**Title**  
Actin-Induced Structure in the Beta-Thymosin Family of Intrinsically Disordered Proteins

**Author(s)**  
Xue, B.; Robinson, Robert Charles

**Citation**  

**Date**  
2016

**URL**  
http://hdl.handle.net/10220/41562

**Rights**  
© 2016 Elsevier. This is the author created version of a work that has been peer reviewed and accepted for publication by Vitamins and Hormones, Elsevier. It incorporates referee's comments but changes resulting from the publishing process, such as copyediting, structural formatting, may not be reflected in this document. The published version is available at: [http://dx.doi.org/10.1016/bs.vh.2016.04.007].
Actin-induced structure in the Beta-thymosin family of intrinsically disordered proteins

Bo Xuea and Robert C. Robinsona,b,c,d,e,f

aInstitute of Molecular and Cell Biology, A*STAR (Agency for Science, Technology and Research), Biopolis, Singapore 138673.
bDepartment of Biochemistry, National University of Singapore, 8 Medical Drive, Singapore 117597.
cDepartment of Biochemistry, Yong Loo Lin School of Medicine, National University of Singapore, Singapore 117597.
dNTU Institute of Structural Biology, Nanyang Technological University, 59 Nanyang Drive, Singapore 636921.
eSchool of Biological Sciences, Nanyang Technological University, 60 Nanyang Drive, Singapore 637551.
fLee Kong Chan School of Medicine, 50 Nanyang Avenue, Singapore 639798.

Corresponding author:
Bo Xue, Institute of Molecular and Cell Biology, Proteos, 61 Biopolis Drive, Singapore 138673; Tel: +65-6586-9831; Fax: +65-6779-1117; E-mail: bxue@imcb.a-star.edu.sg
Abstract

Thymosin β4 (Tβ4) is a 43-amino acid signature motif peptide that defines the beta-thymosin (βT) family of proteins. βTs are intrinsically unstructured in their free states, and undergo disorder-to-order transitions in carrying out their biological functions. This property poses challenges in determining their 3D structures, mainly favoring structural studies on the complexes formed between βTs and their interaction partners. One of βTs’ primary binding partners is monomeric actin, a major component of the cytoskeleton in eukaryotic cells. Tβ4’s role in this system is to maintain the highly concentrated pool of monomeric actin that can be accessed through profilin by actin filament nucleating machineries. Here, we give an account of the structures of βTs that have been illuminated by nuclear magnetic resonance (NMR) and X-ray crystallography. NMR has been the method of choice for probing regions that have intrinsic conformational preference within the largely disordered βTs in their native states in solution. X-ray crystallography has demonstrated at atomic detail how βTs interact with actin. Detailed analysis of these structures highlights the disorder-to-order transition of Tβ4 in binding to actin and its isoform specificity.

Keywords:
Beta-thymosin, thymosin β4, intrinsically disordered/unstructured proteins, actin, structure, crystallography, NMR, protein hybrid,
Introduction

Beta-thymosins (βTs) are a family of proteins that are comprised of one or more of a ~43 amino acid signature motif (βT repeat) that was first defined for the prototypical single motif protein, thymosin β4 (Tβ4). Tβ4 was discovered in the 1970s in an effort to extract hormones from thymus tissue (Goldstein, 2007). In the early 1990s, Tβ4 was found to bind and sequester monomeric actin, a major component of the cytoskeleton in eukaryotic cells (Safer et al., 1991; Safer et al., 1990). Around that time, the first nuclear magnetic resonance (NMR) structural studies on isolated Tβ4 began, and later on the Tβ4:actin complex (Czisch et al., 1993; Domanski et al., 2004; Zarbock et al., 1990). X-ray crystallography studies were only possible once strategies were developed to stabilize the intrinsically disordered Tβ4 in its complex with actin (Aguda et al., 2006; Hertzog et al., 2004; Irobi et al., 2004; Xue et al., 2014). The accumulation of these structural data, together with the discovery of homologs through protein and nucleotide sequencing, allowed for a precise definition of the features of the βT repeat: it has a consensus LKKT motif at the center of the sequence, which is flanked by two moderately conserved segments that tend to weakly form amphipathic α-helices in isolation, yet become stabilized when bound to actin. Since similar LKKT motifs, N-terminal α-helices and actin-binding properties can also be found in the Wiskott-Aldrich syndrome protein (WASP) homology domain 2 (WH2)-containing proteins, βTs and WH2s are often grouped in the same functional superfamily (Paunola et al., 2002), whether or not they arose from a common ancestor (Edwards, 2004).

