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G-actin sequestering protein thymosin-β4 regulates the activity of myocardin-related transcription factor

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ABSTRACT

Myocardin-related transcription factors (MRTFs) are robust coactivators of serum response factor (SRF). MRTFs contain three copies of the RPEL motif at their N-terminus, and they bind to monomeric globular actin (G-actin). Previous studies illustrate that G-actin binding inhibits MRTF activity by preventing the MRTFs nuclear accumulation. In the living cells, the majority of G-actin is sequestered by G-actin binding proteins that prevent spontaneous actin polymerization. Here, we demonstrate that the most abundant G-actin sequestering protein thymosin-β4 (Tβ4) was involved in the regulation of subcellular localization and activity of MRTF-A. Tβ4 competed with MRTF-A for G-actin binding; thus, interfering with G-actin–MRTF-A complex formation. Tβ4 overexpression induced the MRTF-A nuclear accumulation and activation of MRTF–SRF signaling. The activation rate of MRTF-A by the Tβ4 mutant L17A, whose affinity for G-actin is very low, was lower than that by wild-type Tβ4. In contrast, the β-actin mutant 3DA, which has a lower affinity for Tβ4, more effectively suppressed MRTF-A activity than wild-type β-actin. Furthermore, ectopic Tβ4 increased the endogenous expression of SRF-dependent actin cytoskeletal genes. Thus, Tβ4 is an important MRTF regulator that controls the G-actin–MRTFs interaction.

1. Introduction

MAL/MKL1/Myocd-related transcription factor-A (MRTF-A) and MAL16/MKL2/MRTF-B are robust coactivators of the serum response factor (SRF) [1]. They regulate various biological processes such as the rearrangement of the actin cytoskeleton [2,3], development of mammary myoepithelial cells [4], epithelial–mesenchymal transition [5,6], and cell motility [7,8]. MRTFs have three tandem RPEL repeats, which are monomeric globular actin (G-actin) binding motifs, at their N-terminus, and their activities are regulated by G-actin binding [1]. G-actin-binding MRTFs are found in the cytoplasm in an inactive state. MRTFs interact with the nuclear transport receptor importin α/β via their basic domains that overlap with the RPEL repeats [9,10]. G-actin interferes with the MRTF–importin interaction; thus, inhibiting the MRTFs nuclear translocation. The stimuli that induce actin polymerization lead to a depletion of the cytoplasmic G-actin pool, resulting in the nuclear translocation and transcriptional activation of G-actin-free MRTFs. Thus, a change in the amount of cytoplasmic G-actin pool as a result of actin polymerization/depolymerization is a critical event for the regulation of MRTF activity, although post-translational modifications also contribute to it [11–13].

Under physiological conditions, free G-actin spontaneously polymerizes to form microfilaments in vitro. However, in the living cells, several types of actin binding proteins such as thymosin β4 (Tβ4), profilin, DNAse I, and capping proteins sequester free G-actin, thereby prevening spontaneous actin polymerization [14]. Tβ4 is a small peptide comprising 43 amino acids that strongly binds to G-actin but very weakly to filamentous actin (F-actin) [15–17]. Tβ4 prevents actin polymerization by inhibiting the exchange of the G-actin-bound nucleotide [18]. Because Tβ4 is abundantly expressed in a wide variety of cell types, it is believed that >80% G-actin is sequestered by Tβ4 and that free G-actin is scarce in the cytoplasm [14,19]. Previous studies demonstrate that DNAse I is present in the G-actin-MRTF-A complex [20], whereas profilin-binding G-actin cannot bind to MRTF-A [21]. In contrast to Tβ4, profilin facilitates actin polymerization by enhancing the exchange of actin-binding nucleotides [18], leading to the activation of MRTFs [1,22]. Although Tβ4 is the most abundant G-actin sequestering protein, its role in the regulation of MRTF activity remains unknown. Here, we demonstrate that Tβ4 competed with MRTF-A for G-actin binding, and that it induced the nuclear accumulation and transcriptional activation of MRTF-A. To the best of our knowledge, this is the first report of Tβ4 as a regulatory factor of MRTF activity.

