Metal coordination of thymosin β 4: chemistry and possible implications

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Abstract

Thymosin β 4 (T β 4) was first isolated in the '60ies from calf thymus and was initially perceived as a thymic hormone with immunological effects on lymphocytes. It was then identified as a G-actin binding protein, featuring numerous functions in the human body including blood clotting, tissue regeneration, angiogenesis and tumor metastasis. T β 4 is also involved in anti-inflammatory and neurodegenerative processes. The exact mechanisms of action of T β 4 are still unknown, and the binding of the G-actin protein cannot itself explain the multi-activity of T β 4. We hypothesize that the property of T β 4 regulating the numerous physiological processes involving T β 4, is its essential metal - binding ability.

1. Introduction

The function of a protein is defined by its native conformation. Yet, over 30% of human proteins have no obvious tertiary structure and contain long, disordered regions [1, 2], especially those regulating gene transcription, cell signaling, and those linked to cancer and cardiovascular diseases [3-5]. Disordered proteins are flexible and undergo fast interconversion [6, 7]. They often contain multiple, distinct molecular recognition elements along their sequences [8-10] that allow them to interact with different target proteins or metal ions. Alternatively, such proteins can undergo fast conformational changes that allow a single binding site to interact with several different proteins [11]. As summarized in [12] disordered proteins can adopt a tertiary structure upon binding to their targets, even if the entropy associated with this transition is high. As a consequence, the interactions between an ordered protein and its target have a low affinity but a high specificity, and are of paramount importance in cell regulation [12].

Timbetasin, the name recommended by the World Health Organization [13] for T β 4, is encoded in humans by gene *TMSB4X* [14-16] (Figure S1). *TMSB4* is also present on chromosome Y, where it encodes a slightly different amino-acid sequence (Figure S1). The amino-acid sequence of T β 4 is the same for a great variety of species (Figure S1). Additionally, different human thymosin peptides exhibit the same N-terminal sequence (SDKPD) and actin binding motifs (KLKK) (Figure S1). T β 4 mRNA is expressed in many tissues, but mostly in those with high concentrations in calcium, iron, copper and zinc ions (Figure 1). T β 4 levels vary between different cells, and their intracellular concentrations may reach up to 0.5 mM [17]. Different diseases have been associated with the *TMSB4X* gene, such as osteoarthritis and invasive malignant thymoma, for which possible pathology pathways are ERK signaling and a response to elevated levels of platelet cytosolic calcium ions.

Figure 1. Tb4 mRNA expression (based on data from Su AI, Wiltshire T, Batalov S, et al (2004). "A gene atlas of the mouse and human protein-encoding transcriptomes". Proc. Natl. Acad. Sci. U.S.A. 101 (16): 6062–7) and essential metal ion concentrations (according to Report of the Task Group on Reference Man, International Commission on Radiological Protection publication 23, 1992, Pergamon Press), in selected human tissues.

AcSDKP is the N-terminal end of Tb4 that can be cleaved enzymatically [18]; it regulates different cellular processes such as angiogenesis, cell differentiation and proliferation (further discussed in section 4).

Human T β 4 protein is composed of 43 amino acids (Figure S1), and contains 9 lysine residues (Figure 2, yellow color); however, it is classified as an acidic protein (pI 5.1) [19] due to the presence of 11 amino-acids with carboxylic acid groups in their side- chains (Figure 2, red color). As shown in table 1, lysine, glutamic and aspartic groups are natural metal- binding sites.

Table 1. Amino acids with metal- binding sites in T β 4 and other peptides or proteins.

Ligand	Number of residues in T β 4	Essential metal- binding	Toxic metal-binding	Reference
Glutamic acid	8	Ca ²⁺ , Mg ²⁺ , Fe ³⁺ , Fe ²⁺	La ³⁺ , Yb ³⁺	[20-22]
Aspartic acid	3	Ca ²⁺ , Mg ²⁺ , Fe ³⁺ , Fe ²⁺ , Cu ²⁺ , Ni ²⁺ , Zn ²⁺ , Cd ²⁺	La ³⁺ , Yb ³⁺	[22, 23]
Lysine	9	Cu ²⁺	Ni ²⁺ , Co ²⁺ , Cd ²⁺ , Al ³⁺	[22, 23]

T β 4's structure was characterized for the first time in 1990 :[24]. NMR and CD spectra in water showed no stable regular structure; however, the addition of Trifluoroethanol (TFE) led to the formation of a stable tetriary structure consisting of two α -helices. However, TFE is known to foster the formation of helical secondary motifs in the polypeptide chains. The structural data that were obtained were not deposited in PBD and Figure 2 shows the X-ray structure of T β 4 extrapolated from the complex with actin (PDB: 4PL7). The 3D structure of T β 4 (Figure 2) has the same structural characteristics as those reported in 1990 [24] for T β 4. It consists of two helices: the first α -helix ranges from Pro4 to Lys16 and has an asymmetrical charge distribution, both in the first and last helical turns; the second α -helix ranges from Ser30 to Ala40. Figure 2 shows that the first helix has an asymmetrical charge distribution, both in the first and last helical turns. The tertiary structure, shown in Figure 2, may be the result from T β 4's

binding to actin, or may simply be due to the mode of preparation of the crystal, in a buffer consisting of a solution of citric acid (pH 4.5) and 15% (wt/vol) of PEG 3350, at 15°C [25].

Figure 2. X-ray structure of Tβ4 extrapolated from the complex with actin (PDB 4PL7). Asp and Glu residues are marked in red; Lys residues are indicated in blue. Amino acids in parentheses were not defined by the X-ray structure.

Proteins that specifically bind to Ca^{2+} can have different specific structural motifs. One of them is the helix-loop-helix EF-hand motif, in which the two helices are inclined at approximately 90° , with Ca^{2+} ions being bound to side chains and carbonyl oxygens of amino-acid being in the loop [26]. The related calcium-binding amino-acid sequence is often DxDxDG [27]. In all EF-hand proteins, the calcium ion has seven binding sites: three monodentate Asp or Asn residues, one bidentate Glu residue, one bound water molecule and one peptide carbonyl group. As shown in Figure 2, the folded structure of Tβ4 contains a comparable helix-loop-helix motif and numerous Asp and Glu residues, meaning that one Tβ4 may be coordinated to up to three calcium ions.

Free iron ions are toxic for cells and tissues; however, when bound to proteins, they are often essential for their biological activity. In hemoproteins, iron is bound to porphyrin, where it transports O₂ or activates it in electron transfer reactions. Iron-sulfur proteins are involved in electron transfer as well, whereas ferritin and transferrin participate in the storage and transport of iron ions, respectively. Compared to binding by porphyrin, iron ions are bound less tightly to carboxylic groups of the amino acid side chains in transferrin and ferritin in order to facilitate release from the protein, when necessary. Multiple Asp or Glu residues in close spatial proximity constitute iron-binding motifs, the REXXE iron-binding motif that is present in ferritin is also found in other metal-binding proteins, such as APP [28].

