradiographed.

Culturing and Staining of Cells

PtK2 cells, from a rat kangaroo epithelial cell line (American Tissue Type Collection, Rockville, MD), were grown on glass bottom dishes (MatTek, Ashland, MA) in Eagle's minimal medium supplemented with 10% fetal bovine serum and 1.5% glutamine (Life Technologies, Gaithersburg, MD) as described previously (Sanger *et al.*, 1983; Danowski *et al.*, 1992). Cardiac myocytes and skeletal myotubes were isolated from 5- to 6-d-old and 11- to 12-d-old chick embryos, respectively (Sanger *et al.*, 1984, 1986), and were grown on glass-bottomed dishes with collagen as a substrate for the myotubes. Cardiomyocytes were fixed and stained with a muscle-specific myosin II antibody as described previously (Rhee *et al.*, 1994), rhodamine-phalloidin (Molecular Probes, Eugene OR), or a sarcomeric α-actinin antibody (Sigma, St. Louis, MO).

Transfection, Microinjection, and Observation of Cells

A 1.1-kb zeugmatin cDNA was subcloned into the pEGFP-N1 plasmid (Clontech, Palo Alto, CA), resulting in the Z1.1GFP construct. Plasmid DNA was purified with a Qiagen column (Qiagen, Chatsworth, CA) and transfected into cells 24 h after subculturing of PtK2 or 48 h after isolating primary chicken cardiac cells. Ten microliters of Lipofectamine (Life Technologies) and 1 μg of DNA were used per 35-mm glass-bottomed Petri dish. The DNA and Lipofectamine mixture were added to OPTI-MEM (Life Technologies) serum-free medium, placed over the cells for 12 h, then removed, and replaced with the standard medium for each cell type. For microinjection, the Z1.1GFP plasmid was diluted to 0.1 μg/μl in water and microinjected into cultured chicken myotubes. All cells were observed in glass-bottomed dishes under 5% CO_2 with a Nikon Diaphot 200 microscope with a phase-contrast 100× objective. Images were acquired with a liquid-cooled charge-coupled device (cCCD) camera (Photometrics, Tuscon, AZ) and processed with a Metamorph image processing system (Universal Imaging, West Chester, PA).

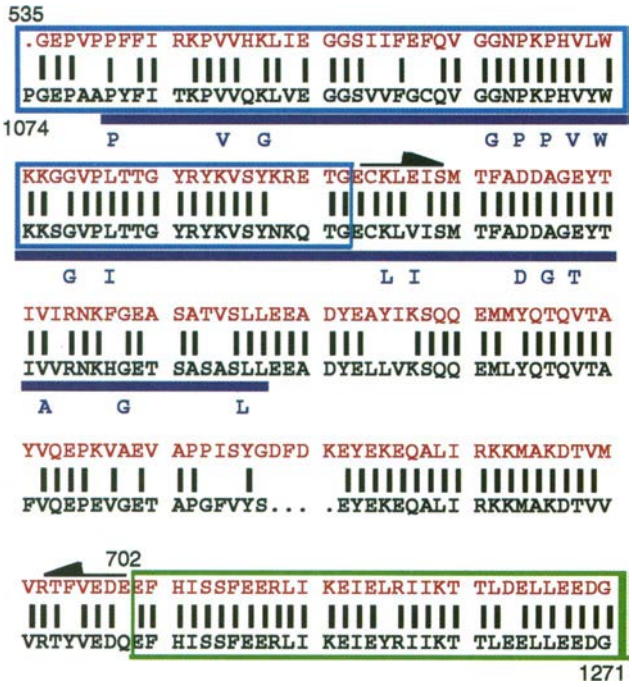

Figure 1. Linking of zeugmatin Z1.8 cDNA (GenBank accession number U51476) with the chicken connectin I cDNA (GenBank accession number D16844). Predicted amino acid sequence of a segment of the N-terminal region of human cardiac titin cDNA (Labeit and Kolmerer, 1995a) is shown in black starting at position 1074, and the chicken cardiac titin cDNA is shown in red. The C-terminal segment of Z1.8 (blue box) and the N-terminal part of connectin I (green box) are aligned with the human sequence. Arrowheads define the 0.32-kb RT-PCR product that fills the gap between Z1.8 (Turnacioglu *et al.*, 1996) and connectin I (Maruyama *et al.*, 1994) sequences. This spanning region is 79% identical, with one gap, to the human sequence. The underlined segment denotes an Ig domain. The most commonly conserved amino acids, those that occur in 6 of the 10 human Z-band cardiac Ig domains, are indicated in blue letters. Numbers indicate the amino acid position of human and chicken titin sequences. The sequence data of the 0.32-kb segment are available from GenBank under accession number U64829.

RESULTS

Linkage of Zeugmatin and Connectin I

To extend prior evidence that zeugmatin is a proteolytic fragment of the N-terminal region of titin and not a novel Z-band protein (Turnacioglu *et al.*, 1996), we proceeded to connect the 1.8-kb zeugmatin cDNA (Z1.8) to the 3.3-kb connectin I (Maruyama *et al.*, 1994), the chicken titin sequence with the highest homology to the N terminus of the human titin. A comparison of these chicken sequences with the full-length human titin sequence (Labeit and Kolmerer, 1995a) predicted a gap of 320 nucleotides between the two chicken titin sequences (Figure 1). A RT-PCR with total chicken heart RNA, a sense primer from the 3′ end of the Z1.8, and an antisense primer from the 5′ end of the connectin I sequence resulted in a 0.32-kb product (Figure 1). This 0.32-kb sequence has a 79% identity at the amino acid level, with one gap, to a Z-band region of the human titin sequence connecting two segments that are homologous to the chicken titin sequences. The 0.32-kb spanner region also contains the remainder of an Ig domain (Figure 1, underlined in blue) that begins in the Z1.8 sequence. The 19 amino acids that are most frequently conserved in the 10 Ig motifs of the Z-band are shown in Figure 1 in blue.

A Southern blot analysis of chicken genomic DNA, hybridized with a ^{32}P-labeled 1.1-kb zeugmatin cDNA fragment (Figure 2), revealed only two bands in each lane after autoradiography. This simple banding pattern suggests that zeugmatin is most likely the product of a single gene. Combined with the high sequence homology of zeugmatin to titin and the presence of

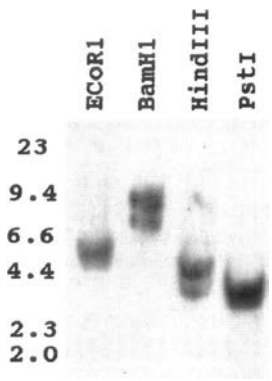

Figure 2. Southern blot analysis of digested chicken cardiac genomic DNA. Genomic DNA was isolated and digested with restriction enzymes shown above, electrophoresed on a 1.0% agarose gel, and transferred to Hybond nitrocellulose. The blot was hybridized to a 1.1-kb cDNA derived from an anti-zeugmatin antibody screening of a cardiac chicken expression library. The simple banding pattern suggests that zeugmatin is probably the product of one gene.

only one titin gene in human (Labeit and Kolmerer, 1995a), it is inferred that zeugmatin is not a novel protein but rather part of the N-terminal region of titin.

Expression of the Z1.1GFP Fusion Protein in Nonmuscle Cells

The 1.1-kb cDNA was subcloned into a GFP expression plasmid, pEGFP-N1, and transfected into nonmuscle cells. After 24–48 h, the fluorescence in expressing cells ranged from dim to intensely bright. In the dim cells, the punctate fluorescence of the Z1.1GFP fusion protein was localized in the dense bodies and adhesion plaques of PtK2 cells (Figure 3). This pattern was not visible in the very bright cells because intense fluorescence obscured any subcellular localization. The fluorescent Z1.1GFP fusion protein is located, almost exclusively, in the α-actinin containing dense bodies, the adhesion plaques, and the cell junctions of the transfected PtK2 cells. The spacings between the dense bodies were 1.2 μm on average, which is within the range of dense body spacings in epithelial cells (Sanger *et al.*, 1986).