βT repeats appear within proteins either singly or as multiple repeats. Tβ4, and close homologs, such as Tβ10 and Tβ15, contain one βT repeat, whereas other members contain from 2 to 27 such repeats (Xue and Robinson, 2013). One widely studied multi-βT repeat protein is the three βT repeat Drosophila ciboulot, which was the first protein to yield an actin-bound βT repeat structure by X-ray crystallography (Hertzog et al., 2004). Generally, single- and multi-repeat βTs appear to be mutually exclusive in their phylogenetic distributions (Van Troyes et al., 2007), with few exceptions (Dehal et al., 2002; Dhaese et al., 2009). Single- and multi-repeat βTs also show differences in their actin-binding functionality: all of the studied single-repeat βTs act like Tβ4 in inhibiting their bound actin from forming or joining a filament, whereas multi-repeat βTs, such as ciboulot and the four-repeat TetraThymosinβ, often allow their bound actin to join the fast-growing, barbed end of a preexisting actin filament, a profilin-like activity (Hertzog et al., 2004; Van Troyes et al., 2004). Recent studies have attributed the functional difference to the stability of the C-terminal helices within the βT repeats (Didry et al., 2012; Xue et al., 2014).

From a structural point of view, βTs belong to a class of proteins termed intrinsically disordered/unstructured proteins (IDPs/IUPs) (Andreeva et al., 2014; Oldfield and Dunker, 2014; van der Lee et al., 2014). Members of this class of proteins contain regions, or in the case of βTs, the entire protein, that lack ordered structure in their native states, but undergo disorder-to-order transitions in carrying out their biological functions (Uversky, 2002). A large collection of IDPs/IUPs can be found in DisProt, the curated database of disordered proteins (Sickmeier et al., 2007), among which is Tβ4 and other actin-binding proteins, such as WASP, tropomodulin, and troponin. Despite being generally unstructured, IDPs/IUPs do have regions with intrinsic conformational preferences, which are further classified into two motifs by SCOP2 (Andreeva et al., 2014), flexible disordered helical regions and extended binding regions. We give a brief historical account of the structural studies on βTs and present a detailed analysis of the interaction between Tβ4 and actin, which reveals that βTs contain both of the IDPs/IUPs motifs.
Beta-thymosins in solution

Tβ4 in mixed organic-aqueous solvent

Due to the small size and unstructured nature of βTs, solution NMR has been the method of choice to study their isolated structures. The first βT NMR structure determined was that of bovine Tβ4, which is identical in amino acid sequence to human Tβ4 (Zarbock et al., 1990). The experiments were conducted in solutions of 60% (v/v) trifluoroethanol-d3 or 50% (v/v) hexafluoroisopropanyl-d2 in water, because no obvious structure could be detected by circular dichroism and 1H NMR spectra for Tβ4 in aqueous solutions. The presence of alcohols in the solution promoted the formation of ordered helical structures in Tβ4 at residues 4-16 and 30-40. Due to insufficient nuclear Overhauser enhancement (NOEs) signals for residues 17 and 18, the relative orientation of these two helices could not be determined. In addition to the helices, there was also a loop region between residues 24-29 of Tβ4.

Tβ4 in aqueous solution

The NMR study was subsequently continued to probe the influences of temperature and buffer pH on the structure of Tβ4 in aqueous solutions (Czisch et al., 1993). The measurements were performed at 1, 4, 14 and 29°C for samples at pH 3.0, and at 1 and 14°C for samples at pH 6.5, which were the conditions favorable for minimizing amide exchange. Temperature was found to have a significant effect on the secondary structure Tβ4. Residues 5-16 had a tendency to form an α-helix, which was stable at up to 14°C and became destabilized at higher temperatures. Residues 31-37 also showed a propensity to adopt a helical conformation at 1 and 4 °C, albeit less well defined than residues 5-16; however, residues 31-37 became completely random at 14°C. In agreement, the helical content percentages determined from the circular dichroism spectra were 15, 9 and 7% for 4, 9 and 20 °C, respectively. The first four residues at the N-terminus of Tβ4 were always disordered irrespective of the temperature.