Prothymosin α (ProT α) is only peptide of the thymosin family for which experimental studies for metal ion binding in the presence of zinc ions have been published so far. ProT α 's is similar in amino acid sequence and structure to T β 4, and can thus serve as a model for metal ion binding of the latter one. Previous studies have shown that the binding of ProT α to target -proteins such as Keap1 [29], cytochrome c [30], and Rev [31] is mediated by zinc ions. Choy et al. [32] demonstrated that Zn²⁺ binds at carboxyl groups of Glu and Asp [31, 33] at the C-terminal end of ProT α , leading to local folding while the N-terminal part (of ProT α) remains unstructured. The formation of a helical structure on the C-terminal end upon zinc binding improves the recognition of targets by the ProT α peptide, and thus allows zinc ions to have switch control of the ProT α interactions.

To our knowledge, no chemical or molecular dynamics study of the formation of metal complexes has been performed with T β 4 despite there are numerous potential metal-binding sites. Yet, the identification and characterization of metal ion-binding sites in T β 4 would be a very important step forward in the recognition of peptide function and, consequently, the development of molecular drugs to treat human diseases involving T β 4.

2. Cellular localization of Thymosin β 4

At the subcellular level, T β 4 is localized in the cytoplasm [34, 35], nucleus [36, 37] or inside organelles, and can be translocated between compartments after specific induction [38, 39]. Moreover, T β 4 can be excreted from the cells and take part in signaling; however, its mechanism of action remains unknown. It is possible that T β 4 takes part in the extracellular transport of exosomes, where, together with miRNAs, it mediates the communication between cells [40].

In HepG2 cells, T β 4 is present mainly in the cytoplasm and, in the nuclear compartment, T β 4 is present only inside the nucleolus [41]. It is likely that T β 4 regulates the polymerization of actin filaments in the nucleolus, and consequently modulates nucleolar transcription processes [41]. T β 4 undergoes various conformational changes, and translocates from one cytoplasmic domain to another, from the cytoplasm to the nucleus and back, under different environmental stimuli (Figure 3). In mitotic cells, T β 4 moves from vacuoles to the cytoskeleton; In HepG2 cells, the scarcity of nutrients results in a lower expression of T β 4 and a translocation of the peptide from vacuoles to the trans-Golgi network [28]. Transmission immunoelectron microscopy studies have shown that T β 4 translocates to the nucleoplasm in starving cells, where it may interact with nuclear actin and remodel chromatin [41] .

Figure 3. Calcium uptake to cells is controlled by different channels, e.g. ORAI (1 and 2) and P2X4, which is an ATP gated channel. Moreover, cytosolic calcium levels are increased by the release of calcium ions from the ER, which is mediated by STIMs (1 and 2) calcium sensors. High cytosolic calcium levels lead to reorganization of actin cytoskeleton and increase of TNF- α , IL- β and NO production, which provoke inflammation. In addition, calcium ions regulate the permeability of nuclear pores. T β 4 has the ability to bind different targets: ATP synthase and G-actin as well as metal ions like calcium. Upon binding to the targets, the unstructured T β 4 assumes tertiary structure. T β 4 is localized mainly in the cytoplasm, but after induction may translocate to different cellular compartments e.g. nucleus. [Images produced by KAUST scientific illustrators This image is original and designed specifically for the targeted publication. It should not be cropped, distorted or in any way edited without the expressed consent of Research Publication Services. Because KAUST owns the copyright of the original image, you may cause a copyright conflict between the journal and KAUST if you modify the illustration in any way before submitting it to the journal. Any other use of the image apart from your paper (e.g., in a presentation, poster, or website) should be accompanied by credits to the journal in which it is published and to the illustrator as follows: Ivan Gromicho/KAUST].

Full-length T β 4, and partial polypeptides T β 4¹⁷⁻²³ (KLKKTET, actin-binding motif) and T β 4¹⁴⁻¹⁹ (DKSKLK, nucleus translocation motif^{32,38}) were extensively studied by Zoubek et al. [42]. They showed that the nuclear localization of the representative fragments T β 4¹⁻²⁶, T β 4²⁷⁻⁴³ and T β 4⁷⁻⁴³, in living cells, had an equal distribution between the cytoplasm and the nucleoplasm, regardless of the basic cluster of lysine residues, or their affinity to G-actin [42]. In this case, T β 4 is probably translocated to the nucleus through the nuclear pore, or transported by a single transport protein (Figure 3). Molecules smaller than 45-60 kDa (depending on the type of cell) may pass through the nuclear pore complex (NPC) by passive diffusion, whereas bigger molecules with a nuclear localization signal (NLS) sequence require an energy-dependent multi-step mechanism in order to enter or exit the nucleus [43-45]. In 2004, Thorogate et al. [46] showed that the passive diffusion of small molecules (10-20 kDa) was regulated by calcium ions. The release of calcium from the endoplasmic reticulum (ER) regulates the permeability of the pore and prevents the passive diffusion of molecules larger than 10 kDa [44, 47-50]. In contrast, the addition of Ca²⁺ and ATP to an isolated nuclei bath reverses the blockade of medium-sized molecules [50].

We thus hypothesize that calcium ions may bind to T β 4, resulting in structural changes of T β 4 that facilitate its translocation to the nucleus (Figure 3). It is also possible that T β 4 channels calcium ions into the periplasm of the nucleus, and mediates calcium signaling on the cytoplasm ↔ nucleus route.

3. Actin binding

T β 4 is a main actin-sequestering protein in many cell types; it plays an important role in the organization of the cytoskeleton and the cell motility. T β 4 sequesters G-actin and prevents the formation of F-actin filaments. Profilins, proteins with a molecular mass of ~ 16 kDa, also sequester G-actin, preclude the nucleation or participation in the elongation of pointed-end filaments. In contrast to T β 4, they dissociate from the actin monomers, as the monomer gets incorporated into the filament, where it

plays a role in the elongation of barbed-end filaments (Figure 4). T β 4 competes with profilin for actin binding through the formation of a tertiary complex T β 4-actin-profilin.

Figure 4. Polymerization scheme of actin monomers of G-actin into filaments of F-actin. The following PDB structures were used for the scheme preparation: 3HBT, 4PL7, 2PBD

In 2014, two crystal structures of the T β 4:actin complex were obtained by Robinson et al. using X-ray diffraction analysis and then compared with the structures of the profilin:actin complex. This structure comparison, together with molecular dynamic simulations, biochemical assays and principal component analysis of allowed the authors to propose a mechanism for the exchange between T β 4 and profilin in actin binding (Figure 4). In this model, protein-protein binding reciprocally stabilizes different actin conformations that are suboptimal for the other competing protein for profilin:actin (lower the distance between sub-domain 1 and 3) and T β 4:actin (recedes sub-domains 2 and 4 and closer 1 and 3) complexes.

