

Vertebrate tropomyosin: distribution, properties and function

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Abstract

Tropomyosin (TM) is widely distributed in all cell types associated with actin as a fibrous molecule composed of two α -helical chains arranged as a coiled-coil. It is localised, polymerised end to end, along each of the two grooves of the F-actin filament providing structural stability and modulating the filament function. To accommodate the wide range of functions associated with actin filaments that occur in eucaryote cells TM exists in a large number isoforms, over 20 of which have been identified. These isoforms which are expressed by alternative promoters and alternative RNA processing of four genes, TPM1, 2, 3 and 4, all conform to a general pattern of structure. Their amino acid sequences consist of an integral number, six or seven in vertebrates, of quasiequivalent regions of about 40 residues that are considered to represent the actin-binding regions of the molecule. In addition to the variable regions a large part of the polypeptide chains of the TM isoforms, mainly centrally located and expressed by five exons, is invariant. Many of the isoforms are tissue and filament specific in their distribution implying that the exons expressed in them and the regions of the molecule they represent are of significance for the function of the filament system with which they are associated. In the case of muscle there is clear evidence that the TM moves its position on the F-actin filament during contraction and it is therefore considered to play an important part in the regulation of the process. It is uncertain how the role of TM in muscle compares to that in non-muscle systems and if its function in the former tissue is unique to muscle.

Discovery of tropomyosin

Up to the early 1940s myosin was the only protein of the muscle contractile system that had been isolated and characterised. It was not until a procedure was employed to selectively denature this protein with an organic solvent that the other major myofibrillar proteins were isolated. Straub was the first to apply this method with the isolation of actin in 1942 and few years later Bailey (1946) using a similar procedure was able to obtain tropomyosin (TM). Although clearly an asymmetric protein TM readily crystallised to form fragile plates containing about 90% water (Bailey, 1948). It also possesses the property of being heat stable, the thermal unfolding of smooth muscle TM for example being completely reversible up to 60°C (Orlov *et al.*, 1998). At low ionic strength solutions of TM are very viscous and were shown to contain long fibrils of relatively uniform diameter, 200–300 Å, (Astbury *et al.*, 1948) that were depolymerised by increasing the ionic strength. Hydrodynamic methods indicated a particle weight at high ionic strength of 90 kDa which was almost halved by dissociating agents (Bailey *et al.*, 1948; Tsao *et al.*, 1951). Bailey concluded therefore that the molecule was probably dimeric although due to the lack of a free N-terminal, this could not be confirmed at the time by end group assay (Bailey, 1951). Although the

molecular weight values originally reported were higher than the now accepted figures, Bailey's conclusion about the dimeric nature of the molecule was correct. Thus soon after its discovery, over 50 years ago, the biochemical and physical properties of TM were well defined but its biological function was not. It was clear to Bailey that TM was of myofibrillar origin and presumably of structural importance in view of its fibrous properties but there was no clear indication as to its role in muscle. Because of some similarities in its amino acid composition and physical properties, and the higher yields he obtained from the muscles of young animals, Bailey believed that it might be related to myosin. For this reason he named it with a prefix suggesting it was a precursor of this protein. Subsequent research has shown that it is not the case and that TM is the product of an independent gene family.

A few years later Bailey (1956, 1957) and Kominz *et al.* (1957) isolated from the smooth adductor muscles of bivalves large amounts of a crystalline TM-like protein. Although this protein had many similar properties to striated muscle TM its solubility was very different and it was named TM A to distinguish it from the striated muscle protein which was designated TM B. It was later shown that TM A was identical with paramyosin, the protein that is responsible for the characteristic striated filaments first identified in adductor

muscles by Bear (1944) and Hall *et al.* (1945). TM B is also present in whole adductor muscle, probably associated with the striated portion of this tissue. Subsequently the name TM without alphabetical qualification has been retained for the family of proteins (see Cummins and Perry, 1973) with similar properties and amino acid sequences to the striated muscle protein.