Tβ10 in mixed organic-aqueous solvent

Following these studies on bovine Tβ4, the NMR study was extended to bovine Tβ10 which is a close homolog of Tβ4 and was originally called Tβ9 (Stoll et al., 1997). Two helical regions at residues 4-27 and 32-41 of Tβ10 were detected in a solution of 40% (v/v) 1,1,1,3,3,3-hexafluoro-2-propanol-d2 in water, which were separated by a poorly defined loop region between residues 28-31 (Fig. 1A). The first helix of Tβ10 was, however, twice the length of its counterpart in Tβ4. Similar to Tβ4, the lack of medium or long-range NOEs for the loop region impeded the determination of the relative orientation of the two helices in Tβ10. Moreover, the N-terminus of Tβ10 also showed random-coil structure.

Tβ4 mutants in mixed organic-aqueous solvent

To further correlate Tβ4’s structure with its actin binding-activity, NMR spectra were recorded in 60% (v/v) trifluoroethanol for wild-type Tβ4 and its mutants (Simenel et al., 2000). Consistent with the previously reported Tβ4 structures in alcohols, the wild-type conformation under these conditions consisted of two α-helices: residues 5-16, of which Lys11 to Lys16 form a more stable core; and the second helix from residues 31-39. Several point mutations were made in the first helix and in the LKKT motif (residues 17-22), the two main regions known to be responsible for actin binding. One such mutation, S15A, caused the first helix to become extended at its C-terminal end, to include the LKKT motif leading to a 50% reduction in its actin-binding activity. Another mutation, K11P, disrupted the formation of the first helix, resulting in complete loss of actin binding. These data
demonstrated that the presence of the first helix and its correct orientation in relation to the LKKT motif are essential for the Tβ4:actin interaction.

Thus, these structural data indicate that βTs are largely unstructured in solution, but have a latent propensity to form two regions of helix, which can be induced by aqueous-organic mixed solvents or low temperatures.

**Beta-thymosins in complex with actin**

**NMR-based Tβ4:actin model**

Chemical cross-linking data had established that Tβ4 adopts an extended conformation when bound to actin, with its N-terminal region in contact to subdomain 1 of actin and its C-terminal region in close vicinity to His40 and Gln41 of actin subdomain 2 (De La Cruz et al., 2000; Reichert et al., 1996; Safer et al., 1997). The first high-resolution model for the structural basis of the Tβ4:actin interaction was constructed from NMR data using selective labeling of Tβ4 (Domanski et al., 2004). The relatively large size of the Tβ4:actin complex (47 kDa) had precluded NMR spectroscopy studies of the whole complex. However, a complete assignment of amide protons and nitrogen resonances for Tβ4 was successfully achieved, while bound to actin, using a combination of selective labeling of Leu, Thr, and Lys of Tβ4 in a three-dimensional 1H,15N NOESY-HSQC experiment. The actin-bound model (PDB ID: 1UY5, Fig. 1B) was constrained in its positioning on actin by the distance restraints arising from the crosslinking data. This model predicts that the entire length of Tβ4 is engaged in binding to actin, causing the Tβ4 backbone to adopt a conformation, with the two terminal helices flanking the extended region. Cleavage of the actin DNase I binding loop by subtilisin did not modify the signal arising from Tβ4 in the complex, suggesting that the Tβ4 backbone is not in the vicinity of actin residues 42-47. These data established that Tβ4 terminal segments form helices that are induced and/or stabilized through binding to actin.