The T β 4:actin's crystal structure showed that the C-terminal α -helix of T β 4, not the actin-binding motif, was, in fact the most important part in the sequestration of G-actin. The C-terminal helix mediates

the interaction between T β 4 and actin in both T β 4:actin and profilin:actin:T β 4 complexes and induces a change in the conformation of actin to its closed, pointed- face cleft conformation. A small fraction of the T β 4:actin and profilin:actin:T β 4 complexes, however, exhibits no interaction of the T β 4 C-terminal α -helix with the actin protomer. In these cases where the T β 4 C-terminal α -helix does not provide steric hindrance, the actin monomer in the T β 4:actin and profilin:actin:T β 4 complexes can only be sequestered by an allosteric mechanism [21]. As outlined above, the C-terminal part of T β 4 consists is rich in Glu and Asp residues that potentially coordinate calcium ions; metal ion binding has, however to this day never been experimentally demonstrated. Even if the available PDB crystal structures of T β 4:actin (PDB: 4PL7 and 4PL8) and profilin:actin (PDB: 2PBD) contain ATP:Ca²⁺ in the center of the complex, the presence of the metal has never been discussed by the authors, nor explored as playing an active role in the elongation process of actin. In our opinion, the role of calcium in the polymerization process of actin has, so far, been greatly underestimated. Yet, a clear understanding of its mechanism of action is necessary to comprehend and control the activity of the cytoskeleton.

During polymerization phases, the profilin–actin complex gives back its actin to the elongating filaments. The free profilin then competes with T β 4 for binding ATP–G-actin, and effectively restores the pool of polymerization- competent actin. However, the underlying mechanisms of this complex process remain unknown due to the dynamic nature of the interactions involved [51-54]. ATP-actin monomers possess a high affinity for Ca²⁺ ions (K_d = 2-8 nM), and the polymerization of actin monomers lowers the metal exchange rate at its binding site by more than 3 orders of magnitude, making the bound calcium ions almost unavailable. F-actin-bound Ca²⁺ can be released by depolymerization and dissociation from Ca²⁺-ADP-actin monomers (K_d =375 nM). Small amounts of profilin release Ca²⁺, both from Ca and Mg/Ca-F-actin; they also inhibit the uptake of Ca²⁺ by Mg/ Ca-F-actin [55]. The exact

mechanism and role of calcium ions in the exchange of G-actin with F-actin has, however, to our knowledge, not yet been elucidated.

4. Tumor growth and metastasis

The growth of solid tumors depends on the formation of new blood vessels, as the supply in oxygen to the tumor cells diminishes [53]. In cells and tissues, hypoxia induces the expression of genes relevant for angiogenesis, vasorelaxation, metabolism, cell proliferation or survival, and the resistance to anti-cancer drugs [56-58]. T β 4 actually contributes to regulate gene expression in hypoxia-dependent cell migration, angiogenesis and tumor metastasis [59-62]. In addition, T β 4 is a possible mediator of extracellular stimulation and downstream signaling molecules, such as HIF-1 α [63, 64].

The potential role of calcium ions in tumor metastasis is a relatively new topic in oncology. Many elements of the action mechanism of metastasis have not yet been investigated in detail, and for those that are already known, the specific roles in migration, invasion and metastasis of different types of cancers are only beginning to be understood [65]. Impaired calcium signaling nevertheless is an important factor in the metastatic process. Variable levels of Ca²⁺-handling proteins and/or expression of Ca²⁺-dependent effectors have been found in tumor cells, where they are implicated to lead to higher turnover of focal adhesions and a more effective proteolysis of extracellular matrix components. Research studies have also shown that the expression patterns of Ca²⁺-permeable ion channels, as well as their degree of functionality, change as the cancer progresses [65].

In most cancer cells, calcium ions are trafficked via a store-operated calcium entry (SOCE) mechanism mediated by calcium sensor STIMs (STIM1 and 2) in the endoplasmic reticulum, and through a plasma- membrane channel formed by the Orais (Orai 1-3) unit (Figure 3). When dysregulated, SOCE

may facilitate the metastatic dissemination and its colonization by reorganizing the actin cytoskeleton, degrading the extracellular matrix and remodeling the tumor's microenvironment [66].

Intracellular Ca^{2+} initiates the transcription of different immediate early genes inducing cell proliferation and motility [67]. Often, an increase in $[\text{Ca}^{2+}]_i$ leads to the production of nitric oxide, which, in turn, promotes the proliferation of endothelial cells in wound healing [68].

The presence of T β 4 in various cellular processes has been well-documented by numerous research studies, but the signaling mechanisms that modulate the T β 4-induced activation of endothelial cells and their migration remain unclear. T β 4 interacts with ecto-ATP synthase in endothelial cells, and increases the ATP levels of surface cells. The ATP-responsive P2X4 receptor that supports the migration of HUVEC cells may be involved in the related extracellular signaling pathway [69]. P2X4 belongs to the family of P2X mammalian receptors; these receptors are ATP-gated ion channels [70] that, with the exception of P2X5, facilitate the entry of ions into the cell, when stimulated by extracellular ATP [69] (Figure 3).

Application of peptide AcSDKP generates angiogenesis *in vitro* and *in vivo* [71]. It has an inhibitory effect on the proliferation and differentiation of hematopoietic progenitor cells, and a protective effect on the early hematopoietic cells [18, 64]. AcSDKP further stimulates the release of Ca^{2+} from the pool of $[\text{Ca}^{2+}]_i$, and releases a potent extracellular calcium influx in HL-60 cells [18].

Nevertheless, in 2012, the migration of HUVEC cells and the formation of tubular structures in Matrigel (a complex protein mixture required for optimal growth of cell culture) was shown to improve by an external treatment with T β 4 and its mutant forms without the N-terminal tetrapeptide AcSDKP (T β 4_{AcSDKPT/4A}), and without the actin-binding sequence KLKKTET (T β 4_{KLKKTET/7A}). In contrast to observations reported by prior studies [18], T β 4 initiated cellular processes, without changes in intracellular Ca^{2+} concentration [72].

The exact nature of the relationship between calcium and T β 4 thus is still unclear, but there is limited indirect evidence in support of the involvement of calcium ions in T β 4 interactions inside the cell. Understanding these interactions would allow us to elucidate the extent to which they contribute to tumor metastasis. Given the multitude of disease-specific and other critical pathways that can be modulated by calcium and T β 4, their pharmacological targeting may be a therapeutic approach in slowing the progression of tumor metastasis in the long run.

5. Tissue regeneration

Numerous cellular and whole-animal studies have confirmed the important role of T β 4 in tissue regeneration [73], which has led to numerous therapeutic applications successful enough to be extended to human clinical trial [74]. The broad regenerative effects of T β 4 are unlikely to be exerted only via intracellular G-actin sequestration, as many affected cells already exhibit high intracellular T β 4 concentrations, and would not need to uptake additional amounts of T β 4. This means that there should be targets other than actin, probably receptors with extracellular ligand-binding domains that can contribute to tissue regeneration [75]. The β sub-unit of cell surface-located ATP synthase, which has a high affinity for T β 4, would allow T β 4 to signal via a purinergic receptor [61]. In addition, AcSDKP inhibits the proliferation of haematopoietic stem cells from bone marrow, and may mediate some of T β 4's roles, other than actin binding [64].