Abbreviations: MRTF, myocardin-related transcription factor; SRF, serum response factor; Tβ4, thymosin-β4; G-actin, monomeric globular actin; F-actin, filamentous actin.

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2. Materials and methods

2.1. Cell culture and transfection

The NIH 3T3, HeLa, and HEK293T cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. The cultured cells were transfected for 24–48 h using Lipofectamine LTX and Plus Reagent (Invitrogen).

2.2. Construction of expression plasmids

The coding regions for human Tβ4 and β-actin and mouse MRTF-A were amplified by PCR and subcloned into the highly efficient mammalian expression plasmid pCAGGS. A FLAG- or myc-tag sequence was fused to the 5′- or 3′-end of each coding sequence. Expression plasmids for Tβ4 (L17A) and β-actin (3DA) were constructed by site-directed mutagenesis using PrimeSTAR Max DNA Polymerase (Takara Bio, Inc.). MRTF-A (123-1A) was constructed by site-directed mutagenesis as described previously [20].

2.3. Luciferase reporter assay

The 3× CArG-Luc vector was used to measure the MRTF-A transcriptional activity [6]. 3× CArG-Luc and the indicated expression plasmids were cotransfected into HeLa cells along with pSV-βGal (Promega) that was used to normalize the transfection efficiency. Two days after transfection, luciferase and β-galactosidase activities were measured using the Luciferase Assay System (Promega) and Luminescent β-Galactosidase Detection Kit II (Clontech Laboratories, Inc.), respectively.

2.4. Coimmunoprecipitation

The HEK293T cells transfected with the expression plasmids were lysed with an NP-40 buffer [0.5% NP-40, 5 mM $MgCl_2$, 1 mM ATP, and protease inhibitor cocktail for use with mammalian cell and tissue extracts (Nacalai Tesque) in 1× PBS (Nacalai Tesque)]. The cell lysates were briefly sonicated and centrifuged to remove cell debris. The resultant supernatants were incubated with FLAG Affinity Gel (Sigma) for 3 h at 4 °C with gentle rotation. Subsequenctly, the beads were washed with NP-40 buffer and boiled in SDS sample buffer to elute the immunocomplexes.

2.5. Immunocytochemistry

The NIH 3T3 cells were cultured on coverslips and transfected with the expression plasmids. On the following day, the cells were fixed using 4% paraformaldehyde and then incubated with a blocking solution (0.1% Triton X-100, 0.2% BSA, 10% normal goat serum in 1× PBS). The cells were incubated with anti-FLAG (Sigma) and/or anti-myc (Santa Cruz Biotechnology) antibodies diluted in the Can Get Signal immunostain reagent (TOYOBO). After washing with 1× PBS, the cells were incubated with Alexa Fluor-conjugated secondary antibodies (Invitrogen) diluted in the blocking solution. To label the nuclei and F-actin, Hoechst 33342 (Invitrogen) and Alexa Fluor 568 phalloidin (Invitrogen) were added to the secondary antibody solution, respectively. The stained cells were mounted with Fluoromount (Diagnostic BioSystems) and observed using a BZ-9000 model fluorescence microscope (Keyence).

2.6. RT-PCR

Total RNA was extracted from the cells using RNAiso Plus (Takara Bio, Inc.), and reverse transcribed with SuperScript III Reverse Transcriptase (Invitrogen) and a random hexamer primer. cDNA was amplified using gene-specific PCR primer pairs. Primer sequences are based on a previous report [2].