**X-ray structures**

**C-terminal segment of Tβ4**

Further breakthroughs in understanding the Tβ4:actin interaction came with novel strategies to stabilize the complex for X-ray crystallography. Through creating a hybrid protein between domain 1 of gelsolin (G1), a protein that severs and caps actin filaments (Nag et al., 2013), and the C-terminal segment of Tβ4 (hereafter referred to as G1-Tβ4), the structure of the G1-Tβ4:actin complex was elucidated at 2 Å resolution (PDB ID: 1T44, Fig. 1C) (Irobi et al., 2004). This structure revealed that the C-terminal segment of Tβ4 forms a helix which bridges, and narrows, the gap between subdomains 2 and 4 of actin, in contrast to the NMR model where it only contacts subdomain 2. The new conformation provided an explanation for the biochemical evidence that Lys38 of Tβ4, located at the Tβ4 C-terminus, could be crosslinked to Gln41 in the DNase I loop of actin (Safer et al, 1997). In the G1-Tβ4:actin structure, the flexible actin DNase I loop (residues 39-50) is, like in most other actin structures, disordered. The distance between the mainchain Cα carbon atoms of Tβ4’s Lys38 and actin’s Pro38, which is the closest residue to Gln41 that was resolved in the G1-Tβ4:actin structure, is 15.1 Å. Given the length of the side chains of Lys (6.4 Å) and Gln (4.4 Å), the flexibility of actin’s DNase I loop, and the unstructured nature of Tβ4 before becoming fully bound to actin, it is reasonable to envisage that these two residues may be accessed by a zero-length crosslinker. Therefore, this crystal structure revealed the binding geometry of Tβ4’s C-terminal segment to actin that induces and/or stabilizes the helical conformation of Tβ4.
N-terminal segment of ciboulot’s first βT repeat

In the same year as the determination of the G1-Tβ4:actin X-ray structure (2004), Hertzog et al. determined the X-ray structure of a 25-residue peptide from the three-repeat β-thymosin protein, ciboulot, in complex with actin at 2.5 Å (PDB ID 1SQK, Fig. 1D) (Hertzog et al., 2004). The peptide in the structure corresponds to the N-terminal segment of ciboulot’s first βT repeat (Asp10 to Ser34, hereafter referred to as Cib1N), which has moderate sequence similarity to Tβ4. This X-ray structure is of considerable significance because it was the first to reveal how the N-terminal helix and the central LKKT motif of the βT/WH2 superfamily of proteins interact with actin, and the mode of interaction was later confirmed repeatedly by other complex structures of actin-bound WH2 motifs (Chereau et al., 2005; Lee et al., 2007). The N-terminal α-helix of Cib1N in the structure was extended relative to later βT/WH2:actin structures. Later it became apparent that the helix was extended by stabilization through crystal packing, and that the actual actin-bound helix is one turn shorter (Didry et al., 2012). Nevertheless, this work demonstrated that actin binding induces and/or stabilizes the βT/WH2 N-terminal helix and revealed the binding geometry of this helix and the conserved LKKT motif.

C-terminal segment of ciboulot’s second βT repeat

Utilizing a similar strategy employed for the G1-Tβ4 hybrid, a hybrid protein comprised of G1 and ciboulot’s second and third βT repeats (hereafter referred to as G1-Cib23), was used in an attempt to determine the structure of this hybrid bound to actin (PDB ID: 2FF6, Fig. 1E) (Aguda et al., 2006). Although the hybrid contained nearly half of ciboulot (Glu72 to Ala129), the structure revealed only a short stretch of 11 residues from the C-terminal segment of ciboulot’s second β-thymosin repeat (Glu72 to Lys82). This visible portion closely resembled Tβ4, in that it bound to the same place on the surface of actin. The last visible ciboulot residue, Lys82, corresponded to Lys31 in Tβ4, the first residue of the C-terminal helix. The absence of an ordered C-terminal helix in the G1-Cib23:actin structure may be attributed to the sequence differences between Tβ4 and ciboulot: Ser31 and Thr34, in Tβ4, are Asp81 and Ala84 in ciboulot, respectively. These substitutions were reasoned to cause destabilization of the helix, leading to partial loss of the actin binding by the C-terminal segment of ciboulot’s second βT repeat. In agreement with such a hypothesis, the second repeat in isolation is the weakest in binding to actin when compared with the first and the third ciboulot repeats (Chereau et al., 2005). Thus, this work established that actin stabilization of the C-terminal helix is βT/WH2 module specific.

