Nuclear localization of the titin Z1Z2Zr domain and role in regulating cell proliferation

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1Flexcell International Corporation, Hillsborough, North Carolina; 2Joint Department of Biomedical Engineering, University of North Carolina at Chapel Hill and North Carolina State University, Raleigh, North Carolina; and 3Institute for Anesthesiology and Intensive Operative Care, University Hospital, Mannheim, Germany

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Qi J, Chi L, Labeit S, Banes AJ. Nuclear localization of the titin Z1Z2Zr domain and role in regulating cell proliferation. Am J Physiol Cell Physiol 295: C975–C985, 2008. First published August 6, 2008; doi:10.1152/ajpcell.90619.2007.—Titin (also called connectin) is a major protein in sarcomere assembly as well as providing elastic return of the sarcomere postcontraction in cardiac and striated skeletal muscle tissues. In addition, it has been speculated that titin is associated with nuclear functions, including chromosome and spindle formation, and regulation of muscle gene expression. In the present study, a short isoform of titin was detected in a human osteoblastic cell line, MG-63 cells, by both immunostaining and Western blot analysis. Confocal images of titin staining showed both cytoplasmic and nuclear localization in a punctate pattern. Therefore, we hypothesized that human titin may contain a nuclear localization signal (NLS). A functional NLS, 200-PAKKTKT-206, located in a low-complexity, titin-specific region between Z2 and Z repeats, was found by sequentially deleting segments of the NH2-terminal sequence in conjunction with an enhanced green fluorescent protein reporter system and confirmed with site-directed mutagenesis. Overexpression of titin’s aminoterminal fragment (Z1Z2Zr) in human osteoblasts (MG-63) increased cell proliferation by activating the Wnt/β-catenin pathway. RT-PCR screens of tissue panels demonstrated that residues 1–206 were ubiquitously expressed at low levels in all tissues and cell types analyzed. Our data implicate a dual role for titin’s aminoterminal region, i.e., a novel nuclear function promoting cell division in addition to its known structural role in Z-line assembly.

Wnt; β-catenin; osteoblast

TITIN [also called connectin (22)] is a giant protein expressed in cardiac, skeletal, and smooth muscle tissues and is responsible for muscle elasticity as well as providing a scaffold for assembly of sarcomeric proteins (20). Analysis of the full-length sequence of the human titin gene indicates that titin contains 363 exons that encode a 4,200-kDa protein with 38,138 amino acid residues (4). Structural analysis showed that titin is mainly composed of immunoglobulin (Ig), fibronectin III (Fn-III) domains, and PEVK repeats (an ~28-residue, PEVK-enriched motif), which contribute to the elasticity of titin (4). Different combinations of these domains determine the stiffness of titin and therefore the stiffness of muscle tissue (20). Additional sarcomeric protein binding sites have been found on titin, which confirmed the key role that titin plays in the assembly of sarcomeric units (13, 15, 23, 33, 38). In addition to its structural and elastic functions, the titin filament may also modulate myofibrillar signaling pathways in a stretch-dependent manner by sensing mechanical load and by controlling muscle gene expression and turnover (20).

While the regulatory roles of titin in striated muscle are well established, titin’s potential signaling roles in nonmuscle tissues are more controversial. Several studies have reported the nuclear localization of titin and implicated a nuclear isoform of titin in the organization and maintenance of the structures of chromosomes, spindles, and nuclear membranes (12, 21, 39). However, proteomics studies involving samples from rapidly dividing cells failed to identify titin, at least in the form of a major structural component for chromosomes (32). Nuclear actin and myosins have been reported recently as titin-binding partners (27–29).

In the present study, it was hypothesized that if a nuclear isoform of titin exists, it may require a nuclear import signal for transport. By performing a search with 30 different primer pairs covering the entire transcriptional unit of titin, we identified that the 5’ 600 bp are transcribed ubiquitously (S. Labeit, unpublished data). In the present study, we report that a short isoform of titin is expressed in the human osteoblastic cell line, MG-63, by immunohistochemistry and Western blot techniques. By using an enhanced green fluorescence protein (EGFP) reporter system, immunostaining, and confocal microscopy techniques, we identified that the motif 200-PAKKTKT-206 within this ubiquitously transcribed segment is a functional nuclear localization signal (NLS) that could direct the NH2 terminus of titin and EGFP fusion proteins into the nucleus in different cell lines, including MG-63, BHK-21, mouse osteoblasts (MC3T3-E1), and COS-7 cells. Mutation of K203 to alanine disrupted the function of this NLS and resulted in cytoplasmic localization of a mutant titin amino fragment (Z1Z2Zr domain that includes Z1, Z2, and Z repeats). This is the first report of a functional NLS in the human titin protein, thus providing a model of how titin can be transported to the nucleus.