T β 4 is also highly expressed in human heart, although its role there still remains poorly understood. In 2018, Wilson et al. [76] proved expression of T β 4 in the developing human heart, and demonstrated that T β 4 was primarily localized in endothelial cells of the cardiac microvasculature, coronary vessels, and endothelial-like cells of the endocardium and epicardium [76]. The tissue-regenerating effects of T β 4 were recently studied for the repair of human heart muscle after myocardial

infarction or other pathologies of the heart. Mice treated with T β 4 started to grow new heart- muscle cells from inactive precursor cells in the outer lining of adult heart [77], which would then migrated to the inside of the heart muscle and form new blood vessels [78] . Exogenous treatment with recombinant T β 4 reduced infarct sizes and fibrotic scarring, thus improving cardiac output in various *in vivo* models of myocardial infarction [79-81].

In 2012, Riley et al. [82] demonstrated that T β 4 activates epicardium-derived coronary vasculogenesis and leads to the activation of quiescent adult epicardial cells (EPDCs). Once stimulated, EPDCs facilitate the neovascularization of ischemic adult heart and contribute to the regeneration of cardiomyocytes. EPDC-derived cardiomyocytes structurally and functionally integrate with the resident muscle to regenerate functional myocardium. This limits pathological remodeling, resulting in an improvement of the cardiac function. Moreover, the functional integration of YFP+ cardiomyocytes with existing myocardium was proven upon measurements of intracellular calcium [Ca²⁺]_i transient with neighboring YFP– cardiomyocytes. These results suggest that T β 4-primed EPDC-derivatives get associated with surviving cardiomyocytes following an injury, and form a functional syncytium with the resident myocardium. A MRI analysis over a 28-day period after myocardial infarction revealed that a treatment with T β 4 results in a significant improvement of the ejection fraction and diastolic/systolic volumes over time [82].

6. Anti-inflammatory activity against neurodegeneration.

It is a well-known paradigm in neurodegeneration that an excess of free redox- active metals (e.g. copper and iron) is leading to ROS production and inflammation. Brain tissue is highly sensitive to ROS species due to a high consumption of oxygen and low prevalence of anti-oxidative defense mechanism. Biomolecules damaged by ROS' may become misfolded, aggregate, and accumulate within intracellular inclusion bodies. Such inclusions have been found in different neurodegenerative disorders, such as

Alzheimer's disease (AD), Parkinson's disease (AD), amyotrophic lateral sclerosis (ALS) and Huntington's disease (HD) [83].

T β 4 is present in the brain in high quantities, yet its function in the central nervous system (CNS) in molecular terms is unknown [84]. T β 4 shapes the development of CNS, regulates the proliferation of progenitor cells, neurogenesis, tangential expansion, and neurite outgrowth [85, 86]. During the chronic stage after neural injury or clinical onset of symptoms, T β 4 provides neurological benefit by amplifying neurovascular remodeling [87]. A treatment with T β 4 may lead to recovery in different neurological diseases by promoting plasticity and neurovascular remodeling of CNS and the peripheral nervous system (PNS) [36]. T β 4 improves angiogenesis, neurogenesis, neurite and axonal outgrowth, and oligodendrogenesis, and thereby significantly improves functional and behavioral outcomes even if T β 4 treatment is started 24 h or more after an injury or the onset of disease [40]. A continuous 7-day- intra-hippocampal infusion of AcSDKP in mice has shown a permanent improvement in spatial memory, after 5 weeks [88].

In the brain of Huntington's and Alzheimer's patients, T β 4 is found to be elevated in the cell bodies and cell processes of reactive microglia in the brain regions affected by the neurodegeneration of T β 4's immunoreactivity in microglia, at the early stage of Huntington's disease [89]. Considering gliosis as a first protective mechanism in neuropathological diseases, it is likely that increasing T β 4 concentration has anti-inflammatory and repair functions in microglia [90]. Indeed, reactive gliosis provokes the induction of T β 4, which, in turn suppresses proinflammatory TLR signaling [91, 92], and the effects of exogenous application of T β 4 are the same as those of T β 4 expression inside microglial cells. The study of Zhang et al. [92] conducted on T β 4-knockdown microglial cells showed an increase of pro-inflammatory cytokines (tumor necrosis factor α (TNF- α) and Interleukin-1 β (IL1 β)) concentrations in

ethanol-induced inflammatory conditions, and reversal of the inflammatory process by exogenous treatment with Tβ4 [93]. Tβ4 can modulate microglial activity, both intracellularly and extracellularly. When applied exogenously, Tβ4 decreases the expression of inflammatory mediators and the secretion of hypoxia-induced TNF-α, IL-1β and nitric oxide (NO), *in vivo*. *In vitro* experiments by Zhou et al. also showed that microglial activation was decreased after treatment with Tβ4 [94]. Intracellular calcium ions are involved in the inflammatory process, and their concentration increases in the presence of TNF-α, IL-1β, and nitric oxide (NO) [95] (Figure 3). A delay of the increase in the amount of $[Ca^{2+}]_i$ ions, observed shortly before a cell dies, can be prevented by removal of extracellular Ca^{2+} [96].

Despite in-depth studies on Tβ4 in recent years, the putative receptors of Tβ4 in the cell membrane and the participation of Tβ4 in different signaling pathways are still poorly understood [92]. Tβ4 could be also sequestering calcium, as well as iron, copper or zinc ions, and thus prevent inflammatory processes and cell death.

Conclusions

Tβ4 is present in numerous tissues where it is involved in physiological as well as pathological processes. Its high mRNA expression in specific cells is correlated with high calcium, iron, zinc and copper metal ion concentrations in those cells. Tβ4 is an unstructured peptide with different protein- and potential metal- binding sites that, upon binding to its target, assumes a tertiary structure. The exact mechanism of action of Tβ4 in numerous cellular processes is still elusive, and the binding of metal ions to Tβ4 has never been directly studied. We hypothesize that the metal coordination is a driving force in Tβ4 cellular translocation and actin binding, with further influences on tissue regeneration, tumor metastasis and neurodegeneration. As shown in our review, there are many premises that calcium and other metal ions are coordinated by thymosin in biological processes, but there is clear need for more research. We believe

that elucidating the mechanisms that govern the metal coordination by T β 4 will likely be an important step forward in the recognition of peptide function, and consequently the control of metal content as a tool to switch T β 4 pathological functioning into physiological processes with multiple medical implications.