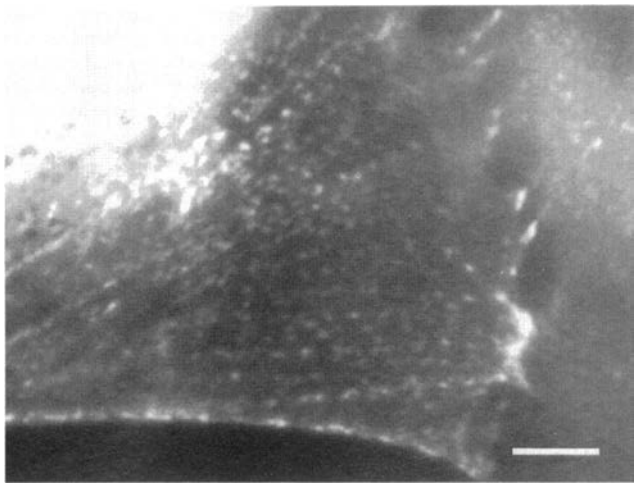

Figure 3. Fluorescent image of a live PtK2 cell transfected 48 h previously with the Z1.1GFP plasmid (a fusion construct of a 1.1-kb zeugmatin cDNA sequence with GFP). The focus is on the distal regions of the cell where the fluorescent Z1.1GFP is more easily resolved than in the thicker intensely fluorescent perinuclear region. The fluorescence corresponds to the dense bodies of the stress fibers. Bar, 5 μm.

Expression of the Z1.1GFP Fusion Protein in Muscle Cells

Within 24 h after transfection of an actively contracting chick embryonic cardiomyocyte that had been in culture for 6 d, all of the Z-bands were fluorescent (Figures 4A and 5). In contrast to the nonmuscle cells, the expression of the Z1.1GFP protein was much easier to discern in myocytes probably, in part, due to the large Z-bands of muscle cells that provide a greater volume for the incorporation of the fusion protein than the small dense bodies of nonmuscle cells. Because of the high intensity of Z-band fluorescence, the exposure times were short enough to capture the myocyte between contractions. When transfected cells were fixed and stained with a sarcomeric α-actinin antibody, the staining colocalized with the GFP fluorescence in the Z-bands (Figure 5). In actively spreading chick embryonic myocytes such as the one shown 3 d after isolation (Figure 4B), Z1.1 GFP fluorescence localized to centrally positioned Z-bands (arrows) spaced 2.1 μm apart and to punctate arrays (arrowheads) at the cell periphery that were spaced about 1.0 μm apart. The small size of these punctate arrays and their spacing suggest that they delineate the Z-bodies of pre- and nascent myofibrils forming at the spreading edge of the cardiomyocyte (Rhee *et al.*, 1994).

Localization of the Z1.1GFP protein product to the Z-bands was also observed when cultured embryonic chick myotubes were directly microinjected with Z1.1GFP plasmid (Figure 6). Fluorescence in these cells was evident just 8 h after microinjection. The fluorescent spacings were 2.1 μm, corresponding to the sarcomere length of the myotubes. As in our earlier transfection experiments, the intensity of fluorescence varied from cell to cell indicating a variable amount of expressed Z1.1GFP. The presence of the fluorescent truncated titin in these Z-bands at these levels of expression did not inhibit the spontaneous contractions normally observed in these cultured myotubes.

Time-Lapse Observation of Z1.1GFP Localization in Phase-Dense Z-Bands

Z1.1GFP expression could be followed in the same cell over several days to track changes in the Z-bands. Figure 7 shows a cardiac myocyte expressing Z1.1 on d 3 in culture (Figure 7, A, C, and E) and the same

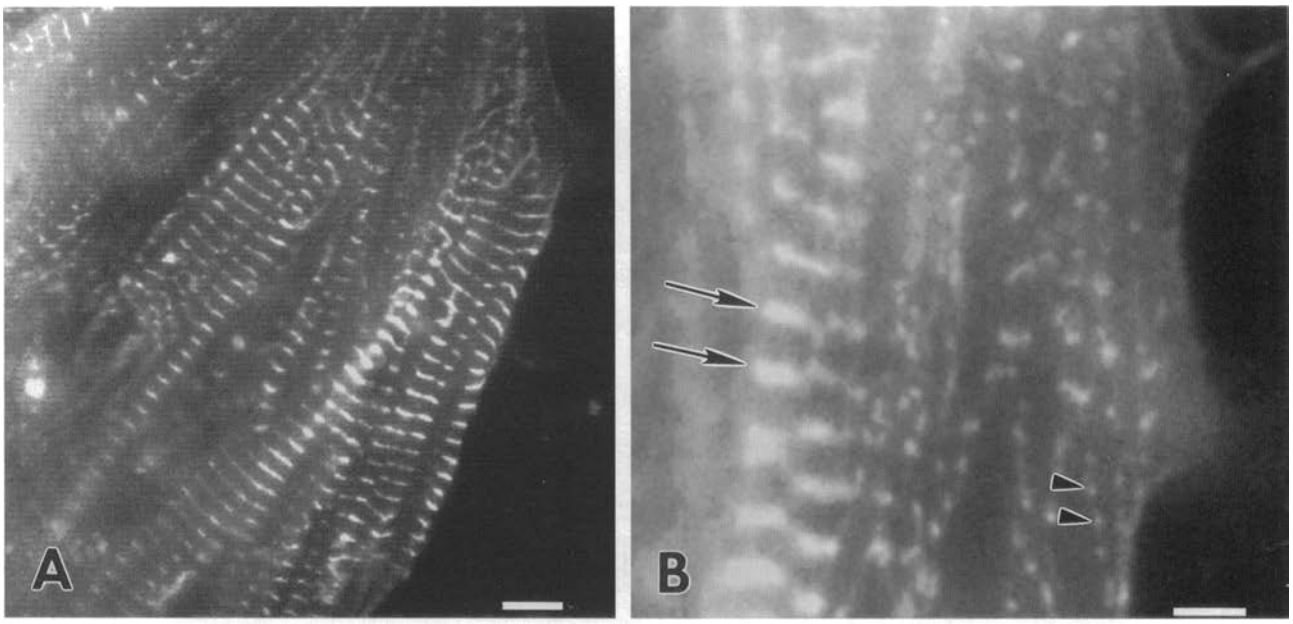

Figure 4. Fluorescent image of live embryonic chicken cardiac muscle cells in culture transfected with Z1.1GFP. (A) The fusion protein localizes to the Z-bands of contracting myofibrils. (B) The fusion protein localizes to the mature myofibrils with an average spacing of 2.1 μm and to the Z-bodies at the edge of the spreading cell with an average spacing of 1.2 μm. Bars: A, 5 μm and B, 2 μm.

region of the myocyte again 14 h later (Figure 7, B, D, and F). In this cell, the Z-bands were also visible with phase-contrast optics. As expected, the fluorescence of the fusion protein was localized to the Z-bands (2.4-μm average spacings) and also to the much thinner Z-bodies or dense bodies (1.2-μm average spacings; Figure 7, A and B).