As a major structural protein in muscle tissues, titin plays an important role in sarcomere assembly and maintenance of muscle structure. The reported functions of nuclear titin are mainly the extension of its functions as a structural protein in muscle tissues, such as the elastic functions in spindles and chromosomes and the structural function in the nuclear envelope (12, 39). In the present study, we report that overexpression of the titin Z1Z2Zr domain in MG-63 cells increased cell proliferation by activating the Wnt/β-catenin pathway. These data implicate a dual role for titin’s amino terminal region, i.e., a novel nuclear function in addition to its known structural role...
in Z-line assembly. Since the Wnt/β-catenin pathway is an important component in bone maintenance (31, 37), nuclear titin may play a critical role in bone remodeling.

EXPERIMENTAL PROCEDURES

Antibodies. A rabbit affinity-purified polyclonal anti-titin antibody ZI22 was raised to the ZI22 region of titin; M8M9 antibody was raised to the M89 region of titin (for more details, see Ref. 8). AlexaFluor 568-conjugated goat anti-rabbit IgG (H+L) was obtained from Molecular Probes (Eugene, OR).

Primers. All primers used in the present study were synthesized by MWG Biotech (High Point, NC).

Cell culture. MG-63 cells, a human osteoblastic cell line, were maintained in MEM medium (Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum (Hyclone, Logan, UT). Mouse osteoblast MC3T3-E1 cells were maintained in DME M (Invitrogen) containing 10% bovine calf serum (Hyclone, Logan, UT). BHK-21 and COS-7 cells were maintained in DME M containing 10% fetal bovine serum. A growth curve of MG-63 cells was determined by plating the cells at 20,000 per well in 12-well plates, and cell numbers were determined with a particle counter (Beckman-Coulter, Fullerton, CA) at the indicated time points.

Reverse transcription polymerase chain reaction of human titin. The total RNAs of human cardiac muscle, skeletal muscle, kidney, brain, and liver were obtained from Ambion (Austin, TX). The total RNAs of bone, cartilage, meniscus, ligament, and tendon were isolated from tissues with TRI reagent (Sigma, St. Louis, MO). The cDNAs were synthesized using SuperScripII (Invitrogen). PCR conditions were as follows: 94°C for 5 min; 35 cycles of 94°C for 30 s, 65°C for 60 s, and 72°C for 30 s; 72°C for 5 min. The primer pair recognizing titin amino terminus (N) was used (Table 1). All of the primers have been tested on skeletal muscle samples, and the PCR products have been confirmed by sequence analysis.

RNA silencing. To knock down titin mRNA, small interfering (si)RNAs recognizing titin-specific sequences were designed on Ambion’s website (http://www.ambion.com/techlib/misc/siRNA_finder.html). One of them was able to knock down titin mRNA in MG-63 cells and was cloned into pSUPER.neo+gfp vector (OligoEngine, Seattle, WA). MG-63 cells were transfected with titin siRNA construct using lipofectamine (Invitrogen) according to the manufacturer’s protocol. The stable transfectants of MG-63 were selected using G418 (Invitrogen) 48 h posttransfection at 500 μg/ml.

Table 1. Primer sequences

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<th>Reverse (5’ to 3’)</th>
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The COOH-terminal residue of enhanced green fluorescent protein was mutated from lysine to serine (AAG to AGC) in NF-11 and NF-12 (underlined).

Prediction of NLSs. The potential NLSs within the NH2- and COOH- termini of human titin protein were predicted using three web-based programs: NucPred, PredicNLS, and PSORT II (10, 24).