List of abbreviations

[Ca²⁺]_i Intracellular calcium ions concentration

AD Alzheimer's disease

ALS amyotrophic lateral sclerosis

APP Amyloid Precursor Protein

ATP Adenosine triphosphate

CNS central nervous system

EPDCs quiescent adult epicardial cells

ER endoplasmic reticulum

ERK extracellular signal-regulated kinase

HD Huntington's disease

HL-60 human leukemia cell line

HUVEC Human Umbilical Vein Endothelial Cells

IL1 β Interleukin-1 β

MRI magnetic resonance imaging

NLS nuclear localization signal

NO nitric oxide

NPC nuclear pore complex

PD Parkinson's disease

PEG Polyethylene Glycol
 PNS peripheral nervous system
 ROS reactive oxygen species
 SOCE store-operated calcium entry
 STIM stromal interaction molecule
 TLR Toll-like receptors
 TNF- α tumor necrosis factor α
 T β 4 thymosin beta 4
 YFP yellow fluorescent protein

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Figure S1. Human Thymosin $\beta 4$ encoded by *TMSB4X* gene alignment with A) its homolog on *TMSB4Y* gene, B) other human thymosine sequences and C) Thymosin $\beta 4$ of chosen species. The amino-acid sequence differences are signed in red.

Metal coordination chemistry of Thymosin β 4 and its possible implications.

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Abstract

Thymosin β 4 (T β 4) was isolated in the '60s from calf thymus and was initially perceived as a thymic hormone with immunological effects on lymphocytes. It was then identified as a G-actin binding protein, featuring numerous functions in the human body including blood clotting, tissue regeneration, angiogenesis and tumor metastasis. T β 4 is also involved in anti-inflammatory and neurodegenerative processes. The exact mechanisms of action of T β 4 are still unknown, and the binding of the G-actin protein cannot itself explain the multi-activity of T β 4. We hypothesize that the property of T β 4 regulating the numerous physiological processes involving T β 4, is its essential metal - binding ability.

1. Introduction

The function of a protein is defined by its native conformation. Yet, over 30% of human proteins have no tertiary structure and contain long, disordered regions[1, 2], especially those regulating gene transcription, cell signaling, and those linked to cancer and cardiovascular diseases[3-5]. Disordered proteins are flexible and undergo fast interconversion[6, 7]. They often contain multiple, distinct molecular recognition elements, along their sequences[8-10], that allow them to interact with different target proteins or metals. Alternatively, proteins can undergo fast conformational changes that allow a single binding site to interact with several different proteins[11]. Numerous studies have shown that disordered proteins adopt a ternary structure, upon binding to their targets[12], even if the entropy associated with this transition is high. As a consequence, the interactions between an ordered protein and its target have a low affinity but a high specificity, and are of paramount importance in cell regulation[12].

Timbetasin, the official generic, non-proprietary name recommended by the World Health Organization[13] for T β 4, is encoded in humans by the *TMSB4X* gene[14-16] (Figure S1). *TMSB4* is also present on chromosome Y, where it encodes a slightly different amino-acid sequence (Figure S1). The amino-acid sequence of T β 4 is the same for a great variety of species (Figure S1). Additionally, different human thymosin peptides exhibit the same N-end sequence (SDKPD) and actin binding motifs (KLKK) (Figure S1). T β 4 mRNA is expressed in many tissues, but mostly in those with high concentrations in calcium, iron, copper and zinc ions (Figure 1). T β 4 levels vary between different cells, and their intracellular concentrations may reach up to 0.5 mM[17]. Different diseases have been associated with the *TMSB4X* gene, such as osteoarthritis and invasive malignant thymoma, for which

possible pathology pathways are ERK signaling and a response to elevated levels of platelet cytosolic calcium ions.

Figure 1. Tβ4 mRNA expression and essential metal ion concentrations in selected human tissues.

AcSDKP is the N-terminal end of Tβ4 that can be cleaved enzymatically[18]; it regulates different cellular processes such as angiogenesis, cell differentiation and proliferation (further discussed in section 4).

Human T β 4 protein is composed of 43 amino acids (Figure S1), and contains 9 lysine residues (Figure 2, yellow color); however, it is classified as an acidic protein (pI 5.1)[19], due to the presence of its 11 amino-acids with carboxylic groups in the side- chains (Figure 2, red color). As shown in table 1, lysine, glutamic and aspartic groups are natural metal- binding sites.

Table 1. Amino acids with metal- binding sites in T β 4 and other peptides or proteins.

Ligand	Number of residues in HSA	Essential metal- binding	Toxic metal-binding	Reference
Glutamic acid	8	Ca ²⁺ , Mg ²⁺ , Fe ³⁺ , Fe ²⁺	La ³⁺ , Yb ³⁺	[20-22]
Aspartic acid	3	Ca ²⁺ , Mg ²⁺ , Fe ³⁺ , Fe ²⁺ , Cu ²⁺ , Ni ²⁺ , Zn ²⁺ , Cd ²⁺	La ³⁺ , Yb ³⁺	[22, 23]
Lysine	9	Cu ²⁺	Ni ²⁺ , Co ²⁺ , Cd ²⁺ , Al ³⁺	[22, 23]

T β 4's structure was characterized for the first time in 1990[24]. NMR and CD spectra in water showed no regular structure; however, the addition of Trifluoroethanol (TFE) led to the formation of a ternary structure consisting of two α -helices. However, TFE is known to foster the formation of helical secondary motifs in the polypeptide chains. The structural data that were obtained were not deposited in PDB and Figure 2 shows the X-ray structure of T β 4 extrapolated from the complex with actin (PDB 4PL7). The 3D structure of T β 4 (Figure 2) has the same structural characteristics as those reported in 1990[24] for T β 4. It consists of two helices: the first α -helix ranges from Pro4 to Lys16 and has an asymmetrical charge distribution, both in the first and last helical turns; the second α -helix ranges from Ser30 to Ala40. Figure 2 shows that the first helix has an asymmetrical charge distribution, both in the first and last helical turns. The ternary structure, shown in Figure 2, may be the result from T β 4's binding

to actin, or may simply be due to the mode of preparation of the crystal, in a buffer consisting of a solution of citric acid (pH 4.5) and 15% (wt/vol) of PEG 3350, at 15°C[25].

Figure 2. X-ray structure of Tβ4 extrapolated from the complex with actin (PDB 4PL7). Asp and Glu residues are marked in red; Lys residues are indicated in blue. Amino acids in brackets were not defined by the X-ray structure.

Proteins that specifically bind to Ca^{2+} have different specific structural motifs. One of them is the helix-loop-helix EF-hand motif, in which the two helices are inclined at approximately 90° , Ca^{2+} ions are bound to side chains, and carbonyl oxygens of amino-acid are in the loop[26]. The calcium- binding amino-acid sequence is often DxDxDG [27]. As shown in Figure 2, the folded structure of Tβ4 contains an helix-loop-helix motif and numerous Asp and Glu residues. In all EF-hand proteins, the calcium ion has seven binding sites: three monodentate Asp or Asn residues, one bidentate Glu residue, one bound water molecule and one peptide carbonyl group. This means that one Tβ4 may be coordinated to up to three calcium ions.