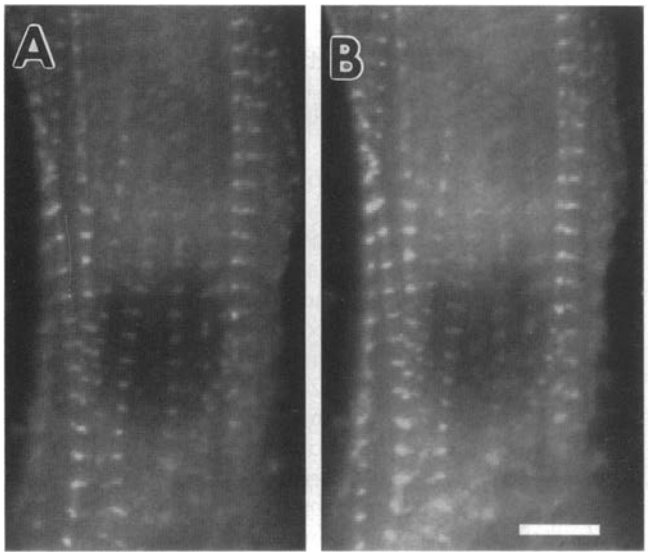

Figure 5. Fluorescent image of a cardiomyocytes expressing Z1.1GFP (A) that was later fixed and stained with a sarcomeric α-actinin antibody (B). The Z1.1GFP fusion protein localizes to the Z-bands that are rich in α-actinin. Bar, 10 μm.

Over the 14-h observation period, the Z-bands became more compact (compare Figure 7, A and B) and less punctate in appearance. This compaction of Z-bands over several hours was observed in other transfected cardiac myocytes as well, suggesting that Z-bodies may be fusing laterally to form larger Z-bands. Z-body fusion was previously observed in cardiac myocytes microinjected with α-actinin (Sanger *et al.*, 1984). At both time points shown in Figure 7, the

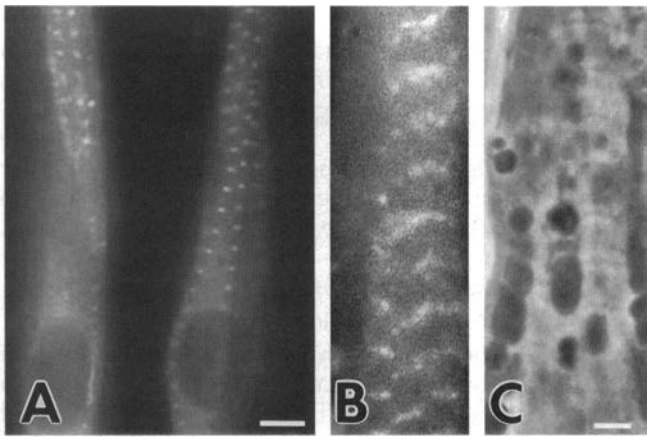

Figure 6. Fluorescent image of an embryonic chicken skeletal myotube in culture microinjected with the Z1.1GFP construct. (A) The Z1.1GFP is localized at the Z-bands. (B) In a myotube shown at higher magnification, the Z1.1GFP protein localizes to the Z-bands seen in the phase-contrast micrograph (C). Bars: A, 5 μm and B and C, 2 μm.

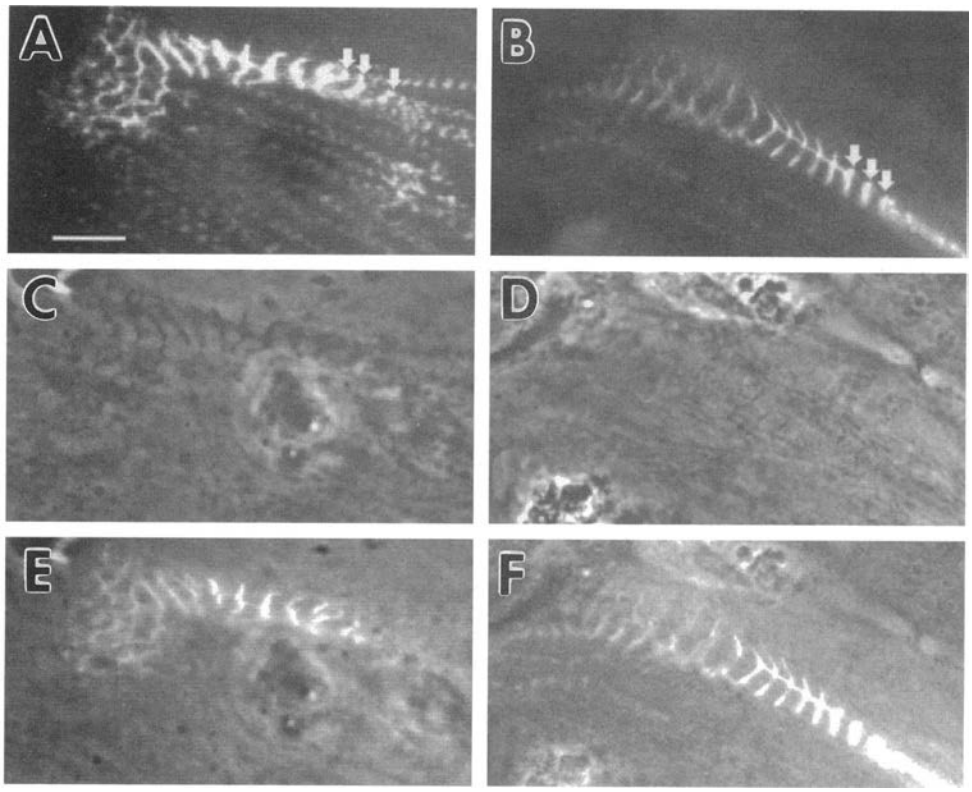

Figure 7. Region of a contracting cardiomyocyte from a culture of embryonic chicken cardiac cells transfected with the Z1.1GFP construct. The micrographs on the right (B, D, and F) were taken 14 h after those on the left (A, C, and E). (A and B) Fluorescent images showing the Z1.1GFP fusion protein incorporating into Z-bands and Z-bodies (arrows). Most of the Z-bodies have disassembled in B, but the Z-bands are more compact and ordered. The granular appearance of the Z-bands in A become smooth, and more compact Z-bands in B. (C and D) Phase micrographs of the cell showing the Z-bands. (E and F) Summation of images in A and C and in B and D, respectively, demonstrating Z1.1GFP protein incorporation in the phase-dense Z-bands. Bar, 10 μm.

prominent myofibrils in this myocyte were contracting regularly. In cells with low levels of background fluorescence (Figure 7, A and B), the Z-bands of the mature sarcomeres remained stable. However, with increasing time after transfection, the small Z-bodies in forming myofibrils (Figure 7A) were greatly reduced in number without being replaced by mature myofibrils (Figure 7B), suggesting that the smaller Z-bodies of pre- and nascent myofibrils are easier to disrupt than the larger Z-bands of mature myofibrils.

Effects of Various Levels of Z1.1GFP Fusion Protein Expression on the Integrity of the Z-Band

To examine the effects of high levels of expression of Z1.1GFP fusion protein, cardiac myocytes with high levels of unincorporated fluorescence were followed for several days and contrasted with cells that had reduced levels of background fluorescence. Figure 8 shows two embryonic cardiac myocytes in culture that were transfected with Z1.1GFP 6 d after isolation. The culture dish was placed under circulating 5% CO_2 on the microscope stage and followed for several days with an image acquired every 2 h. Both cardiac myocytes were actively contracting at the initial observation. In more than 36 h of observation, one myocyte was essentially unchanged and continued to contract regularly (Figure 8, A and B). Note the low level of unincorporated or background fluorescence in this myocyte. In contrast, the myocyte with a high level of background fluorescence became almost completely depleted of muscle and ceased beating after 12 h of observation (Figure 8, C and D).