Genes, isoforms and distribution

The TMs make up a large complex family of 100% α -helical fibrous proteins that are widely distributed in eucaryotes, with those present in vertebrates classified into two major groups. These are the high molecular weight (HMW) group containing 284–281 amino acid residues and the low molecular weight (LMW) group containing 245–251 amino acid residues. The two major TM isoforms present in yeast have even lower molecular weights, containing 199 and 161 amino acid residues respectively (Drees *et al.*, 1995). The number of amino acids in all of the TM isoforms so far isolated consist of multiples of approximately 40 residues, each of which when TM is complexed with F-actin is considered to interact with an actin monomer subunit. So far there is no evidence of TM in procaryotes. The most significant property of TM is its ability to bind to actin filaments and it is usually found associated with these structures in cells. It is invariably found in the thin filaments of vertebrate striated and smooth muscles where it is presumed to have a role in the regulation of the contractile process. Relatively smaller amounts are found in non-muscle cells associated with the cytoskeleton and actin-based motor systems.

So far four TM genes (Figure 1) have been characterised in mammals from which at least 20 isoforms (Pittenger *et al.*, 1994; see Figure 10) are expressed by alternative promoters and alternative RNA processing. The four genes so far identified in the human genome have been named TPM1, TPM2, TPM3 and TPM4

(Cutticchia and Pearson, 1993). TPM1 and TPM2 correspond to the α - and β -genes respectively that give rise to the α - and β -TMs found in striated muscle (Helfman *et al.*, 1986; Ruiz-Opazo and Ginard, 1987). The α -gene contains 15 exons of which exons 3, 4, 5, 7 and 8 are expressed in all isoforms (Figure 2). In addition to skeletal muscle α -TM, by alternative splicing of the other exons at least eight other isoforms, including smooth muscle α -TM, are produced from this gene in mammalian and avian species. The α -TM isoforms of skeletal and smooth muscle both contain 284 amino acid residues.

The β -TM gene contains nine exons in the rat of which exons 3, 4, 5, 7 and 8 are expressed in all known isoforms. In addition to coding for skeletal β -TM this gene codes for the smooth muscle β -TM and fibroblast TM1 isoforms. Despite the differences in sequences, all these β -TM isoforms are 284 amino acid residues in length (Figures 2 and 10). In the chicken the β -gene codes for a LMW TM in fibroblasts, TM-3b (Smillie, 1996).

The TPM3 gene (sometimes referred to as the γ -TM gene) which was previously known as the hTM_{nm} or the hTM_{30nm} gene (Lin *et al.*, 1997), codes for fibroblast TM_{30nm} (TM5) of LMW consisting of 248 residues (Clayton *et al.*, 1988) and TM3, a slow twitch skeletal muscle HMW α -TM of 284 residues (Gunning *et al.*, 1990; Smillie, 1996). This isoform has been referred to as α -TM slow because of its homology with the chicken slow twitch muscle isoform but has also been called sk α -TM2 (MacLeod and Gooding, 1988; Lees-Miller and Helfman, 1991; Muthuchamy *et al.*, 1997). Products of this gene have not so far been identified in smooth muscle.

Gene TPM4, (also known as the δ or TM4 gene), which contains eight exons in the rat (Lees-Miller *et al.*, 1990a, b) has so far only been shown to code for one isoform, fibroblast TM4. This gene is very similar to a human fibroblast cDNA corresponding to the gene coding for human fibroblast TM_{30pl} (MacLeod *et al.*, 1987).

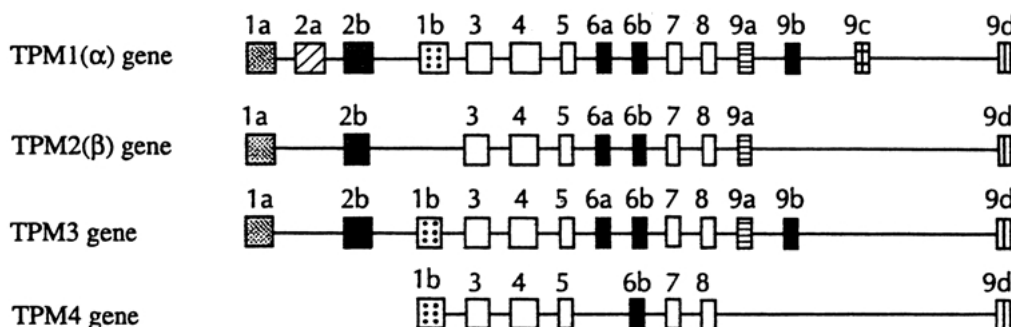


Fig. 1. The intron-exon organisation of the mammalian TM genes. Boxes represent the coding portions of the exons and introns are represented by horizontal lines. The empty boxes depict exons common to all genes. The TPM1, (α), and TPM2, (β), genes shown are those of the rat (Helfman *et al.*, 1986; Ruiz-Opazo and Nadal, 1987; Lees-Miller *et al.*, 1990a; Goodwin *et al.*, 1991). The TPM3 gene is that of the human (Clayton *et al.*, 1988) and the structure of the TPM4 gene has been determined in the rat (Lees-Miller *et al.*, 1990b). Reproduced, slightly modified, with permission from Pittenger *et al.* (1994).