Free iron ions are toxic for cells and tissues; however, when bound to metallo-proteins, they are responsible for their biological activity. In haemoproteins, the iron is bound to porphyrin, where it transports or activate O₂ in electron transfer reactions. Iron-sulfur proteins are involved in electron transfer, whereas ferritin and transferrin participate in the storage and transport of iron ions, respectively. Compared to porphyrin, iron ions are bound less tightly to carboxylic groups of the amino acid side chains (transferrin and ferritin), in order to be released from the protein, when necessary. Numerous Asp or Glu residues in close spatial proximity constitute the iron-binding motifs. The REXXE iron-binding motif that is present in ferritin was also found in other metal-binding proteins, such as APP[28].

The only peptide of the thymosin family that has been studied in the presence of zinc ions is Prothymosin α (ProT α). ProT α 's sequence of amino-acids and structure are similar to those found in T β 4, and can be used as a zinc-binding model. Previous studies have shown that the binding of ProT α to target-proteins such as Keap1[29], cytochrome c[30], and Rev[31] is mediated by zinc. Choy et al.[32] demonstrated that Zn²⁺ binds, via the carboxyl groups of Glu and Asp[31, 33], to the C-terminal of ProT α , and leads to its folding, whereas the N-terminal part (of ProT α) remains unstructured. The formation of a helical structure on the C-end, upon zinc binding, improves the recognition of targets by the ProT α peptide, and allows zinc ions to have switch control of the ProT α interactions.

To our knowledge, no chemical or molecular dynamics study of the formation of metal complexes has been performed, even though there are numerous metal-binding sites in T β 4. Yet, the identification and characterization of metal ion-binding sites in T β 4 would be a very important step forward in the recognition of peptide function and, consequently, the development of molecular drugs to treat human diseases involving T β 4.

2. Cellular localization of Thymosine β 4

At the subcellular level, T β 4 is localized in the cytoplasm[34, 35], nucleus[36, 37] or inside organelles, and can be translocated after specific induction[38, 39]. Moreover, T β 4 can be excreted from the cells and take part in signaling pathways; however, its mechanism of action remains unknown. It is possible that T β 4 takes part in the extracellular transport of exosomes, where, together with miRNAs, it mediates the communication between cells[40].

In HepG2 cells, T β 4 is present mainly in the cytoplasm, whereas, in the nuclear compartment, T β 4 is present only inside the nucleolus[41]. It is likely that T β 4 regulates the polymerization of actin filaments in the nucleolus, and consequently modulates nucleolar transcription processes[41]. T β 4 induces various conformational changes, and translocates from one cytoplasmic domain to another, from the cytoplasm to the nucleus and back, under different environmental stimuli (Figure 3). In mitotic cells, T β 4 moves from vacuoles to the cytoskeleton; In HepG2 cells, the scarcity of nutrients results in a lower expression of T β 4 and a translocation of the peptide from vacuoles to the trans-Golgi network[28]. Transmission immunoelectron microscopy studies have shown that T β 4 translocates to the nucleoplasm of starving cells, where it may interact with nuclear actin and remodel chromatin[41].

Figure 3. Calcium cell uptake is controlled by different channels, e.g. ORAI (1 and 2) and P2X4, which is an ATP gated channel. Moreover, intracellular calcium levels are increased by the release of calcium ions from ER, which is mediated by STIMs (1 and 2) calcium sensors. High intracellular calcium levels lead to reorganization of actin cytoskeleton and increase of TNF- α , IL- β and NO production, which provoke inflammation. In addition, calcium ions regulate the permeability of nuclear pores. T β 4 has the ability to bind different target molecules: ATP synthase and G-actin as well as metal ions like calcium. Upon binding to the targets, the unstructured T β 4 assumes ternary structure. T β 4 is localized mainly in the cytoplasm, but after induction translocates to different cellular compartments e.g. nucleus.

Full-length T β 4, T β 4¹⁷⁻²³ (KLKKTET, actin-binding motif) and T β 4¹⁴⁻¹⁹ (DKSKLK, nucleus translocation motif^{32,38}), were extensively studied by Zoubek et al.[42]. They showed that the nuclear localization of the representative fragments T β 4¹⁻²⁶, T β 4²⁷⁻⁴³ and T β 4⁷⁻⁴³, in living cells, had an equal distribution between the cytoplasm and the nucleoplasm, regardless of the basic cluster of lysine residues, or their affinity to G-actin[42]. In this case, T β 4 is probably translocated to the nucleus through the nuclear pore⁴², or transported by a single transport protein⁴² (Figure 3). Molecules smaller than 45-60

kDa (depending on the type of cell) go through the nuclear pore complex (NPC) by passive diffusion, whereas bigger molecules with a nuclear localization signal (NLS) sequence require an energy-dependent multi-step mechanism in order to enter or exit the nucleus[43-45]. In 2004, Thorogate *et al.*[46] showed that the passive diffusion of small molecules (10-20 kDa) was regulated by calcium ions. The release of calcium from the endoplasmic reticulum (ER) regulates the permeability of the pore and prevents the passive diffusion of molecules larger than 10 kDa[44, 47-50]. In contrast, the addition of Ca^{2+} and ATP to an isolated nuclei bath reverses the blockade of medium-sized molecules[50].

We hypothesize that calcium binds to T β 4, resulting in structural changes of T β 4 that facilitate its translocation to the nucleus (Figure 3). It is also possible that T β 4 channels calcium ions into the nucleus periplasm, and mediates the signaling of calcium on the cytoplasm $\leftrightarrow$ nucleus route.

3. Actin binding

T β 4 is one of the main actin-sequestering proteins in many types of cells; it plays an important role in the organization of the cytoskeleton and the cell motility. T β 4 sequesters G-actin and prevents the formation of F-actin filaments. Profilins (molecular mass $\sim$ 16 kDa) also sequester G-actin, preclude the nucleation or participation in the elongation of pointed-end filaments. In contrast to T β 4, they dissociate from the actin protomer, as the protomer gets incorporated into the filament, where it plays a role in the elongation of barbed-end filaments (Figure 4). T β 4 competes with profilin for actin binding through the formation of a ternary complex T β 4-actin-profilin.

Figure 4. Polymerization scheme of actin monomers of G-actin into filaments of F-actin.

In 2014, two crystal structures of the Tβ4:actin complex were revealed by Robinson et al., who compared them with the structures of the profilin:actin complex, using X-ray spectroscopy. This structure comparison, together with molecular dynamic simulations, biochemical assays and an analysis of the principal component allowed the authors to determine the exchange mechanism between Tβ4 and profilin in actin binding (Figure 4). The mechanism proposed by the authors involved steric and allosteric interactions, in which the binding of profilin to actin (lower the distance between sub-domain 1 and 3) and Tβ4 (recedes sub-domains 2 and 4 and closer 1 and 3) reciprocally stabilize different conformations of the actin that are suboptimal for the other competing protein.