3. Results and discussion

3.1. Tβ4 induces the activation of MRTF–SRF signaling

According to a previous in vitro study, Tβ4 prevents the exchange of the G-actin-bound nucleotide, resulting in the inhibition of actin polymerization [18]. In the living cells overexpressing exogenous Tβ4, G-actin dissociated from actin filaments is sequestered by excess Tβ4; thus, Tβ4-binding G-actin remains in the cytoplasmic G-actin pool [23,24]. We confirmed that Tβ4 overexpression induced disassembly of F-actin bundles in the HeLa cells (Fig. 1A). To determine whether Tβ4 contributes to the

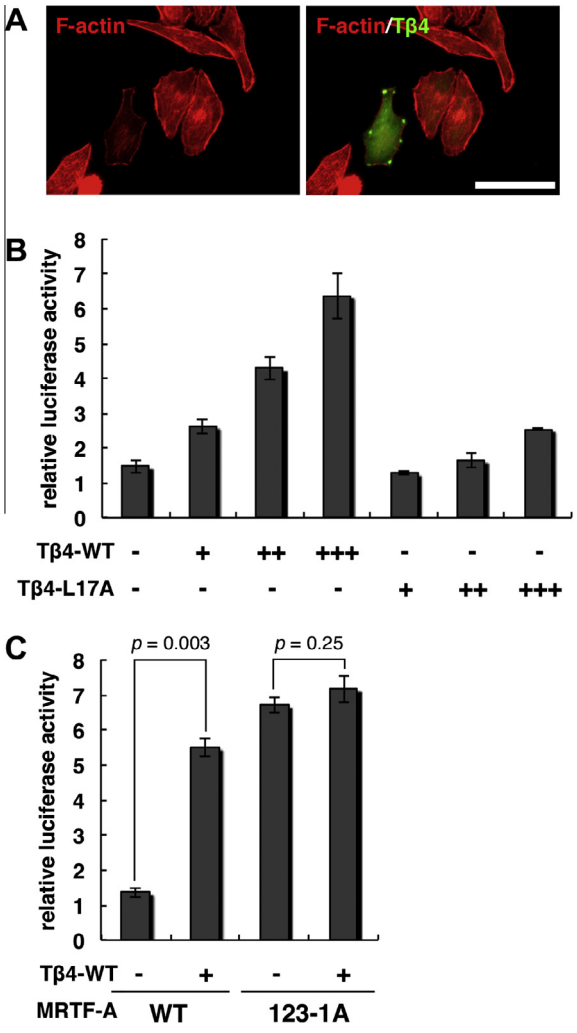

Fig. 1. Activation of MRTF–SRF signaling by Tβ4. (A) The HeLa cells were transfected with the Tβ4–myc expression plasmid. On the following day, the cells were fixed and stained with an anti-myc antibody (green) and phalloidin (red). Bar = 50 μm. (B) The HeLa cells were transfected with the SRF reporter 3× CArG-Luc and increasing amounts [15 (+), 80 (++), and 250 ng (+++)] of the Tβ4 expression plasmid, together with the MRTF-A expression plasmid and pSV-βGal. Luciferase and β-galactosidase activities were measured 2 days after transfection. Data represent the mean ± SEM of three independent experiments. (C) The HeLa cells were transfected with 3× CArG-Luc, the Tβ4 expression plasmid, the MRTF-A expression plasmid [wild-type (WT) or 123-1A], and pSV-βGal. Luciferase and β-galactosidase activities were measured 2 days after transfection. Statistical analysis was performed by paired Student's *t*-test. Data represent the mean ± SEM of three independent experiments.

regulation of MRTF activity, we performed a luciferase reporter assay using 3× CArG-Luc vector containing three copies of the SRF binding cis-element CArG box. Ectopic Tβ4 unexpectedly induced the activation of MRTF–SRF signaling in a dose-dependent manner (Fig. 1B).

In addition, Tβ4 has been reported to be secreted and function as a multi-functional trophic factor [25]. The secreted Tβ4 appears to function via an extracellular receptor in a different way from G-actin sequestering [26]. Point mutation of Tβ4 at position 17 leucine to alanine [Tβ4 (L17A)] significantly reduces its affinity for G-actin [27,28]. Tβ4 (L17A) more weakly activated the MRTF–SRF signaling pathway compared with wild-type Tβ4 (Fig 1B), indicating that G-actin binding is important for Tβ4-induced MRTF-A activation. MRTF-A (123-1A) mutant reportedly has no ability to bind to G-actin because the critical arginine sites in all three RPEL motifs are replaced with alanine [20]. MRTF-A (123-1A) functioned as a constitutively active form and was not additively activated by ectopic Tβ4 (Fig. 1C). These results suggested that Tβ4 bound to G-actin and modulated MRTF-A activity by regulating the G-actin–RPEL interaction within MRTF-A.