Z1.1GFP always localized to the Z-bands but its appearance in the cells occurred in one of two distinct patterns: static low level and continuously accumulating. The continuously accumulating pattern of expression always resulted in a dominant negative phenotype (i.e., myofibril disassembly), whereas the static low level of expression resulted in myofibrils that were unperturbed and continued to contract unaffected by the Z-band incorporation of Z1.1GFP. Increased levels of expression of Z1.1 GFP results in increased levels of fluorescence in transfected cells, allowing cells with different levels of Z1.1GFP to be

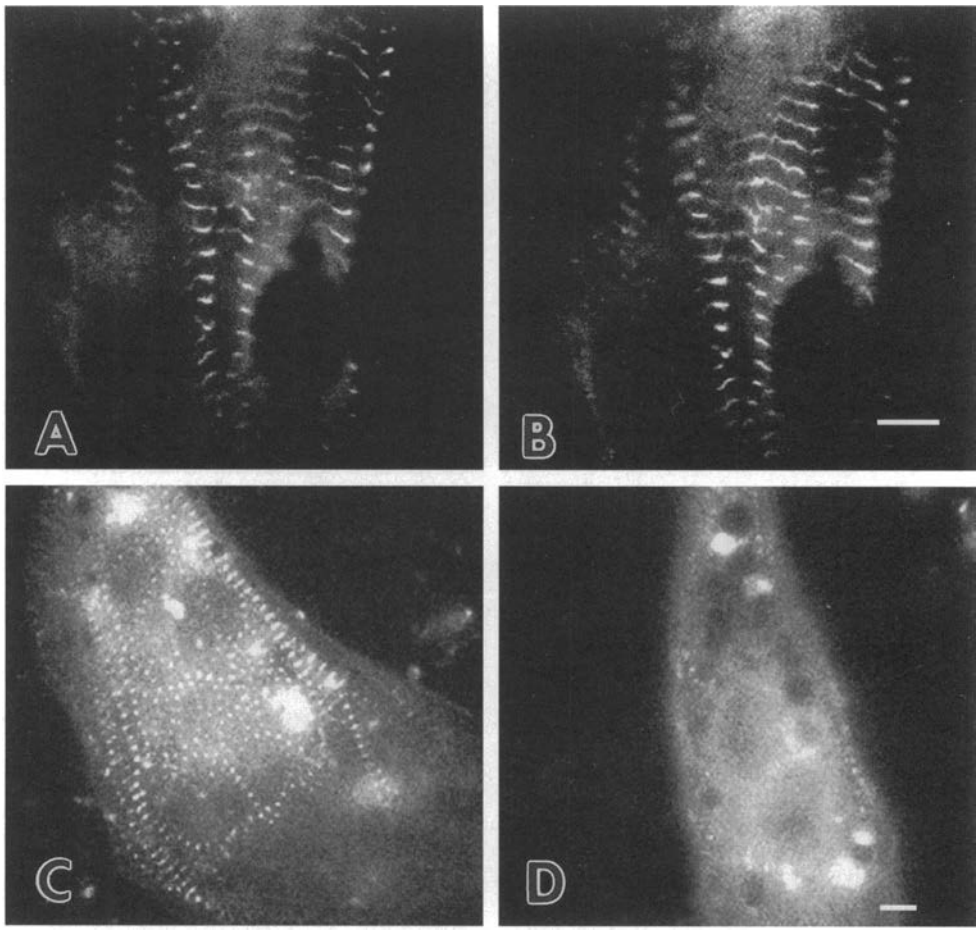

Figure 8. Fluorescent images of two embryonic chicken cardiac muscle cells transfected with Z1.1GFP and followed for several days. A beating cardiac cell in A is shown 36 h later in B still contracting with intact Z-bands. (C) Another cardiac cell with a high level of background fluorescence shows a depletion of Z-bands 12 h later (D), and the cell has begun to collapse. Our interpretation is that a high level of background fluorescence is indicative of a high level of Z1.1GFP fusion protein expression and results in the collapse of Z-bands. This Z-band depletion and cell collapse were not observed in A and B where the level of background fluorescence is much lower. Bars: A and B, 5 μm and C and D, 2 μm.

compared. With high levels of expression, fluorescence is present not only in the Z-bands but also throughout the cytoplasm as excess Z1.1GFP accumulates. To compare the levels of expression in different cells, we measured the intensity of fluorescence in the Z-band and in the cytoplasm between the Z-bands. The ratio of Z-band fluorescence to cytoplasmic fluorescence remained level over several days in the cells with low levels of fluorescence, whereas in the cells continuously accumulating Z1.1GFP, this ratio decreased with time (Figure 9). In a cardiomyocyte with no discernible loss of Z-bands (Figure 8, A and B), the Z-band fluorescence remained about threefold greater than the static low level of background fluorescence (Figure 9, open squares). In a cardiomyocyte with a loss of Z-bands (Figure 8, C and D), the background intensity rose to levels approximating the Z-band intensity (Figure 9, solid squares). Observations on other transfected cardiomyocytes always supported this correlation: myofibril diassembly is accompanied by high levels of background fluorescence. Fourteen transfected cardiomyocytes were subjected to extended time-lapse observation of at least 36 h. Ten cells exhibited a continously accumulating pattern of Z1.1GFP expression with the concomitant disassembly of Z-bands and myofibrils. Four cells had no significant increase in background fluorescence, with Z-bands remaining fluorescently labeled yet well organized.

Another cardiomyocyte that expressed high levels of Z1.1GFP fusion protein (Figure 10A) was still beating with only the remnants of a myofibril (Figure 10B, area between the arrowheads); however, as the level of background fluorescence increased, the cardiomyocyte became rounder and was no longer contractile (Figure 10C).

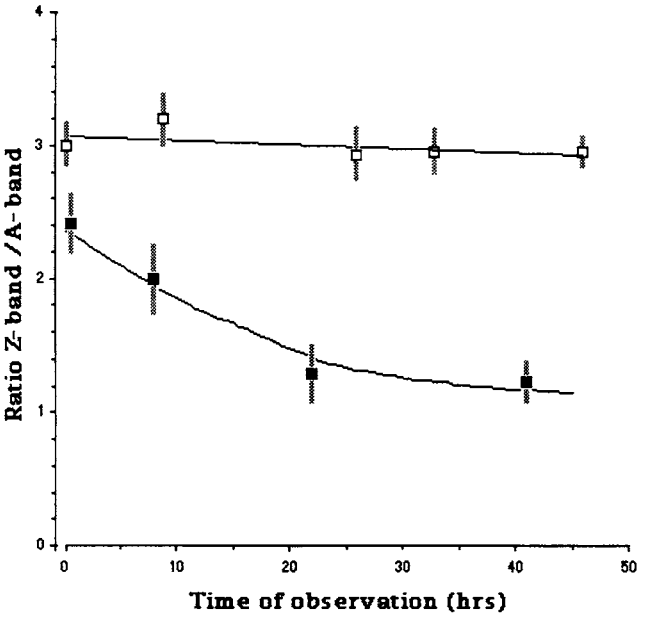

Figure 9. Graph of ratios of Z-band fluorescence intensities to A-band intensities plotted versus times of observation for the two cardiomyocytes shown in Figure 8. The pixel intensity of the same Z-band and region between the Z-bands (A-band region) was measured, after background subtraction, in cCCD images that were acquired every 2 h. The cardiomyocyte in Figure 8, A and B, that showed no loss of Z-bands or contractility over 50 h of observation yielded a linear plot that showed no increase in background fluorescence (small diamonds). The cardiomyocyte in Figure 8, C and D, experienced Z-band disassembly concomitant with a rise in background fluorescence. This yielded a tapering curve plot (large boxes) as background fluorescence rose to Z-band levels. Four pairs of pixel intensities were acquired for each time points shown and the gray bars are equal to 1 SD unit.