The Tβ4:actin's crystal structure showed that the C-terminal α-helix, not the actin-binding motif, was, in fact, the most important fragment in the sequestration of G-actin. After binding to actin, the C-terminal helix changes the conformation of actin and stabilizes its closed, pointed-face cleft conformation, as well as the interaction between Tβ4 and actin, in both Tβ4:actin and profilin:actin:Tβ4 complexes. During the allosteric action of actin, a small fraction of the Tβ4:actin and profilin:actin:Tβ4

complexes exhibits no interaction of the T β 4 C-terminal α -helix with the actin protomer. When the T β 4 C-terminal α -helix does not provide the steric hindrance, the actin monomer in the T β 4:actin and profilin:actin:T β 4 complexes can only be sequestered by an allosteric mechanism²¹. Previous studies have shown that the C-terminal of T β 4 consists of fragments rich in Glu and Asp residues that can coordinate calcium ions, but a metal- ion action has, to this day, never been proposed. Even if the available PDB crystal structures of T β 4:actin (PDB: 4PL7 and 4PL8) and profilin:actin (PDB: 2PBD) contain ATP:Ca²⁺ in the center of the complex, the presence of the metal has never been mentioned, nor explored as playing an active role in the elongation process of actin. In our opinion, the role of calcium in the polymerization process of actin has, so far, been greatly underestimated. Yet, a clear understanding of its mechanism of action is necessary to comprehend and control the activity of the cytoskeleton.

During polymerization phases, the profilin–actin complex gives back its actin to the elongated filaments. The free profilin then competes with T β 4 for its bound ATP–G-actin, and effectively restores the pool of polymerization- competent actin. However, the underlying mechanism of this complex process (due to the dynamic nature of the interactions involved) remains unknown, despite numerous investigations on this topic[51-54]. ATP-actin monomers possess a high affinity for Ca²⁺ ions (K_d = 2-8 nM), and the polymerization of actin monomers lowers the metal exchange rate, at its binding site, by more than 3 orders of magnitude, making the bound calcium ions almost unavailable. F-actin-bound Ca²⁺ can be released by depolymerization and dissociation from Ca²⁺-ADP-actin monomers (K_d =375 nM). Small amounts of profilin release Ca²⁺, both from Ca and Mg/Ca-F-actin; they also inhibit the uptake of Ca²⁺ by Mg/ Ca-F-actin[55]. The exact mechanism and role of calcium ions in the exchange of G-actin with F-actin has, to our knowledge, never been elucidated.

4. Tumor metastasis

The growth of solid tumors depends on the formation of new blood vessels, as the supply in oxygen to the tumor cells diminishes⁵³. In cells and tissues, hypoxia induces the expression of angiogenesis, vasorelaxation, metabolism, cell proliferation or survival, and the resistance to anti-cancer drugs genes[56-58]. T β 4 regulates the gene expression of hypoxia-dependent cell migration, angiogenesis and tumor metastasis[59-62]. In addition, T β 4 is a possible mediator of extracellular stimulation and downstream molecules, such as HIF-1 α [63, 64].

The role of calcium in metastasis is a relatively new topic in oncology. Many elements of the action mechanism of metastasis have not yet been investigated, and for those that are already known, the specific roles in migration, invasion and metastasis of different types of cancers are only beginning to be understood[65]. Impaired calcium signaling is an important factor in the metastatic process. Different levels of Ca²⁺-handling proteins, and/or an expression of Ca²⁺-dependent effectors have been found in tumor cells, where they lead to the higher turnover of focal adhesions and a more effective proteolysis of extracellular matrix components in cancer cells. Research studies have shown that the expression patterns of Ca²⁺-permeable ion channels, as well as their degree of functionality, change as the cancer progresses[65].

In most cancer cells, calcium ions are trafficked via a store-operated calcium entry (SOCE) mechanism mediated by calcium sensor STIMs (STIM1 and 2) in the endoplasmic reticulum, and through a plasma- membrane channel formed by the Orais (Orai 1-3) unit (Figure 3). The SOCE mechanism is dysregulated to stimulate the migration and invasion of cancer cells, as well as the metastasis inside the cells. When hyperactive, SOCE may facilitate the metastatic dissemination and its colonization by reorganizing the actin cytoskeleton, degrading the extracellular matrix and remodeling the tumor's microenvironment[66].

Intracellular Ca^{2+} initiates the transcription of different immediate early genes inducing cell proliferation and motility[67]. Often, an increase in $[\text{Ca}^{2+}]_i$ leads to the production of nitric oxide, which, in turn, promotes the proliferation of endothelial cells in wound healing[68].

The presence of T β 4 in various cellular processes has been well-documented by numerous research studies, but the downstream molecules or signaling mechanisms that modulate the T β 4-induced activation of endothelial cells and their migration remain unclear. T β 4 interacts with ecto-ATP synthase in endothelial cells, and increases the ATP levels of surface cells. The ATP-responsive P2X4 receptor that supports the migration of HUVEC cells may be involved in the extracellular signaling pathway[69]. P2X4 belongs to the family of P2X mammalian receptors; these receptors are ATP-gated ion channels[70] (with the exception of P2X5) that facilitate the entry of ions into the cell, when stimulated by extracellular ATP[69] (Figure 3).

AcSDKP generates angiogenesis *in vitro* and *in vivo*[71]. It has an inhibitory effect on the proliferation and differentiation of hematopoietic progenitor cells, and a protective effect on the early hematopoietic cells[18, 64]. AcSDKP stimulates the release of Ca^{2+} from the pool of $[\text{Ca}^{2+}]_i$, and releases a potent extracellular calcium influx in HL-60 cells[18].

In 2012, the migration of HUVEC cells and the formation of tubular structures in Matrigel was improved by an external treatment with T β 4 and its mutant forms without the N-terminal tetrapeptide AcSDKP (T β 4_{AcSDKPT/4A}), and without the actin-binding sequence KLKKTET (T β 4_{KLKKTET/7A}). In contrast to observations reported by prior studies[18], T β 4 initiated cellular processes, without changes in intracellular Ca^{2+} concentration[72].

The exact nature of the relationship between calcium and T β 4 is still unclear, but there is limited evidence supporting the involvement of calcium ions in T β 4 interactions, inside the cell. Understanding these interactions would allow us to elucidate the extent to which they contribute to tumor metastasis.

Given the multitude of disease- specific and other critical pathways that can be modulated by calcium and T β 4, their pharmacological targeting may be a therapeutic approach in slowing the progression of tumor metastasis.