3.2. Tβ4 prevents the G-actin–MRTF-A interaction

Posern et al. report that profilin-binding G-actin cannot interact with MRTF-A [21], raising the possibility that Tβ4 also disrupts the G-actin–MRTF-A interaction. A coimmunoprecipitation assay clearly demonstrated that Tβ4 prevented the G-actin–MRTF-A binding in a dose-dependent manner (Fig. 2A). Furthermore, Tβ4 did not coimmunoprecipitate with the G-actin–MRTF-A complex, indicating that Tβ4 competed with MRTF-A for G-actin binding. In the NIH 3T3 cells, exogenous MRTF-A was predominantly localized in the cytoplasm, and excess Tβ4 induced the MRTF-A nuclear accumulation (Fig. 2B). Taken together, these results illustrated that Tβ4 dissociated the G-actin–MRTF-A complex by competing with MRTF-A for G-actin binding, resulting in the nuclear accumulation and transcriptional activation of G-actin-free MRTF-A.

3.3. Tβ4-low-affinity mutant of β-actin 3DA more effectively inhibits MRTF-A activity

Safer et al. report that lysine 18 of Tβ4 makes contact with N-terminal acidic residues of the skeletal muscle actin [29]. We constructed the β-actin mutant 3DA, in which three aspartic acid residues at the N-terminus were replaced with alanine. This mutant actin exhibited lower affinity for Tβ4 than wild-type β-actin (Fig. 3A). A previous study indicates that excessive expression of ectopic β-actin suppresses MRTF-A activity caused by an increase in the cytoplasmic G-actin pool; thus, non-polymerized mutants of β-actin more effectively suppress MRTF–SRF activity [30]. When 3DA actin was expressed in the NIH 3T3 cells, it polymerized and co-localized with F-actin in the same manner as wild-type β-actin (Fig. 3B). However, 3DA actin more effectively suppressed MRTF-A activity compared with wild-type β-actin (Fig. 3C). These results suggest that ectopic wild-type β-actin was partially sequestered from MRTF-A by binding to endogenous Tβ4, whereas 3DA actin was unable to do so. Considering that the majority of G-actin binds to Tβ4 and profilin, MRTF-A activity is likely to be regulated by the remaining free G-actin.

3.4. Tβ4-overexpression increases the expression levels of SRF-dependent cytoskeletal genes

We previously reported that MRTFs are critical regulators of actin cytoskeleton organization [2,3,6]. Overexpression of MRTFs markedly facilitates the formation of thick stress fibers with increased expression of SRF-dependent actin cytoskeletal genes, whereas knockdown of MRTFs results in the disappearance of stress fibers [2]. To examine whether excess Tβ4 enhances the