Molecular cloning. The cDNA encoding the NH2-terminal and COOH-terminal fragments of human titin were amplified and cloned into pcDNA3.1 (Invitrogen) and pEGFP (BD Biosciences Clontech, Mountain View, CA) vectors, respectively. The NH2 terminus was composed of residues 1–790, including domains Z1, Z2, Z, and Z repeats. The COOH terminus was composed of residues 33,791–34,350, including domains M7–10. For pcDNA3.1 constructs, the NH2- and COOH-terminal fragments of the human sequence were amplified using the following primers: NH2 terminus, 5’-aaa atg att ctg aca aca caa gca ccc agc agt ttt-3’ (forward) and 5’-AAA AGT CTA CGA TGA TTT CTG TGA TAT CAT GAT CAT CCC ATT CTT CTT CTT CTT CTT CTT CTT-3’ (reverse); COOH terminus, 5’-aaa atg att ctg aca aca caa gca ccc agc agt ttt-3’ (forward) and 5’-AAA AGT CTA CGA TGA TTT CTG TGA TAT CAT GAT CAT CCC ATT CTT CTT CTT CTT CTT CTT CTT-3’ (reverse). For pEGFP constructs, the primers were as follows: NH2 terminus, 5’-aaa atg att ctg aca aca caa gca ccc agc agt ttt-3’ (forward) and 5’-AAA AGT CTA CGA TGA TTT CTG TGA TAT CAT GAT CAT CCC ATT CTT CTT CTT CTT CTT CTT CTT-3’ (reverse). In the constructs of the COOH terminus, a start codon was added at the 5’-end. In the pcDNA3.1 constructs, a stop codon (TAA) was included at the end of both NH2- and COOH-terminal fragments of titin due to the lack of stop codons in the pcDNA3.1 plasmid. The titin cDNAs were amplified from human skeletal muscle total RNA (no. 7982; Ambion) and cloned into pcDNA3.1 at BamHI sites. NLS localization constructs NFs 1–7 were cloned in pcDNA3.1 at BamHI sites. The primers used for making these NF constructs are listed in Table 1. To reduce the effects of the COOH-terminal lysine residue of GFP on the nuclear localization of NFs 8–10, the lysine (K) was mutated to serine (S) in these three constructs (Table 1). pEGFP-N1-α-actinin-1 was from Dr. Carol Otey at the University of North Carolina at Chapel Hill (11). α-Actinin-2
cDNA was amplified from human skeletal muscle total RNA (Ambion) and cloned into pEGFP-N1 at EcoRI/XhoI sites.

Site-directed mutagenesis. The residue K203 of human titin was mutated to alanine (A) by using a modified PCR-based mutagenesis technique (19). Two pairs of primers were used: 5′-aaa agg atc cct atg aca act caa gca ccg acg ttt-3′ (1), 5′-TAC CTG CTA AAG CGA CAA AGA CAA T-3′ (2), 5′-ATT GTC TTT GTC GCT TTA GCA GGT A-3′ (3), and 5′-AAA ACT CGA GAA TTA CTG TGA TGA TAT GTG CAT TCC CTT-3′ (4). The mutation was introduced in primers 2 and 3 (underlined). Three PCR reactions were carried out as follows: the first two PCR cycles were performed by using primer pairs of 1/3 (producing PCR product 1) and 2/4 (producing PCR product 2). PCR products were purified, diluted 1,000-fold, combined in equal proportions, and used as the template in the third PCR reaction. The third PCR was performed with primer pair 1/4 using the mixture of PCR products 1 and 2 as the template. The PCR products from the third PCR were purified and digested with BamHI-XhoI and cloned into pcDNA3.1. Presence of the mutation was confirmed by DNA sequencing.

Transfection of mammalian cells. Plasmids were transfected into MC3T3-E1 cells, BHK-21 cells, COS-7, or MG-63 cells using lipofectamine (Invitrogen) according to the manufacturer’s protocol. The stable transfectants of MG-63 were selected using G418 (Invitrogen) 48 h posttransfection at 500 μg/ml. The cells were incubated with G418 for up to 3 wk until individual colonies were formed. The colonies with green fluorescence were selected using an Olympus BH61 fluorescence microscope. The colonies expressing NH2- or COOH-terminal fragments of titin were selected by immunostaining. The stable transfectants of MG-63 were kept in MEM medium (Invitrogen) containing 100 μg/ml G418.

Immunostaining. Cells were fixed with 3.7% formaldehyde at room temperature (RT) for 30 min and permeabilized with 0.1% Triton X-100 at RT for 15 min. After being washed with phosphate-buffered saline (PBS; Invitrogen), the cells were blocked with 5% bovine

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Fig. 1. Transcription of human titin’s 5′-end region as detected by RT-PCR using an exon2-sense and exon5-reverse primer pair. Lanes 1 to 10 are bone (1), cartilage (2), meniscus (3), ligament (4), tendon (5), cardiac muscle (6), skeletal muscle (7), kidney (8), brain (9), and liver (10). Top bands are 18S ribosomal RNA (rRNA, 489 bp), and bottom bands are titin PCR products (300 bp). Std, standard.