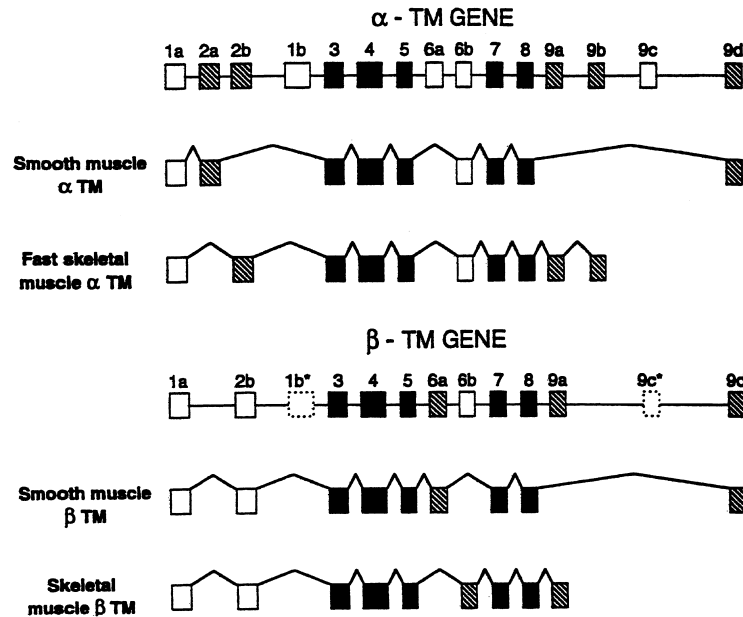


Fig. 2. Expression of the TPM1, (α), and TPM2, (β), genes in mammalian smooth and striated muscles. Boxes represent the coding portions of the exons and introns are represented by lines. The black boxes represent exons that are expressed in all isoforms. Alternative splicing of exons 2a/2b and 9d/9ab produce smooth muscle and fast twitch skeletal α -TM chains respectively. Likewise alternative splicing of exons 6a/6b and 9d/9a produces smooth muscle and fast twitch skeletal β -TM chains respectively. The intron-exon organisation of the α and β genes of chicken and quail are similar. β -TM gene exons 1b* and 9c* have been detected in the chicken but not in the rat. This figure is taken with permission from Smillie (1996) which is adapted from Figure 1 of Helfman (1994) and Figure 2 of Lees-Miller and Helfman, (1991).

In view of the close functional relationship between actin and TM it is of interest to compare the regulation and expression of the genes involved. Both proteins exist in muscle and non-muscle cells in a number of isoforms. In the case of actin this is achieved by the differential expression of the α -, β - and γ -actin genes, whereas the much larger number of TM isoforms is obtained by alternative splicing of the genes. A wide variety of cells possess the capacity of expressing both HMW and LMW isoforms of TM (MacLeod *et al.*, 1985) but it is uncertain whether all the actin and TM genes are expressed in muscle progenitor cells. Myogenesis involves replacement of the non-muscle proteins with the muscle forms, with the result that in striated muscle the actin becomes the α -isoform and most of the TMs are the HMW isoforms, products of the TPM1 and TPM2 genes. Low level expression of non-muscle contractile proteins, β - and γ -actin and LMW TMs can also be detected in adult striated muscle. It is concluded that these non-muscle isoforms are not derived from mononucleated cells in the muscle but are present in the general cytoskeletal architecture of the adult muscle fibre (Otey *et al.*, 1988; Gunning *et al.*, 1990). No two muscle specific TM mRNAs or two non-muscle TM mRNAs are coordinately repressed during myogenesis (Gunning *et al.*, 1990). This suggests that each TM isoform has its own unique determinants of accumulation.

In the yeast, *Saccharomyces cerevisiae*, two genes, TPM1 and TPM2, that code for the 199 and 161 residue isoforms respectively, have been identified (Drees *et al.*, 1995).