Evidence that intermittent imaging at 2-h intervals is not harmful to cells is provided by cells followed for more than 36 h of observation with no loss of Z-bands. To demonstrate that our observations were not due to the presence of GFP, cells were transfected with a plasmid expressing GFP only. Cardiomyocytes expressing GFP alone became intensely fluorescent yet continued to contract as it was intermittently observed over a 48-h period. The cell was then fixed, stained with rhodamine-conjugated phalloidin, and revealed well-maintained myofibrils. The cardiomyocyte was transfected with GFP 48 h after their initial observation (Figure 11).

A muscle-specific myosin II antibody was used to stain the A-bands in cardiac myocytes expressing different levels of Z1.1GFP fusion protein (Figure 12). When the level of fusion protein was low, as in the cardiac myocyte shown in Figure 12A at the lower center, discrete 1.6-μm A-bands were localized between the thin fluorescent Z-bands that were spaced at 2.5-μm intervals along the myofibrils (Figure 12B). However, when a cardiac myocyte expressed high levels of Z1.1GFP and, therefore, became intensely fluorescent (Figure 12A), the A-bands were disrupted (Figure 12B). Linear arrays of Z-bands were irregularly spaced at distances closer than 2.5 μm apart, with the Z-bands narrow and sometimes bent. Single isolated Z-bands also could be detected in some of the overexpressing cells (Figure 12A, inset). In regions where the Z-bands were scattered, A-bands were not evident (Figure 12B).

DISCUSSION

In this article, we have shown that a small portion of the Z-band region of titin (Z1.1) by itself is able to target to the Z-bands of live cardiac and skeletal muscle cells where intact endogenous titin is found. This titin fragment coupled to GFP was used to examine the role of the Z-band portion of titin in myofibrillogenesis. Overexpression of the titin fragment leads to sarcomere disassembly, suggesting that titin is important in the assembly and maintenance of myofibril structure.

Titin-GFP Fragment Localizes To Z-bands In Vivo

Our in vivo data showed that the Z1.1 protein product, fused at its C terminus to the 27-kDa GFP, was able to localize to α-actinin–rich structures in nonmuscle cells as the untagged Z1.1 had localized in cultured PtK2 and REF-52 cells (Turnacioglu *et al.*, 1996). In cardiomyocytes transfected after 3 d in culture with Z1.1GFP, the fluorescent Z-bands often appeared to be composed of aggregates of Z-bodies that changed to become smooth compact Z-bands at later time points (Figure 6). Every Z-band within the contracting myocytes and the Z-bodies of the pre- and nascent myofibrils found at the edges of the cardiomyocytes incorporated the fluorescent fusion protein. The results also indicate that our GFP fusion construct that contains only 19% of the 2000 amino acids in the Z-band region of titin and only 1.3% of the total amino acids in titin is able to localize to the Z-band where the N-terminal region of full-length titin inserts.

There was a direct correlation between the levels of Z1.1GFP expression and the integrity of Z-bands. When the level of fusion protein expression was low, as determined by a low level of unincorporated fluorescence in the cytoplasm, the Z-bands were stable for many days. The Z-bodies found at the periphery of spreading cardiomyocytes often disassembled when Z-bands in the same cell remained intact (Figure 7). In myocytes with high levels of expression (i.e., a high level of unincorporated fluorescence), a dominant negative phenotype of Z-band disassembly and loss of myofibril contractility occurred within hours (Figures 8, C and D, 10, and 12). One explanation for this phenotype may be that the Z1.1 titin fragment com-

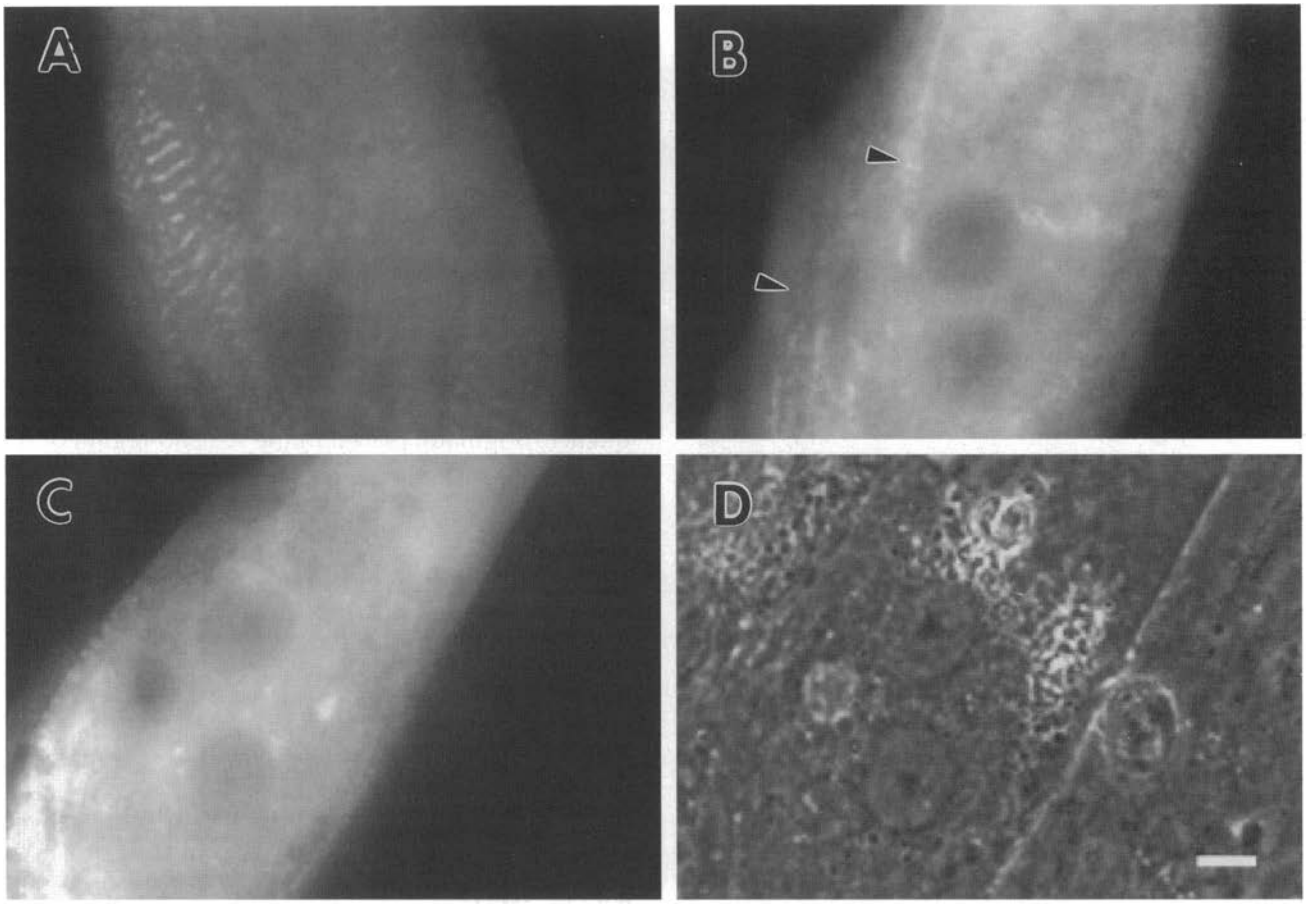

Figure 10. Fluorescent images of an embryonic chicken cardiac muscle cell transfected with Z1.1GFP and observed 24 h after transfection. (A) The cell has a high level of Z1.1GFP fusion protein expression as determined by the high level of background fluorescence, yet the intensely fluorescent Z-bands are visible. (B) At 12 h, The Z-bands are diminished with a few remnants of Z-bands (arrowheads), and the background fluorescence has increased further as Z1.1GFP fusion protein continues to be expressed. (C) By 24 h, the cell has only a few Z-bands visible. (D) A phase micrograph of the cell shown at 24 h. Bar, 5 μm.

petes for the Z-band-binding sites of the native titin thereby inhibiting native titin from stabilizing Z-bands and anchoring myosin in the sarcomeres. It is also possible that the 1.1-kb titin fragment binds α-actinin, and perhaps excess titin fragments may interfere with the normal function of endogenous α-actinin in the Z-bands. The A-bands in overexpressing cells disassemble when the Z-bands become disordered (Figure 12). This occurs despite the fact that myosin-binding proteins are presumably present and thought to help confer order on the myosin filaments aligned in the A-band (Gilbert *et al.*, 1996). In any case, these observations strongly suggest that anchorage of full-length titin into the Z-band is essential for the stabilization and maintenance of the sarcomere.