Fig. 2. Expression of titin in human osteoblast MG-63 cells. A: Coomassie blue staining of the PAGE gel. B: Western blot of titin expressed in human osteoblast MG-63 cells using anti-titin polyclonal antibody, Z1Z2. C: immunostaining of titin in MG-63 with anti-titin antibody Z1Z2. CA: image of immunostaining with Z1Z2 antibody. CB: image of 4′,6-diamidino-2-phenylindole (DAPI) staining. CC: overlap of CA and CB. The arrow in CC shows the nuclear titin. Scale bar is 20 μm. D: results of quantitative RT-PCR using primers recognizing different domains of titin. E: the static state mRNA of titin was knocked down to ~40% of control in clone 6. *Statistical significance (P < 0.05). F: immunostaining images of titin. FA: control cells. FB: titin knockdown cells. Scale bar is 50 μm.
serum albumin (BSA; Fisher Scientific, Suwanee, GA) and 2% goat serum (Sigma) at RT for 2 h or at 4°C overnight and were then labeled with primary antibody at RT for 2 h or at 4°C overnight. Anti-titin antibodies Z1Z2 or M8M9 were diluted at 1:10 in PBS. Anti-catenin antibodies (Invitrogen) were diluted at 1:100 in blocking buffer. After being washed with PBS 3×, 5 min per wash, the proteins were visualized with AlexaFluor-conjugated secondary antibodies at 1:500 diluted in PBS at RT for 1 h. The stained cells were mounted on glass slides using a Slowfade light antifade kit (Molecular Probes) containing 100 ng/ml 4,6-diamidino-2-phenylindole (DAPI; Sigma). The images of the cells were viewed using an epifluorescence microscope (Olympus BX60, OPELCO, Dulles, VA) or a LeicaSP2 AOBS laser scanning confocal microscope (Leica Microsystems, Exton, PA) with a ×40 oil immersion objective.

Real-time RT-PCR. Total RNAs were extracted using RNasy Mini Kits (Qiagen, Valencia, CA). cDNAs were synthesized using SuperScriptII, and quantitative real-time RT-PCR was carried out using Brilliant SYBR green qPCR master mix (Stratagene, La Jolla, CA). PCR conditions were as follows: 95°C for 10 min; 40 cycles of 95°C for 30 s, 60°C for 60 s, and 72°C for 60 s. Primers for α-catenins 1, 2, and 3 were 5′-ATCAGATGGCTGCAGCTAGAGGA-3′ (forward), 5′-ATGCTTTTCCAGACGCTCCTCCA-3′ (reverse); 5′-AGAAGCAGGTCCGAGTGTTGACA-3′ (forward), 5′-AGAAGCTTGGGAAGGAGCTGGAA-3′ (reverse); 5′-TTCTCCAGCTCATCAAGGGCACT-3′ (reverse). Primers for β-catenin were 5′-GGACAGTATGCAATGACTCGAGCT-3′ (forward) and 5′-GAACGCATGATAGCGTGTCTGGA-3′ (reverse). Primers for titin domains are listed in Table 1.

Western blot analysis. Whole cell lysates were prepared with RIPA buffer according to the manufacturer’s protocol (Santa Cruz Biotechnology, Santa Cruz, CA). Protein concentration was determined with Coomassie Plus protein assay reagent (Pierce Biotechnology, Rockford, IL). For the blotting of catenins, 20 μg of total proteins were loaded in each well. Proteins were separated on 8% SDS-PAGE gels and transferred onto a nitrocellulose membrane. The membrane was blocked with blocking buffer (5% milk powder dissolved in Tris-buffered saline with 0.5% Tween 20) at RT for 1 h, then probed with first antibodies at RT for 1 h (1:1,000 for α-catenin and 1:2,000 for β-catenin, diluted in blocking buffer). Horseradish peroxidase-conjugated secondary goat anti-mouse IgG (Pierce Biotechnology) was used at 1:2,000 dilution. After exposure to X-ray film, the membrane was stripped with Restore Plus Western blot stripping buffer (Pierce Biotechnology) and reprobed with anti-actin antibody (Sigma) to verify the equal loading of protein in each well. For the titin Western blot analysis, 60 μg of total proteins were loaded in each well and separated on a PAGE gel consisting of 8% T acrylamide, 2.34% C diallyltartardiamide, 10% vol/vol glycerol, 0.5 M Tris-HCl.

Potential NLS

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Fig. 3. Transient expression of enhanced green fluorescence fusion proteins (EGFP) in four different mammalian cell lines. The pEGFP-C1 constructs were transfected into cells using lipofectamine, and the cells were fixed with formaldehyde 48 h post-transfection. Cell images were recorded with an Olympus FV-II digital camera. All of the titin NH2-terminal EGFP fusion proteins showed nuclear localization, whereas EGFP and titin COOH-terminal EGFP fusion proteins were distributed in both the cytoplasm and nucleus. BHK-21 cells were derived from hamster kidney, MG-63 cells were from a human osteosarcoma, MC3T3-E1 cells were from a mouse osteoblast-like cell line, and COS-7 cells were derived from monkey kidney cells. Scale bar is 50 μm.