5. Tissue regeneration

Numerous cellular and animal studies have confirmed the important role of T β 4 in tissue regeneration[73], which has led to numerous therapeutic applications successful enough to be extended to human clinical trial[74]. The broad regenerative mechanisms of T β 4 are unlikely to be exerted only via intracellular G-actin sequestration, as many affected cells already exhibit high intracellular T β 4 concentrations, and do not need to uptake additional amounts of T β 4. This means that there are other receptors, other than actin, with extracellular ligand-binding domains that can contribute to tissue regeneration[75]. The β sub-unit of cell surface-located ATP synthase, which has a high affinity for T β 4, would allow T β 4 to signal, via a purinergic receptor⁶¹. In addition, AcSDKP inhibits the proliferation of haematopoietic stem cells from bone marrow, and may mediate some of T β 4's roles, other than actin binding⁶⁴.

T β 4 is highly expressed in human heart, although its role still remains poorly understood. In 2018, Wilson et al.[76] presented an expression of T β 4 in the developing human heart, and demonstrated that T β 4 was primarily localized in endothelial cells of the cardiac microvasculature, coronary vessels, and endothelial-like cells of the endocardium and epicardium[76]. The tissue-regenerating ability of T β 4 was recently studied for the reparation of human heart muscle, after myocardial infarction or other pathologies of the heart. Mice treated with T β 4 started to grow new heart- muscle cells from inactive precursor cells (in the outer lining of adult hearts)[77], which then migrated to the inside of the heart muscle[78], forming new blood vessels within the muscle[78]. An exogenous treatment with recombinant T β 4 reduced infarct

sizes and fibrotic scarring, thus improving cardiac output in various *in vivo* models of myocardial infarction[79-81].

In 2012, Riley et al.[82] demonstrated that T β 4, activates the epicardium-derived coronary vasculogenesis, and leads to the activation of quiescent adult epicardial cells (EPDCs). Once stimulated, EPDCs facilitate the neovascularization of the ischemic adult heart and contribute to the regeneration of cardiomyocytes. EPDC-derived cardiomyocytes structurally and functionally integrate with the resident muscle to regenerate functional myocardium. This limits pathological remodeling, resulting in an improvement of the cardiac function. Moreover, the functional integration of YFP+ cardiomyocytes with existing myocardium was proven upon measurements of intracellular calcium [Ca²⁺]_i transient with neighboring YFP- cardiomyocytes. These results suggest that T β 4-primed EPDC-derivatives get associated with surviving cardiomyocytes, following an injury, and form a functional syncytium with the resident myocardium. A MRI analysis over a 28-day period, after myocardial infarction, revealed that a treatment with T β 4 results in a significant improvement of the ejection fraction and diastolic/systolic volumes, over time[82].

6. Anti-inflammatory activity against neurodegeneration.

The well-known paradigm, in neurodegeneration, is that an excess of free redox- active metals (e.g. copper and iron) lead to ROS production and inflammation. Brain is highly sensitive to ROS species, due to their high consumption of oxygen and low anti-oxidative defense mechanism. Biomolecules damaged by ROS' misfold aggregate and accumulate within intracellular inclusion bodies. Such inclusions have been found in different neurodegenerative disorders, such as Alzheimer's disease (AD), Parkinson's disease (AD), amyotrophic lateral sclerosis (ALS) and Huntington's disease (HD)[83].

Tβ4 is present in the brain in high quantities, yet its function in the central nervous system (CNS) is unknown[84]. Tβ4 shapes the development of CNS, regulates the proliferation of progenitor cells, neurogenesis, tangential expansion, and neurite outgrowth[85, 86]. During the chronic stage after a neural injury or a clinical onset of symptoms, Tβ4 provides neurological benefit by amplifying neurovascular remodeling[87]. A treatment with Tβ4 leads to recovery in different neurological diseases by promoting plasticity and neurovascular remodeling of CNS and the peripheral nervous system (PNS)³⁶. Tβ4 improves angiogenesis, neurogenesis, neurite and axonal outgrowth, and oligodendrogenesis, and thereby, significantly improves functional and behavioral outcomes, even if the Tβ4 treatment only starts 24 h or more, after an injury, or the onset of a disease[40]. A continuous 7-day- intra-hippocampal infusion of AcSDKP in mice has shown a permanent improvement in spatial memory, after 5 weeks[88].

In the brain of Huntington's and Alzheimer's patients, Tβ4 is found to be elevated in the cell bodies and processes of reactive microglia in the brain regions affected by the neurodegeneration of Tβ4's immunoreactivity in microglia, at the early stage of Huntington's disease[89]. Considering gliosis as a first protective mechanism in neuropathological diseases, it is likely that increasing Tβ4's concentration has anti-inflammatory and repair functions in microglia[90]. Indeed, reactive gliosis provokes the induction of Tβ4, which, in turn suppresses the proinflammatory TLR signaling[91, 92], and the effects of exogenous Tβ4 treatment are the same as those of Tβ4 expressed inside microglial cells. The study of Zhang et al.⁹² conducted on Tβ4 knockdown microglials showed an increase of pro-inflammatory cytokines (tumor necrosis factor α (TNF- α) and Interleukin-1 β (IL1 β)) concentrations, after an ethanol-induced inflammation, and that an exogenous treatment with Tβ4 reversed the inflammatory process[93]. Tβ4 can modulate microglial activity, both intracellularly and extracellularly. When applied exogenously, Tβ4 decreases the expression of inflammatory mediators and the secretion of hypoxia-induced TNF- α , IL-1 β , and nitric oxide (NO), *in vivo*. *In vitro* experiments by Zhou et al. also showed

that microglial activation was decreased after treatment with T β 4[94]. Intracellular calcium ions are involved in the inflammatory process, and their concentration increases in the presence of TNF- α , IL-1 β , and nitric oxide (NO)[95] (Figure 3). A delay of the increase in the amount of [Ca²⁺]_i ions, observed shortly before a cell dies, can be prevented by removal of extracellular Ca²⁺ [96].

Despite in-depth studies on T β 4, in recent years, the receptors of T β 4 in the cell membrane and the participation of T β 4 in different signaling pathways are still poorly understood[92]. T β 4 could be the calcium, as well as iron, copper and zinc ions, a sequestering agent that prevents inflammatory processes and cell death.

Conclusions

T β 4 is present in numerous tissues, where it is involved in physiological as well as pathological processes. Its high mRNA expression in specific cells is correlated with high calcium, iron, zinc and copper metal concentrations in those cells. T β 4 is an unstructured peptide with different protein- and metal- binding sites that, upon binding to its target, assumes a ternary structure. The exact mechanism of action of T β 4 in numerous cellular processes is unknown, and the metal-binding to T β 4 has never been studied. We hypothesize that the metal coordination is a driven force of T β 4 in the cellular translocation and the actin binding, which influence tissue regeneration, tumor metastasis and neurodegeneration. As shown in this paper, there are many premises that calcium and other metal ions are coordinated by thymosin in biological processes, but there is an urgent need for more research in the fields of coordination chemistry focusing on the metal chelation of T β 4, in combination with in-depth analyses of the potential biological and medical implications. We believe that elucidating the mechanisms that govern the metal coordination by T β 4 will likely be an important step forward in the recognition of

peptide function, and consequently the control of metal content as a tool to switch T β 4 pathological functioning into physiological processes.

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