Structure-based models of Tβ4

A structure-based model for the full-length Tβ4:actin complex was constructed by combining the G1-Tβ4:actin and Cib1N:actin structures (Irobi et al., 2004). The model relied on the conserved central LKKT motif common to the βT/WH2 superfamily. By simply superimposing the actins from these two structures, the N-terminal segment of ciboulot’s first βT repeat, from the Cib1N:actin structure, naturally connected, via its LKKT motif, to the C-terminal segment of Tβ4 in the G1-Tβ4:actin structure. Ciboulot residues were then substituted with the corresponding residues from Tβ4 to yield a model of full-length Tβ4. Overall, this model is of good credibility, as its construction was based on X-ray structures and involved minimum structural manipulations. Certain details are, however, expected to be less reliable, due to the sequence differences in these two proteins. The model was later further refined to take into account the presence of a helix-breaking proline (Pro4) at the N-terminus of Tβ4 (Fig. 1F) (Xue et al., 2007). This proline residue effectively restricted the length of Tβ4’s N-terminal helix, making the helix sterically more compatible with the formation of a ternary complex composed of actin, Tβ4 and profilin (Xue et al., 2014; Yarmola et al., 2001). Sequence alignment
indicates that this proline is generally absent in multi-repeat βTs and WH2 motifs, but is common among the single-repeat βTs (Edwards, 2012; Paunola et al., 2002), implying similar functional roles of this residue in other single-repeat βTs.

**Full-length and N-terminal segment of Tβ4**

Recently, we reported two novel X-ray structures of Tβ4:actin complexes, which provided structural information on the actin interactions of full-length Tβ4 and, independently, its N-terminal segment (Xue et al., 2014). The full-length Tβ4:actin structure was made possible by creating a hybrid protein consisting of full-length human Tβ4 fused to *Pichia* actin (PDB ID: 4PL7, Fig. 1G; hereafter referred to as Tβ4FL). In this structure there was interpretable electron density for the majority of Tβ4 (Asp5 to Ala40), but a lack of electron density for the mobile termini, residues 1-4 and 41-43, respectively. The second structure was a complex between rabbit skeletal actin and a hybrid peptide comprising Tβ4’s N-terminal segment prior to the LKKT motif (residues 1-16), the lysine-rich region of cordon-bleu (residues 1095-1106), and full-length Tβ4 (residues 1-43). Clear electron density was apparent for residues 4-25 for the full-length Tβ4 (residues 34-55 in the hybrid, hereafter referred to as Tβ4N), which were unambiguously assigned (PDB ID: 4PL8, Fig. 1H).

In good agreement with the NMR studies and the structure-based models, full-length Tβ4 in the Tβ4FL:actin structure interacts extensively with actin through its two terminal helices and throughout the connecting linker region. The validity of the structure could be crosschecked with the segments of Tβ4 in the G1-Tβ4:actin and Tβ4N:actin structures. Despite the differences in hybrid design and crystallization conditions, the full-length Tβ4 in Tβ4FL:actin corresponded well with its independently determined N- and C-terminal segments taken from Tβ4N:actin and G1-Tβ4:actin, respectively (Fig. 2). Not only did the main chains of Tβ4 in these structures superimpose precisely, but many side chains also adopted nearly identical conformations, suggesting their critical roles in anchoring of Tβ4 to the surface of actin. The first ordered residue in Tβ4FL is Asp5, which lies at the beginning of the N-terminal helix, in agreement with the NMR measurements in aqueous solution at 14°C (Czisch et al., 1993). The N-terminus in Tβ4N:actin was extended by one residue to Pro4; a residue that has a minimal contact with actin. Thus, these structures defined the full length Tβ4 interaction with actin and demonstrated that many of the residues that could be induced to adopt helical conformations in aqueous-organic mixed solvents or at low temperatures are also functional as helices in interacting with actin.

**Tβ4:actin interface analysis**

Here we present details of the Tβ4:actin interface based on the analysis of the three available Tβ4:actin structures using PISA (Krissinel and Henrick, 2007). Of the 36 ordered Tβ4 residues in the Tβ4FL:actin structure, Met6, Ile9, Phe12, Leu17, Pro27, Leu28, Pro29 and Ile34 are the major contributors of hydrophobic interactions, whereas Asp5, Glu8, Glu10, Lys14, Lys18, Thr20, Thr22, Gln23, Lys25, Asn26, Lys31, Glu37 and Lys38 engage actin through hydrogen bonds and salt bridges (Fig. 3A); only eight residues (Ala7, Lys11, Asp13, Ser15, Glu32, Glu35, Gln39 and Ala40) have no contribution in actin binding, judged by the absence of buried surface area for these residues. However, as pointed out earlier (Xue et al., 2007), some of the non-contacting residues, for example Asp13 and Ser15, contribute indirectly by stabilizing the actin-bound conformation of Tβ4.