Fig. 4. Intracellular localization of titin NH2- and COOH-terminal fragments in COS-7 cells. The NH2- and COOH-terminal fragments of titin were cloned into seven pcDNA3.1 vectors and transfected into COS-7 cells. Cells were fixed and costained with anti-titin antibodies (Z1Z2 for NH2-terminal staining and M8M9 for COOH-terminal staining) and DAPI (staining nucleus). The NH2 terminus of titin showed nuclear localization, whereas the COOH terminus was distributed in both the cytoplasm and nucleus. Scale bar is 50 μm.

Fig. 5. Design of subclones of human titin NH2 terminus for searching potential nuclear localization signals (NLSs).
pH 8.8, 0.028% wt/vol ammonium persulfate, and 0.152% vol/vol N,N,N′,N′-tetramethylethylenediamine (35). Titin proteins were probed with anti-titin antibody Z1Z2.

Statistical analysis. All experiments were repeated at least three times. Statistical analyses were performed using Student’s t-test. A value of \( P < 0.05 \) was deemed as significant. The values were expressed as means ± SD.

RESULTS

Expression of titin’s amino terminal fragment in human tissues and cells. As an initial survey for nonmuscle titin isoforms, we performed an RT-PCR screen for titin transcripts. Transcripts coding for titin’s amino terminus were identified in all tested tissues (Fig. 1). The highest transcript levels occurred in cardiac and skeletal muscles and the lowest levels in kidney and liver. Ligament, tendon, and brain showed intermediate transcript levels. Next, we tested with Z1-Z2 specific antibodies for the localization of titin. Consistent with the transcript data, results of both immunostaining and Western blot analysis showed that titin was also expressed in human osteoblast cells, MG-63 (Fig. 2). The confocal images of titin immunostaining showed that titin in MG-63 cells did not form a fibrillar network. Instead, a punctate pattern was observed in both the cytoplasm and nucleus, under the conditions of the experiment (Fig. 2C). Quantitative RT-PCR showed differential expression of titin domains (Fig. 2D). To confirm the specificity of titin immunostaining, titin was knocked down with RNA silencing. Immunostaining in titin-knockdown cells showed reduced fluorescence intensity (Fig. 2, E and F).

Nuclear localization of titin NH2-terminal fragment-GFP fusion proteins in mammalian cells. It has been suggested that titin is localized in the nucleus and plays structural roles in the chromosome and spindle (12, 39). However, there is no published report of potential NLSs in human titin. Titin is a giant protein mainly composed of Ig, Fn-III, and PEVK repeats. Because of its huge size, it is not feasible to determine its nuclear localization by heterogeneous expression of the whole

Fig. 6. Intracellular localization of subclones of human titin NH2 terminus in COS-7 cells. A total of 12 subclones of the human titin NH2-terminal sequences were made in a pEGFP-C1 vector and transiently expressed in COS-7 cells. EGFP alone, as a control, showed both cytoplasmic and nuclear localization similar as constructs also containing the titin COOH terminus. In contrast, constructs containing the titin NH2 terminus (linked either to the NH2 or COOH terminus of EGFP) exclusively showed nuclear localization. The intracellular localizations of constructs NF-1 to 5 identify a potential NLS between residues 180 and 209. Further deletion of residues in constructs NF-6 to 11 assign the NLS to residues 200 to 206. The deletion of residues Pro and Ala almost ablated the NLS function. Scale bar is 50 μm.
protein. Analysis of the complete sequence of human titin revealed that there are several titin-specific sequence insertions at both the NH$_2$ and COOH termini. Therefore, the GFP-titin fusion proteins of NH$_2$- and COOH-terminal fragments were constructed and transiently expressed in several mammalian cells. In all of the cell lines tested, the titin NH$_2$-terminal fusion proteins localized principally in the nucleus in both high- and low-expressing cells, whereas the titin COOH-terminal GFP fusion proteins and GFP only-control proteins distributed in both the nucleus and the cytoplasm (Fig. 3). To confirm that the intracellular localization of the NH$_2$- and COOH-terminal fragments of human titin protein was not due to the effects of GFP fusion, the tag-free fragments were also cloned into pcDNA3.1 and transfected into COS-7 cells. The transiently expressed titin fragments in COS-7 cells were stained with anti-titin antibodies. The results confirmed the finding for GFP fusion constructs (Fig. 4). The NH$_2$-terminal fragment of titin was mainly in the nucleus, whereas the

Fig. 7. Intracellular localization of subclones of human titin NH$_2$ termini in MG-63 cells. The same set of pEGFP constructs was transfected into MG-63 cells. Stably transfected cells were selected with G418 at 500 μg/ml. EGFP as a control showed both cytoplasmic and nuclear localization. The titin COOH terminus linked to the NH$_2$ or COOH terminus of EGFP was distributed in both cytoplasm and nucleus. The titin NH$_2$ terminus linked to the NH$_2$ or COOH terminus of EGFP showed nuclear localization. The intracellular localization of constructs NF-1 to 5 assigns the potential NLS between residues 180 and 209, with further deletions in constructs NF-6 to 11 to residues 200 to 206. Consistent with this deletion mapping, we found that the deletion of residues PA almost ablated the NLS capability (see NF-12). Scale bar is 50 μm.