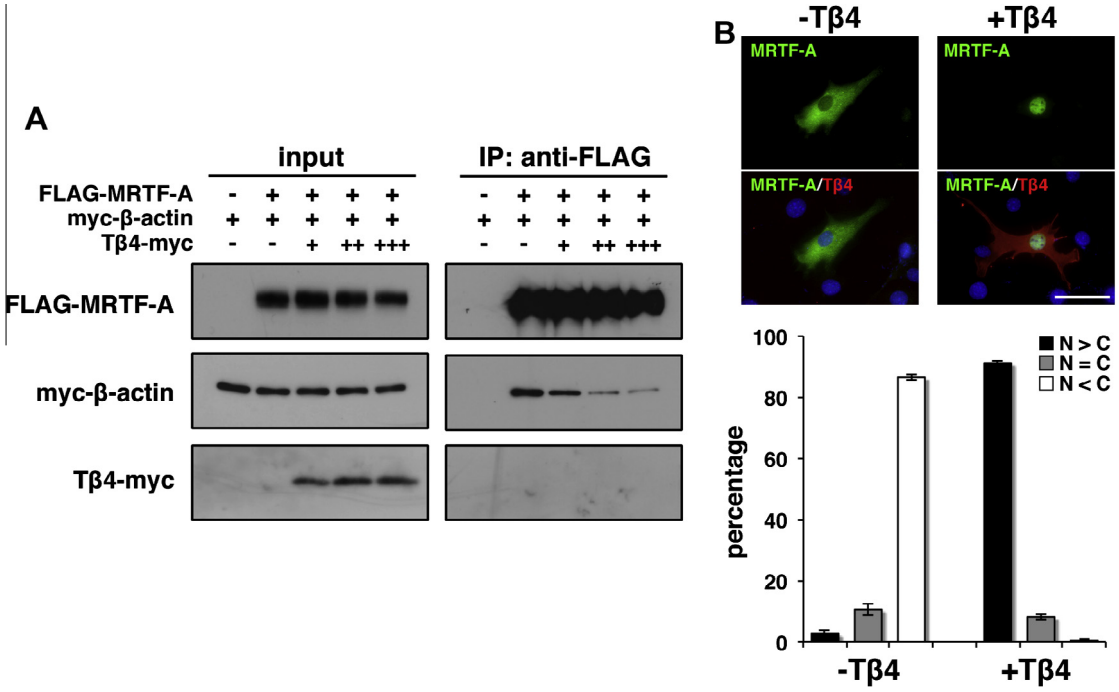

Fig. 2. Interference of the G-actin–MRTF-A complex formation by Tβ4. (A) The HEK293T cells were transfected with increasing amounts [0.3 (+), 0.6 (++), and 1.2 μg (+++)] of the Tβ4–myc expression plasmid, together with FLAG–MRTF-A and myc–β-actin expression plasmids. A coimmunoprecipitation assay was performed using FLAG-affinity gels, followed by a Western blot analysis with anti-FLAG and anti-myc antibodies. (B) The NIH 3T3 cells were transfected with FLAG–MRTF-A and Tβ4–myc expression plasmids. On the following day, the cells were fixed and stained with anti-FLAG (green) and anti-myc (red) antibodies. The nuclei were visualized by Hoechst 33342 (blue). The subcellular localization of MRTF-A was scored as predominantly nuclear (N > C), comparable intensity between the nucleus and cytoplasm (N = C), or predominantly cytoplasmic (N < C) in >100 transfected cells. Data represent the mean ± SEM of three independent experiments.