Transfection of cells with GFP-tagged contractile proteins has some marked advantages over the microinjection of fluorescently labeled proteins. Driven by a constitutive promoter, the GFP fusion protein is continually expressed, and thus, the GFP-tagged protein that is degraded or turned over is renewed with an undiminished level of signal. In addition, the fluorescent part of the GFP is in the interior of the protein and thus protected from the bleaching effects due to oxygen and free radicals. Proteins that are directly labeled and microinjected gradually lose their fluorescence intensity after repeated viewing or after extended periods of time in the cell. However, the disadvantage of the GFP-proteins is that the amount of probe in a cell varies from cell to cell and cannot be regulated or measured, which is possible to do with microinjecion of fluorescently labeled proteins. Another potential disadvantage of the GFP fusion protein is the size of the probe (27 kDa), compared with the size of the dye used on labeled proteins (about 500 Da). The large size of GFP could potentially interfere with the normal function of the protein of study. However, this did not pose a problem in our study as the Z1.1GFP was able to localize to all the sites of α-actinin concentration in nonmuscle cells and muscle cells. Clearly, the two

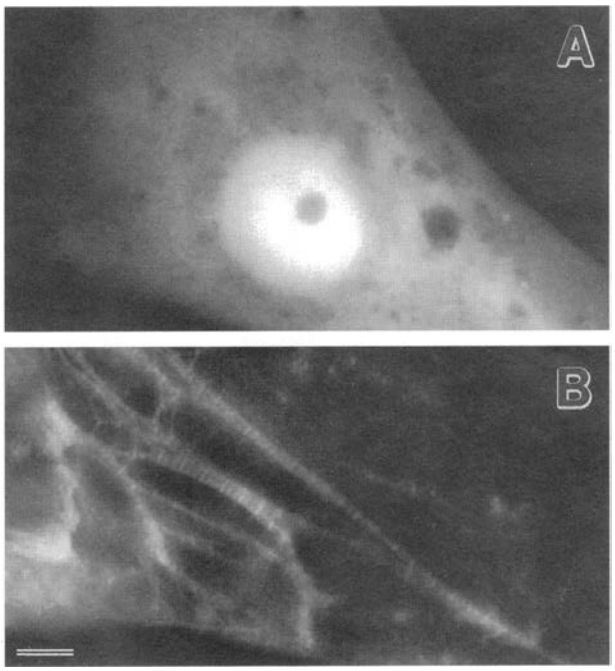

Figure 11. Fluorescent image of a cardiomyocyte expressing just GFP alone (A). The cell remained intensely fluorescent and contractile over 48 h of observation. The cell was then fixed and stained with rhodamine-coupled phalloidin (B) to reveal well-organized myofibrils in the presence of GFP. Bar, 5 μm.

methods do not have to be mutually exclusive as cells transfected with a GFP-tagged protein could be selected and microinjected with a fluorescently labeled protein to follow the distribution of two different cytoskeletal proteins.

Additional Sequence Data Support Our Conclusion That Zeugmatin Is Part of Titin

A comparison of the 1.8-kb chicken zeugmatin cDNA (Turnacioglu *et al.*, 1996) with the human cardiac titin sequence showed zeugmatin to have a 60% identity at the amino acid level with the human titin. By homology with the human titin (Figure 13), the 1.8-kb zeugmatin cDNA is 101 amino acids upstream from a 3.3-kb connectin I cDNA derived from chicken cardiac muscle (Maruyama *et al.*, 1994). The connectin I sequence, originally placed in the A-band region of titin (Maruyama *et al.*, 1994), has a 79% identity at the amino acid level with the segment of human titin that extends from the middle of the Z-band just into the I-band region. The lower identity of 1.8-kb zeugmatin cDNA is probably due to the absence of the multiple Ig motifs that dominate the Z-band region of human titin. By using RT-PCR we obtained a 0.32-kb sequence derived from chicken cardiac RNA (Figure 1, between the arrows) that connects zeugmatin and connectin I. This piece contains the remainder of an Ig domain that begins in the 1.8-kb zeugmatin sequence. At the amino acid level, it has a 79% identity to the corresponding human titin sequence and spans the region between the fourth and fifth Ig domains in human titin. With linkage of the zeugmatin 1.8-kb cDNA to connectin I 3.3-kb cDNA, we are now able to report a 5.4-kb

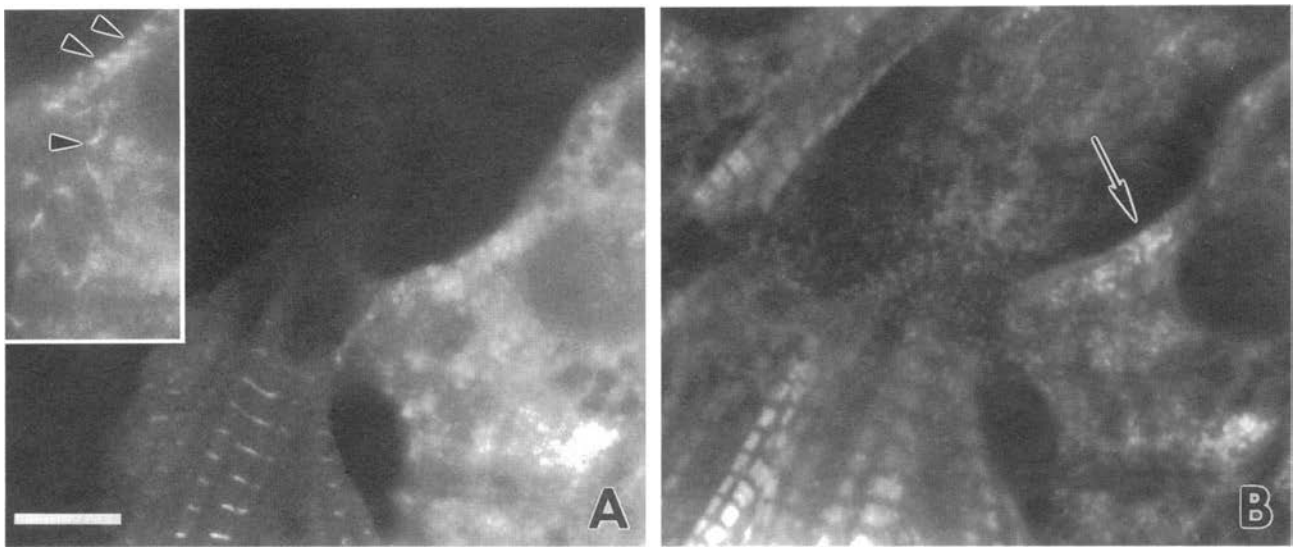

Figure 12. Two cardiomyocytes expressing Z1.1GFP. (A) The intensity of GFP fluorescence indicates a moderate level of expression in the middle cell and high levels of expression in the cell on the right, a portion of which is shown at a different focal plane in the Inset. The Z-bands are well-ordered in the middle cell and disordered in the other cell (inset, arrowheads). Staining with muscle-specific myosin antibodies (B) shows aligned A-bands in the middle cell and in an untransfected cell in the upper left. Only A-band remnants (arrows) are present in the Z1.1GFP-overexpressing cell. Bar, 10 μm.