Fig. 8. Mutation of K203A blocked titin Z1Z2Zr’s nuclear localization in MG-63 cells. The residue 203 was mutated from lysine to alanine, cloned into pcDNA3.1, and transfected in MG-63 cells. A: immunostaining of titin Z1Z2Zr with anti-titin antibody Z1Z2. B: DAPI staining of nucleus. C: combination of A and B. Scale bar is 50 μm.
COOH-terminal fragment of titin was mainly in the cytoplasm. Double labeling with DAPI demonstrated that the titin NH2-terminal fragments were in the nucleus but not in the nucleolus (Figs. 3 and 4).

**Prediction of potential NLSs within the amino and carboxyl termini of human titin.** The sequences of titin NH2 and COOH termini were input to three web-based programs as described in EXPERIMENTAL PROCEDURES. No NLSs were predicted within the COOH terminus of human titin from any of the programs. Negative results on the NH2 terminus were obtained from the programs PredictNLS and NucPred. One potential NLS within the NH2 terminus of human titin was predicted by PSORT II: 200-PAKKTKT-206 (25). The predicted potential NLS is located in the titin-specific sequence between the Z2 domain and Z repeats (arrow, Fig. 5) (4).

PAKKTKT is a functional NLS. Only one potential NLS was predicted by the PSORT II program. However, there may be other nonclassic NLSs in the titin-specific region at the NH2 terminus of human titin protein (9). Moreover, other domains may affect the function of this NLS. To investigate the potential NLSs within the NH2 terminus of titin, 11 constructs were made in the EGFP reporter systems, pEGFP-C1 and pEGFP-N1 (Fig. 5). These constructs were transiently expressed in COS-7 cells (Fig. 6). The deletion of sequences of 568–790, 537–790, and 221–790 did not change the location of EGFP fusion proteins. Results with construct NF-4 further confirmed that

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Fig. 9. The nuclear localization of titin \(Z1Z2Zr\) was not affected by overexpression of \(\alpha\)-actins 1 and 2. GFP fusion proteins (\(\alpha\)-actinin-1 or 2) were cotransfected with pcDNA-\(Z1Z2Zr\) and stained with anti-titin antibody \(Z1Z2\) and DAPI. A: image of \(\alpha\)-actinin-1-GFP fusion proteins. B and E: images of \(Z1Z2\) immunostaining. C and G: DAPI staining. D: overlap of A–C. F: image of \(\alpha\)-actinin-2-GFP fusion proteins. H: overlap of E–G. Scale bar is 50 \(\mu\)m.

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Fig. 10. Overexpression of titin \(Z1Z2Zr\) domain changes cell shape and stimulates cell proliferation. A: wild-type MG-63 cells showed spindle shape and cells aggregated to one another, whereas \(Z1Z2Zr\)-expressing cells showed a polygonal shape and cells evenly distributed. Scar bar is 50 \(\mu\)m. B: cells were plated in 12-well plates at 20,000 per well and grown for 15 days. C: cell numbers were determined using a Beckman-Coulter particle counter at the indicated times. The experiments were repeated three times and representative results are shown.
there is no NLS within residues 209–790. Results with constructs 5–8 showed that residues 1–180 neither imported fusion EGFP proteins into the nucleus nor affected the nuclear location of NLS-containing EGFP fusion proteins. Results with constructs 9–11 further narrowed the NLS to seven amino acid residues. The deletion of residues P-A disrupted the function of this NLS. To eliminate the possibility that the nuclear localization of titin NH2-terminal fragment was due to the overexpression of heterogenous proteins, these constructs were also transfected into MG-63 cells, and stable transfectants for each construct were selected. The results were the same as that in COS-7 cells (Fig. 7). The mutation of residue K203 to A disrupted the integrity of the NLS and resulted in the cytoplasmic distribution of the mutant titin Z1Z2Zr domain (Fig. 8). This result confirmed that PAKKTKT is a functional NLS.