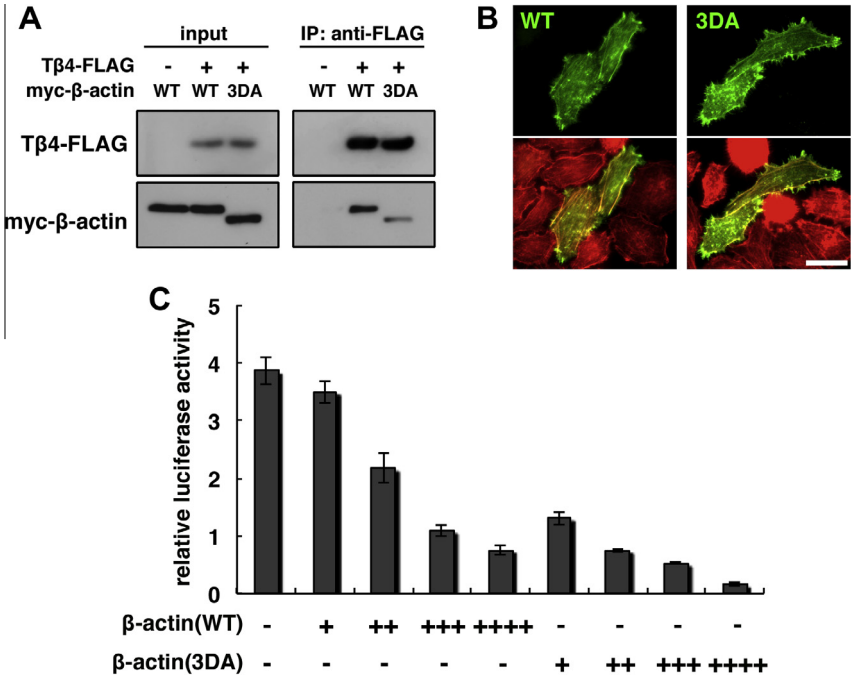

Fig. 3. Suppression of MRTF-A activity by β-actin mutant 3DA. (A) The HEK293T cells were transfected with Tβ4–FLAG and myc–β-actin [wild type (WT) or 3DA] expression plasmids. A coimmunoprecipitation assay was performed using FLAG-affinity gels, followed by a Western blot analysis with anti-FLAG and anti-myc antibodies. (B) The NIH 3T3 cells were transfected with myc–β-actin [wild-type (WT) or 3DA] expression plasmids. On the following day, the cells were fixed and stained with an anti-myc antibody (green) and phalloidin (red). Bar = 50 μm. (C) The HeLa cells were transfected with the SRF reporter 3× CArG-Luc and increasing amounts [30 (+), 60 (++), 120 (+++), and 250 ng (++++)] of the β-actin (WT or 3DA) expression plasmid, together with the MRTF-A expression plasmid and pSV-βGal. Luciferase and β-galactosidase activities were measured 2 days after transfection. Data represent the mean ± SEM of three independent experiments.

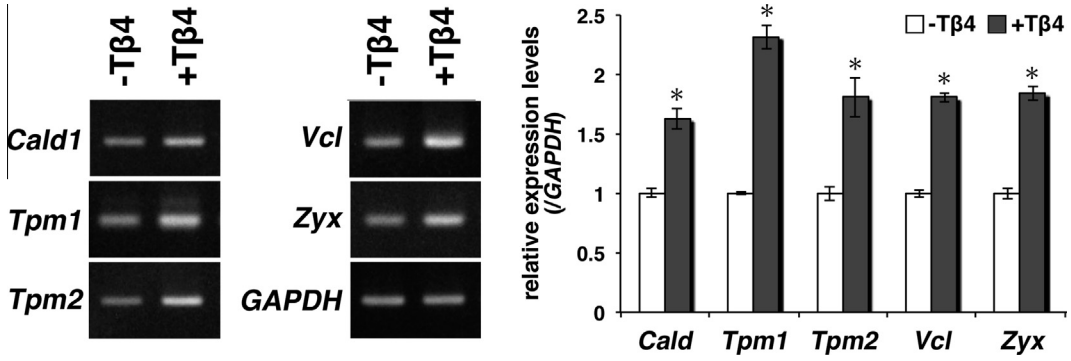

Fig. 4. Increased expression of the SRF-dependent actin cytoskeletal genes by Tβ4 overexpression. The NIH 3T3 cells were transfected with mock control plasmid (−Tβ4) or the Tβ4 expression plasmid (+Tβ4). On the following day, total RNAs were extracted from the cells and RT-PCR was performed using the indicated gene-specific primers. PCR products were quantified by densitometry, values were normalized to *GAPDH* mRNA, and results were statistically analyzed. Data represent the mean ± SEM of three independent experiments. $^{*}p < 0.03$ by paired Student's *t*-test.

activity of endogenous MRTFs, we investigated the effect of Tβ4 overexpression on the expression levels of the actin cytoskeletal genes. The NIH 3T3 cells overexpressing Tβ4 showed increased expression of SRF-dependent actin cytoskeletal genes (Fig. 4), supporting the notion that ectopic Tβ4 induces the activation of endogenous MRTFs. These results are consistent with a previous report that illustrates a twofold increase in the expression of myosin IIA, α-actinin, and tropomyosin proteins in cell lines overexpressing ectopic Tβ4 [31].

In summary, Tβ4 and MRTF-A compete for G-actin binding, and Tβ4 prevents the G-actin–MRTF-A interaction, resulting in the nuclear accumulation and transcriptional activation of MRTF-A. Thus, MRTF activity would be regulated by a small portion of the cytoplasmic G-actin pool that is free from Tβ4 and profilin. To the best of our knowledge, this is the first report to clarify the contribution of Tβ4 to the regulation of MRTF activity, and our results provide new insights into the function of Tβ4 and the regulatory mechanism of MRTFs.

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