An interesting question arose when we considered the Tβ4-bound actins in these structures, which are isoforms from either rabbit skeletal muscle or from the yeast, *Pichia pastoris*. Measured tissue distributions of Tβ4 in mouse indicates that its concentration in muscle is much lower when compared
with some other tissues such as spleen, brain, liver and lung (Hannappel, 2007). Likewise, EST data, which have been shown to correlate with protein concentrations in human and mouse (Xue and Robinson, 2013), show similar patterns of Tβ4 enrichment in mammals and birds (interested readers may check the EST profiles for the individual entries under http://www.ncbi.nlm.nih.gov/unigene/?term=TMSB4X for more details). Therefore, the most biologically relevant binding partners for Tβ4 in mammals are the cytoplasmic β- and γ1-actins. Pichia actin is cytoplasmic. It is, however, only ~85% identical in sequence to mammalian cytoplasmic actins. Moreover, the Pichia actin-Tβ4 interaction is non-endogenous, as no βT family members are found in yeasts.

To address the above-question, we performed a multiple sequence alignment of Pichia, rabbit skeletal muscle, and human cytoplasmic β- and γ1-actins. We then compiled all the interface residues according to the PISA analysis of the three Tβ4:actin structures, and indicated them alongside the aligned sequences (Fig. 4). Particular attention was paid to the residues involved in forming hydrogen bonds and salt bridges with Tβ4, because these interactions are critical in engaging the relatively hydrophilic Tβ4 peptide and in defining the specificity between Tβ4 and actin. There are altogether 59 interface residues on actin. Using the two human cytoplasmic actins as references, which have identical interfaces, the numbers of non-identical interface residues are only 2 and 7 for the rabbit skeletal muscle and the Pichia acts, respectively, indicating the atomic details on Tβ4:actin interaction obtained from the rabbit skeletal muscle and Pichia isoforms are indeed highly relevant to the mammalian cytoplasmic actins. Moreover, the variations in these two isoforms do not completely overlap, but somewhat complement each other. For the two variations in the rabbit skeletal muscle isoform, M16L and T201V, the former is non-existent in the Pichia isoform, whereas the latter becomes a more similar T201S substitution. Conversely, 6 out of the 7 variations found in the Pichia isoform are non-existent in the rabbit skeletal muscle isoform. This complementarity allowed us to examine the interactions between Tβ4 and human cytoplasmic actin by combining the structural information from the three known structures.

We took the Tβ4FL:actin structure as the starting model, and focused on the non-identical interface residues on actin that were involved in hydrogen bonding with Tβ4. One region of interest is at the barbed-face of actin, in which Gly350 in Pichia actin corresponds to Ser350 in both rabbit skeletal muscle and human cytoplasmic actins. As shown by the comparison between the Tβ4N:actin and the Tβ4FL:actin structures (Figs. 3F vs. 3E), the presence of the hydroxyl group on the side chain of Ser350 gave rise to one extra hydrogen bond to Tβ4’s Glu8. Another region of interest lies at the opposite end, the pointed-face of actin, in which Pichia actin’s Thr199, Ser201 and Ser203 are replaced by a serine and two threonines in human cytoplasmic actins. Given the similarity between serines and threonines, these variations are not expected to change the hydrogen bonding patterns observed in the Tβ4FL:actin structure for this region (Fig. 3B). In fact, a threonine at residue 203 had been observed in the G1-Tβ4:actin structure to form a hydrogen bond to Tβ4’s Glu37 (Fig. 3C). It should be pointed out that the overall hydrogen-bonding pattern for the muscle isoform is rather different in this region. The likely cause for the difference is the muscle specific residue, Val201, which eliminates the possibility of a 2.5 Å hydrogen bond observed between Pichia actin’s Ser201 and Tβ4’s Lys38, leading to complete disengagement of Lys38 in G1-Tβ4:actin. A third region of interest is at residue 28 of actin, which harbors a histidine in Pichia, or an arginine in muscle and cytoplasmic actins (Fig. 3D). Despite having longer side chain and greater hydrogen bonding potential, the arginine in Tβ4N:actin did not provide an extra hydrogen bond to Tβ4, because it was tied up by participating in an intra-molecular hydrogen bond to Asp24 of actin. Taken together, the interactions
between mammalian cytoplasmic actins and Tβ4 appear to be captured by the *Pichia* actin isoform structure despite the non-existence of Tβ4 in this species.