Nuclear localization of titin Z1Z2Zr domain is not affected by overexpression of α-actinins. The NH2-terminal fragment of titin containing an NLS was introduced into cardiac myocytes but did not show nuclear localization (15). It was reported that there is an α-actinin binding site close to the NLS (38). Therefore, it is possible that the binding of α-actinin may have blocked the NLS, resulting in the cytoplasmic localization of the titin NH2-terminal fragment in cardiac myocytes. To determine whether the binding of α-actinin could block the nuclear localization of Z1Z2Zr domain of titin, nonmuscle- and muscle-type α-actinins-1 and -2 were cotransfected with Z1Z2Zr into MG-63 cells, respectively. The results showed that neither of the two α-actinins could block the nuclear localization of the Z1Z2Zr domain in MG-63 cells (Fig. 9). Future studies are mandatory to test whether titin-Z1Z2 binding proteins that are expressed in cardiac myocytes (such as T-cap) may block the NLS in myocardial cells.

Overexpression of titin Z1Z2Zr domain in MG-63 cells changed cell shape and cell-cell interaction. Wild-type MG-63 cells showed a spindle shape, and cells aggregated together longitudinally (Fig. 10A). Z1Z2Zr-expressing cells showed a rounded cell shape, and cells did not readily aggregate (Fig. 10A). These results indicated that overexpression of the Z1Z2Zr domain reduced cell-cell adhesion.

Overexpression of titin Z1Z2Zr domain increased cell proliferation. Both pcDNA3.1 and pEGFP-C1 transfected MG-63 cells reached the stationary phase on day 10, whereas Z1Z2Zr- or EGFP-Z1Z2Zr-expressing cells continued growth past day 15 (Fig. 10C). However, GFP-Z1Z2Zr showed less potential, which indicates that GFP may interfere with the interaction of Z1Z2Zr with its target proteins. Analysis of the phase contrast images of the Z1Z2Zr-expressing cells indicated that these cells lost cell-cell contact inhibition (Fig. 10B).

Overexpression of the titin Z1Z2Zr domain downregulated the expression of β-catenin and activated the Wnt/β-catenin pathway. Z1Z2Zr-expressing cells showed less aggregation and increased growth rate (Fig. 10), indicating that the Wnt/β-catenin pathway may be activated (26). Results of immunostaining showed that most of the β-catenin was translocated from the plasma membrane to the nucleus in Z1Z2Zr-expressing cells (Fig. 11). Quantitative real-time RT-PCR and Western blot results showed that the expression of β-catenin was not changed, whereas the expression of α-catenin was downregulated (Fig. 12).

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Fig. 11. Immunostaining of catenins in MG-63 cells. a: immunostaining of α-catenin showed a much stronger fluorescence signal in pcDNA-transfected MG-63 cells compared with that in pcDNA-Z1Z2Zr-transfected cells. Scale bar is 50 μm. b: immunostaining results of β-catenin. bA and bE: DAPI staining. bB and bF: staining with anti-titin antibody Z1Z2. bC and bG: immunostaining of β-catenin. bD: overlap of bA, bB, and bC. H: overlap of bE, bF, and bG. Scale bar is 20 μm.

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DISCUSSION

Titin is a giant filamentous protein, highly expressed in striated muscle tissues (the third most highly expressed protein after actin and myosin in muscle) (34). Titin plays an important role in the assembly of the sarcomere and contributes to the elasticity of muscle tissues (20). A deficiency in titin protein results in severe heart or skeletal muscle diseases (20).