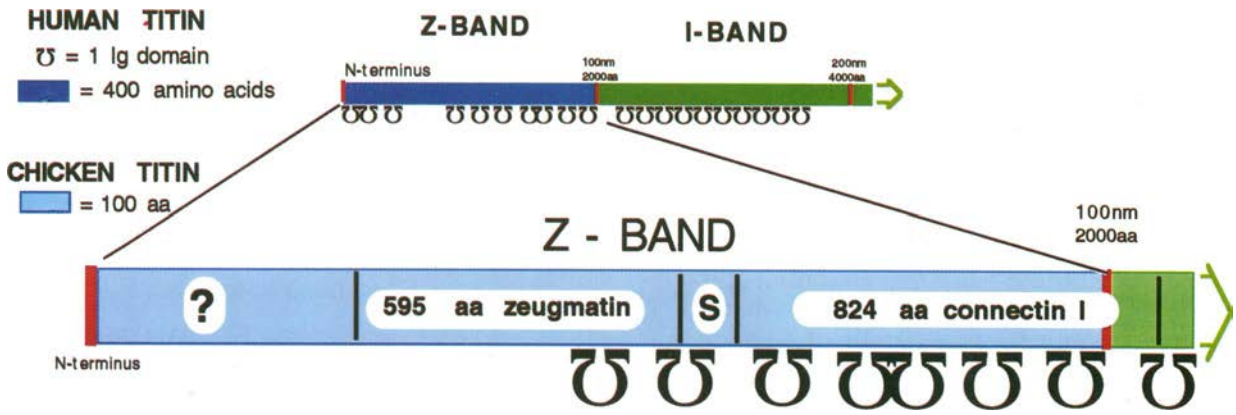

Figure 13. Schematic diagram of the N-terminal 200-nm region of the titin molecule contrasting the fully sequenced human titin with three chicken titin clones shown below it: a 1.8-kb zeugmatin cDNA, a 3.3-kb chicken connectin I sequence, and a 0.3-kb spanner region (S) derived from a RT-PCR with primers in the flanking sequences. The probable Ig domains in titin are shown below the sequence of chicken titin. The region of chicken titin indicated by the question mark (?) has not been isolated but is predicted to be very similar to the most N-terminal region of the human titin.

continuous sequence of chicken cardiac titin with high homology to human titin.

Using Southern blot hybridization of chicken Z1.1 to chicken genomic DNA, we showed that zeugmatin is most likely the product of one gene. The Southern blot data and sequence homology plus the previously reported Western blot demonstrating cross-reactivity of zeugmatin antibodies against titin (Turnacioglu *et al.*, 1996) support our conclusion that zeugmatin is a fragment of titin.

Interestingly, in chicken gizzard, a good source for smooth muscle cells and fibroblasts, we were not able to detect titin in immunoblots with zeugmatin antibody (our unpublished results) or by RT-PCR using primers from the Z1.1 region. It is therefore possible that either a different form of titin is present in the smooth muscle and fibroblasts or the actin-myosin structures are stabilized by another protein.

Implications for Myofibrillogenesis

In a study of spreading cardiac myocytes (Rhee *et al.*, 1994; Turnacioglu *et al.*, 1997; L. Russo *et al.*, 1997), three morphological stages were observed as myofibrils were being assembled: premyofibril, nascent myofibril, and mature myofibril stages. Premyofibrils formed at the edges of spreading cardiomyocytes and contained closely spaced punctate dense bodies of α-actinin (Z-bodies). Titin and zeugmatin were absent from premyofibrils but were first detected by immunofluorescence in the Z-bodies of the nascent myofibrils. It was proposed (Sanger *et al.*, 1984; Rhee *et al.*, 1994) that the Z-bodies of the nascent myofibrils aligned and fused to form the beaded Z-bands of mature myofibrils. Since zeugmatin first localized to the Z-bodies of the nascent myofibrils, it was suggested that this molecule was responsible for joining the α-actinin containing Z-bodies together (Rhee *et al.*, 1994). In the mature myofibrils, the fused Z-bodies were transformed into smooth solid Z-bands, a process that may also require titin.

In contrast to the immunofluorescence studies showing that titin and zeugmatin are absent in the premyofibril, the present study shows that in cells transfected with Z1.1GFP, this truncated titin protein becomes localized in premyofibrils presumably due to the ability of the Z1.1 fragment to bind α-actinin (Turnacioglu *et al.*, 1996). Normally, the large size of the intact titin molecule (3000 kDa) may prevent it from reaching the outer edges of the spreading cardiomyocytes where the premyofibrils are formed.

Titin's role in myofibrillogenesis must involve its two different reactive ends: the 100-nm-long N-terminal Z-band-binding region with α-actinin-binding properties that includes the Z1.1 protein product and the 800-nm-long A-band- binding region with myosin-binding properties. Both ends of titin that interact with the muscle ultrastructure at the M-band and Z-band, respectively, contain about 2000 amino acids with the same arrangement of Ig domains and interdomains (Labeit and Kolmerer, 1995a). Titin is one of the first muscle-specific proteins to acquire a striated pattern in myogenesis (Tokuyasu and Maher, 1987a,b; Furst *et al.*, 1989, Isaacs *et al.*, 1989; Terai *et al.*, 1989; Colley *et al.*, 1990). The 43-nm spacing of the super-repeat region of titin and the associated M-line proteins may play a role in the assembly of the thick myosin filaments (Furst *et al.*, 1989; Trinick, 1994) and full-length titin may pull these filaments into register with the developing Z-bands (Hill *et al.*, 1986; Rhee *et al.*, 1994). Overexpression of the titin fragment resulting in myo-

fibril disassembly supports the hypothesis that intact titin molecules are essential for the assembly and stability of the myofibrils. In nonmuscle cells, overexpression of the titin fragment causes stress fiber collapse, which may support the conclusion made by Eilersten *et al.* (1994) that cellular titin is involved in stabilizing the sarcomeric arrangement of actin, myosin, and α-actinin in stress fibers.

Future work will be needed to demonstrate how titin, in particular the Z-band region of titin, is able to insert into and around the Z-bodies and what role it plays in transforming the fused group of Z-bodies into a smooth solid Z-band as found in mature sarcomeres.

ACKNOWLEDGMENTS

We wish to thank Tracey Hall for her technical assistance in cell culture and Dr. Jeffrey Field for his advice regarding GFP. This work was supported by grants form the Muscular Dystrophy Association (J.W.S.) and from the National Institutes of Health (HL-48954 to J.W.S. and J.M.S.). K.K.T. was supported by a predoctoral fellowship from the American Heart Association. B.M. was a Visiting Scientist partially supported by the Pennsylvania Muscle Institute and a Sanjay Gandhi Post-Graduate Institute of Medical Science intramural grant. G.A.D. was supported by a postdoctoral fellowship from the American Cancer Society.

REFERENCES

Ayme-Southgate, A., Vigoreaux, J., Benian, G.M., and Pardue, M.L. (1991). *Drosophila* has a twitchin/titin-related gene that appears to encode projectin. Proc. Natl. Acad. Sci. USA *88*, 7973–7977.

Benian, G.M., Kiff, J.E., Neckleman, N., Moerman, D.G., and Waterson, R.H. (1989). Sequence of an unusually large protein implicated in regulation of myosin activity in *C. elegans*. Nature *313*, 545–551.

Chomczynski, P., and Sacchi, N. (1987). Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem. *162*, 156–159.

Colley, N.J., Tokuyasu, K.T., and Singer, S.J. (1990). The early expression of myofibrillar proteins in round postmitotic myoblasts of embyronic skeletal muscle. J. Cell Sci. *95*, 11–22.