**Conclusion**

Tremendous progress has been made during the past two and half decades in elucidating the structures of βT family proteins, and in particular that of the family’s archetypal member, Tβ4. A body of evidence has emerged, suggesting that two regions within the largely unstructured βT repeat have a higher propensity towards helix formation, which can be induced when the βT repeat executes its biological function in binding and sequestering monomeric actin. How well the C-terminal helix interacts with actin determines whether the βT repeat acts as a suppressor or facilitator of actin polymerization at the fast growing barbed end of actin filaments. Advances in genomics have brought forth a large number of new members into the βT family, which await further structural and functional characterization.

In addition to sequestering actin, Tβ4 also participates in numerous biological functions such as stimulating wound healing and tissue repair (Malinda et al., 1999), suppressing inflammation (Young et al., 1999), and enhancing cardiomyocyte regeneration (Qian et al., 2012; Smart et al., 2011). However, the mechanisms behind many of Tβ4’s hormonal activities remain elusive (Goldstein et al., 2005). Once the pathways and receptors involved in these processes have been identified, new structural data will be required to compare how closely the disorder-to-order transitions mirror those determined for actin binding, and to help drive drug development in these areas.

**Acknowledgements**

BX and RCR would like to thank the Agency for Science, Technology and Research (A*STAR), Singapore for support.

**References**


Figures

Figure 1 Structures of beta-thymosins. The structures were solved by X-ray crystallography unless stated otherwise. Beta-thymosins are shown as red cartoons; actin is shown as a grey surface, with its bound nucleotide shown as green sticks; gelsolin domain 1 (G1) in two of the structures is shown as a blue cartoon. PDB IDs are given following the abbreviations of the structures, whose meanings can be found in the main text. (A) Solution NMR structure of Tβ10 (1HJ0). (B) NMR-based model of Tβ4:actin (1UY5). (C) Cib1N:actin (1SQK). (D) G1-Tβ4:actin (1T44). (E) G1-Cib23:actin (2FF6). (F) Structure-based model of Tβ4:actin. (G) Tβ4FL:actin (4PL7). (H) Tβ4N:actin (4PL8). This and the subsequent structural figures were prepared with the PyMOL Molecular Graphics System (DeLano Scientific LLC).
Figure 2 Superimposition of the three known Tβ4:actin structures. Tβ4s in Tβ4FL:actin, Tβ4N:actin and G1-Tβ4:actin are colored in green, yellow and violet, respectively. Actin from Tβ4FL:actin is shown as a grey surface.
Figure 3 Detailed interactions between Tβ4 and actin. (A) The Tβ4:actin interface based on the Tβ4FL:actin structure. Tβ4 is shown as green sticks except for its buried hydrophobic residues, which are colored in chocolate. Actin is shown as a grey surface, with its interface residues shown as light blue sticks. Dashed lines in magenta represent hydrogen bonds and/or salt bridges. (B-E) Differences between the *Pichia* and rabbit skeletal muscle isoforms on the pointed-face (B vs. C), side (D), and the barbed-face (E vs. F) of actin. Tβ4 residues from Tβ4FL, Tβ4N and G1-Tβ4 are colored in green, yellow and violet, respectively. Non-identical actin residues from the *Pichia* and the muscle isoforms are colored in light blue and orange, respectively.
Figure 4 Sequence alignment of actin isoforms. hsB & hsG1: Homo sapiens (human) β- & γ1-actins; ocA1: Oryctolagus cuniculus (rabbit) skeletal muscle actin; pp: Pichia pastoris (yeast) actin. Residues lying on the Tβ4:actin interface are generally denoted by an (x), except for those with less than 1.6 Å^2 in buried surface area (w) according to PISA (Krissinel and Henrick, 2007), and for those that are involved in hydrogen bonding with Tβ4 through their main-chain (M), side-chain (S), or both (B). Red boxes highlight non-identical interface residues.