Our group and others have reported that titin may also be expressed in nonmuscle tissues, such as tendons (2, 3, 7, 30). The RT-PCR screening results showed that the amino fragment of titin was detected in all of the tissues tested. In the present study, the expression of titin in human osteoblast cells (MG-63) was confirmed by immunostaining and Western blot analysis. The titin isoform in osteoblasts is much smaller than the full-length titin, and this result was indirectly supported by the results of quantitative RT-PCR using primers recognizing different titin domains, which indicate that I-A junctions and PEVK regions (or part of them) are not expressed in osteoblasts. This observation is consistent with the reported titin isoforms expressed in nonskeletal muscle tissues (17). The immunostaining pattern of titin in MG-63 cells contrasts with that in muscle tissues. There is a punctate staining, but no continuous fibrillar structures in MG-63 cells. The punctate pattern is similar to that of cellular titin (c-Titin) reported by Cavnar et al. (7). This specific staining pattern may indicate different functions of titin in nonmuscle cells. The confocal images of titin in MG-63 cells also showed both cytoplasmic and nuclear localization of titin. Several studies have been performed to show that titin was also involved in nuclear functions (12, 39). Titin has been postulated to be a structural component of the metaphase chromosome (which accounts for its elasticity), and it plays important roles in regulating chromosome condensation and spindle organization (12, 21). Zastrow et al. (39) have reported that nuclear titin plays a role in nuclear organization. However, the conclusion of the existence of nuclear titin is challenged by some recent reports (16, 36). The present study, for the first time, reports that not only is titin ubiquitously expressed in connective tissue and soft tissue cells, but there is a functional NLS within the titin-specific region at the amino terminus of human titin. This observation indirectly supports the finding that titin may be present in the nucleus and offers a mechanism for the process. Deletion of P-A residues disrupts the NLS. This result suggests that a proline residue may be important for maintaining the accessibility and flexibility of the NLS so that the signals may be accessible to the receptor proteins (18). The titin amino fragment has been overexpressed in cardiac myocytes but did not show nuclear localization (15). The mechanism for regulating the importation of classic NLS-containing nuclear proteins has been well investigated (14). The first step is the recognition of NLSs by importin-α. This step can be regulated by the blockage of NLSs by mask domains or partner proteins (14). Since titin is a filamentous protein and the NLS locates outside of Ig domains, it is likely that this step may be regulated by titin-binding proteins. Several titin amino terminus-binding proteins have been reported, such as T-cad/telethonin, obscurin, and α-actinin (1, 15, 38). The potential regulators for the importation of titin into the nucleus may be among them. Among the reported titin-binding proteins, α-actinin is the most likely candidate that binds to Z repeats on titin (1, 38). However, neither nonmuscle-type α-actinin-1 nor muscle-specific α-actinin-2 affected the nuclear localization of the titin Z1Z2Zr domain in MG-63 cells. These data indicate that there may be an undiscovered titin-binding protein that could block the NLS and prevent titin’s nuclear localization in cardiac myocytes. The other possibility is that the importin-α isof orm that could recognize titin’s NLS is not expressed in cardiac myocytes. The nuclear localization of titin in nonmuscle cells indicates that titin may play an additional role in nonmuscle, different from its elastic function in sarcomere return.

While titin’s critical roles as an important architectural and regulatory protein and as an intrasarcomiceric protein in striated muscle are well established, its potential nuclear functions remain less clear. The reported potential functions of nuclear titin isoforms are all structural functions, such as the roles in mitosis/spindles, chromosomal elasticity, and nuclear membrane integrity (12, 39). These are extensions of the functions of striated muscle titin in the nucleus. Titin bears an NLS, is found in the nucleus, but is a low-abundance protein in human osteoblastic cells (MG-63). Taken together, these observations suggest that the nuclear titin isoform in MG-63 cells may play a regulatory role rather than a structural function in the nucleus. Titin Z1Z2Zr-expressing cells showed dramatic cell shape changes. It is well known that cell shape is controlled by adhesion proteins as cells near confluence (6). One of the major cell-cell junction complexes is epithelial (E)–cadherin/β-catenin/α-catenin (6). Therefore, the change of cell shape from spindle shape to polygonal shape may be due to the downregulation of α-catenin (6). The translocation of β-catenin from intercellular adherens junctions to the nucleus further reduced intercellular connections.
The Wnt pathway plays an important role in regulating cell proliferation and differentiation. β-Catenin is the central player in the Wnt signaling pathway (26, 37). β-Catenin is not only a structural protein in intercellular junctions, it is also a transcription cofactor with the T cell factor, TCF/LEF, in the Wnt pathway. Upon activation, β-catenin will relocate from the plasma membrane to the nucleus and regulate the expression of many genes that participate in regulating gene expression, cell adhesion, and migration (26, 37). The regulation of β-catenin occurs mainly at the posttranslational stage by protein kinases (26, 37). That may be the reason why we did not detect the change in the expression of β-catenin. It has been reported that disruption of α-catenin:β-catenin heterodimer may release free β-catenin from E-cadherin complexes (5). Combined with the results in the present study, it suggests that α-catenin may play a key role in stabilizing the E-cadherin complexes. It is believed that deregulation of the Wnt pathway may be one of the factors causing cancer (26). It has been reported recently that the Wnt pathway regulates the physiological responses of bone to mechanical loading (31). Taken together, these observations suggest that titin’s amino-terminal fragment may modulate the Wnt pathway in nonmuscle cells, a pathway that plays important roles during tumorigenesis, the maintenance of bone mass, and potentially, regulation of mechanosensitivity in bone (31).

In conclusion, the amino fragment of titin was expressed ubiquitously in all tissues and cells examined. A short isoform of titin was detected in human osteoblasts, MG-63 cells. Titin may be translocated into the nucleus and regulate cell proliferation by activating the Wnt/β-catenin pathway. This is the first report that suggests that titin may act as a regulatory protein inside the nucleus in addition to its functions as a structural protein in muscle.

GRANTS

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DISCLOSURE

A. J. Banes is president of Flexcell International Corporation and is compensated as such.

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