Danowski, B.A., Imanaka-Yoshida, K., Sanger, J.M., and Sanger, J.W. (1992). Costameres are sites of force transmission to the substratum in adult rat cardiomyocytes. J. Cell Biol. *118*, 1411–1420.

Eilersten, K.J., Kazmierski, S.T., and Keller, C.S.T. (1994). Cellular titin localization in stress fibers and interaction with myosin II filaments in vitro. J. Cell Biol. *126*, 1201–1210.

Einheber, S., and Fischman, D.A. (1990). Isolation and characterization of a cDNA clone encoding avian skeletal muscle C-protein: an intracellular member of the immunoglobulin superfamily. Proc. Natl. Acad. Sci. USA *87*, 2157–2161.

Erickson, P.J. (1994). Reversible unfolding fibronectin type III and immunoglobulin domains provides the structural basis for stretch and elasticity of titin and fibronectin. Proc. Natl. Acad. Sci. USA *91*, 10114–10118.

Fulton, A.B and Isaacs, W.B. (1991). Titin, a huge elastic sarcomeric protein with a probable role in morphogenesis. Bioassays *13*, 157–161.

Furst, D.O., Osborn, M., and Weber, K. (1989). Myogenesis in the mouse embryo: differential onset of expression of myogenic proteins and the involvement of titin in myofibril assembly. J. Cell Biol. *109*, 517–527.

Gilbert, A.U., Kelly, M.G., Mikawa, T., and Fischman, D.A. (1996). The carboxyl terminus of myosin-binding protein-C (MYBP-C, C-protein) specifies incorporation into the A-band of striated muscle. J. Cell Sci. *109*, 101–111.

Granzier, H.L., and Irving, T.C. (1995). Passive tension in cardiac muscle: contribution of collagen, titin, microtubules, and intermediate filaments. Biophys. J. *68*, 1027–1044.

Harpaz, Y., and Chothia, C. (1994). Many of the immunoglobulin superfamily domains in cell adhesion molecules and surface receptors belong to a new structural set which is close to that containing variable domains. J. Mol. Biol. *238.*, 528–539.

Hill, C.S., Duran, S., Lin, Z., Weber, K., and Holtzer, H. (1986). Titin and myosin, but not desmin, are linked during myofibrillogenesis in postmitotic mononucleated myoblasts. J. Cell Biol. *103*, 2185–2196.

Horowits, R., and Podolsky, R. (1987). The positional stability of thick filaments in activated skeletal muscel: evidence for the role of titin filaments. J. Cell Biol. *105*, 2217–2223.

Huxley, H.E. (1972). The Structure and Function of Muscle, vol. 1, ed. G. H. Bourne, New York: Academic Press, 301–387.

Isaacs, W.B., Kim, I.S., Struve, A., and Fulton, A.B. (1989). Biosynthesis of titin in cultured skeletal muscle cells. J. Cell Biol. *109*, 2189–2195.

Labeit, S., and Kolmerer, B. (1995a). Titins: giant proteins in charge of muscle ultrastructure and elasticity. Science *270*, 293–296.

Labeit, S., and Kolmerer, B. (1995b). The complete primary structure of human nebulin and its correlation to muscle structure. J. Mol. Biol. *248*, 308–315.

Lakey, A., Labeit, S., Gautel, M., Ferguson, C., Barlow, D., Leonard, K., and Bullard, B. (1993). Kettin, a large modular protein in the Z-disc of insect muscles. EMBO J. *12*, 2863–2871.

LoRusso, S.M., Rhee, D., Sanger, J.M., and Sanger, J.W. (1997). Premyofibrils in spreading adult cardiomyocytes in tissue culture: evidence for reexpression of the embryonic program for myofibillogenesis in adult cells. Cell Motil. Cytoskeleton. (*in press*).

Maher, P., Cox., G.F., and Singer, S.J. (1985). Zeugmatin: A new high molecular weight protein associated with Z-lines in adult and early embryonic striated muscle. J. Cell Biol. *101*, 1871–1883.

Maruyama, K. (1994). Connectin, an elastic protein of striated muscle. Biophys. Chem. *50*, 73–85.

Maruyama, K., Endo, T., Kume, H., Kawamura, Y., Kanzawa, N., Kimura, S., Kawashima, S., and Maruyama, K. (1994). A partial connectin cDNA encoding a novel type of RSP motifs isolated from chicken embryonic skeletal muscle. J. Biochem. *115*, 147–149.

Nave, R., Furst, D.O., and Weber, K. (1989). Visualization of the polarity of isolated titin molecules: a single globular head on a long thin rod as the M-band anchoring domain. J. Cell Biol. *109*, 2177–2187.

Olson, N.J., Pearson, R.B., Needleman, D.S., Hurwitz, M.Y., Kemp, B.E., and Means, A.R. (1990). Regulatory and structural motifs of chicken gizzard myosin light-chain kinase. J. Biol. Chem. *263*, 10510–10516.

Price, M., and Gomer, R.H. (1993). Skelemin, a cytoskeletal M-disc periphery protein, contains motifs of adhesion/recognition and intermediate filament proteins. J. Biol. Chem. *268*, 21800–21810.

Rhee, D., Sanger, J.M., and Sanger, J.W. (1994). The premyofibril: evidence for its role in myofibrillogenesis. Cell Motil. Cytoskeleton *28*, 1–24.

Sambrook, J., Fritsch, E.F., and Maniatis, T. (1986). Molecular Cloning: A Laboratory Manual. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Sanger, J.M., Mittal, B., Pochapin, M.B., and Sanger, J.W. (1986). Myofibrillogenesis in living cells microinjected with fluorescently labeled alpha-actinin. J. Cell Biol. *102*, 2053–2066.

Sanger, J.W., Mittal, B., and Sanger, J.M. (1984). Formation of myofibrils in spreading chick cardiac myocytes. Cell Motil. *4*, 405–416.

Sanger, J.W., Sanger, J.M., and Jockusch, B.M. (1983). Differences in the stress fibers between fibroblasts and epithelial cells. J. Cell Biol. *96*, 961–969.

Soteriou, A., Clarke, A., Martin, S., and Trinick, J. (1993). Titin folding energy and elasticity. Proc. R. Soc. Lond. B *254*, 83–86.

Terai, M., Komiyama, M., and Shimada, Y. (1989). Myofibril assembly is linked with vinculin, α-actinin, and cell substrate contacts in embryonic cardiac myocytes in vitro. Cell Motil. Cytoskeleton *12*, 185–194.

Tokuyasu, K.T., and Maher, P.A. (1987a). Immunocytochemical studies of cardiac myofibrillogenesis in early chick embryos. I. Presence of Immunofluorescent titin spots in premyofibril stages. J. Cell Biol. *105*, 2781–2793.

Tokuyasu, K.T., and Maher, P.A. (1987b). Immunocytochemical studies of cardiac myofibrillogenesis in early chick embryos. II. Generation of α-actinin dots within titin spots at the time of the first myofibril formation. J. Cell Biol. *105*, 2795–2801.

Trinick, J. (1994). Titin and nebulin: protein rulers in muscle? Trends Cell Biol. *19*, 405–408.

Turnacioglu, K.K., Mittal, B., Sanger, J.M., and Sanger, J.W. (1996). Partial characterization of zeugmatin indicates that it is part of the Z-band region of titin. Cell Motil. Cytoskeleton *34*, 108–121.

Turnacioglu, K.K., Mittal, B., Dabiri, G., Sanger, J.M., and Sanger J.W. (1997). Zeugmatin is part of the Z-band targeting region of titin. Cell Struct. Funct. *22* (*in press*).

Wang, K., McClure, J., and Tu, A. (1979). Titin: major myofibrillar components of striated muscle. Proc. Natl. Acad. Sci. USA *76*, 3698–3702.