Muscle tropomyosins

Skeletal muscle

The TM from rabbit fast skeletal muscle, in which tissue it represents about 3% of the total protein, has been the most widely studied and represents the prototype of the family. The work of Woods (1966, 1967) in particular confirmed that the TM molecule is a dimer composed of subunits of molecular weight about 33,000 in the case of rabbit fast muscle α -TM (Smillie, 1979). Although this preparation was originally considered by Bailey to be homogeneous the application of gel electrophoresis under dissociating conditions (Bodwell, 1967; Weber and Osborne, 1969) suggested that there was heterogeneity in the subunits. Under dissociating conditions skeletal TM can be fractionated into two major isoforms with similar amino acid composition, differing particularly in cysteine content (Cummins and Perry, 1973). The α -subunit contains one cysteine residue and the β -subunit two cysteine residues per 33 kDa but no marked differences in biological properties could be demonstrated in the α_2 and β_2 forms of TM. Although the α - and β -isoforms are the major components of rabbit skeletal muscle TM, additional minor components are also present in this tissue (Cummins and Perry, 1974; Bronson and Schachat, 1982; Salviati *et al.*, 1982; Heeley *et al.*, 1983) which are not phosphorylated forms of the α - and β -subunits. Similar heterogeneity has also been observed in skeletal muscle from the human (Billster *et al.*, 1982), from the cat (Steinbach *et al.*, 1980) and in chick embryonic skeletal muscle (Montarass

et al., 1981). The sequence studies of the Smillie group (Hodges and Smillie, 1970; Sodek *et al.*, 1972) also suggested the presence of at least four different polypeptide chains in rabbit fast skeletal muscle TM, although the α -isoform is the major constituent. The additional minor forms of TM observed in rabbit and rat skeletal muscle have been designated γ - and δ -isoforms (Heeley *et al.*, 1983, 1985). In the rat immediately after birth α - and β -TMs are the major forms present but as differentiation of slow twitch muscle progresses the amounts of α -TM fall until little remains and mainly β -, γ -, and δ -TMs are present in the adult soleus muscle. Development and crossinnervation studies suggest that a reciprocal relationship exists between the extent of expression of the α - and γ -plus δ -isoforms (Heeley *et al.*, 1983, 1985). It is possible that γ - and δ -TMs are alternative RNA processing products of the α -gene although it has been suggested that they may be products of the TPM3 gene (Smillie, 1996).

The relative amounts of α - and β -TMs present in a muscle cell is characteristic for the type and the species and is dependent on the stage of development (Amphlett *et al.*, 1976), type of innervation and probably other factors such as hormone status (Heeley *et al.*, 1985). When α - and β -subunits are present the $\alpha\beta$ dimer is the preferred form. Separation by hydroxyapatite chromatography (Eisenberg and Kielley, 1974) and crosslinking studies (Lehrer, 1975; Bronson and Schachat, 1982) of the undissociated dimers in extracted TM indicate that the distribution of the α - and β -subunits between the dimers is not random, with α_2 and $\alpha\beta$ being the major forms present.

Generally speaking the relative amounts of α -TM are higher in fast fibres (Cummins and Perry, 1974; Heeley *et al.*, 1983). Although the α/β ratio in the whole muscle is relatively constant for the muscle type and species the ratio may vary within a fibre type in a given muscle (Bronson and Schachat, 1982; Heeley *et al.*, 1983; Moore and Schachat, 1985). For example in a given muscle the TM present in type IIB fibres, which are otherwise histochemically indistinguishable, can range from virtually all α_2 to largely $\alpha\beta$ in different fibres (Heeley *et al.*, 1983). Cross innervation studies on soleus and extensor digitorum longus muscles of the rabbit indicated that the changes that were observed when high resolution two-dimensional electrophoresis was employed could be accounted for by the changes in the relative amounts of the α - and the γ - and δ -TM isoforms. Whereas the percentage of the α -isoform present increased with increasing muscle speed, that of the β -isoform was relatively unchanged in both muscles (Heeley *et al.*, 1983).

Without detailed sequence information, it cannot be decided how the γ - and δ -isoforms, which are present in soleus and digitorum longus muscles of the rabbit, relate to TM3, the product of the TPM3 gene. In the mouse there is little or no TM3 in fast twitch skeletal muscle but in slow twitch muscle it represents 31% of the total TM present. It is not expressed in the heart of the

mouse, rat or rabbit whereas in the human it is present in cardiac and skeletal muscle (Pieples and Wiczorek, 1999, 2000). Human TM3, which differs in only three amino acid residues from the equivalent mouse isoform, is of some interest for it undergoes a rare mutation, Met9Arg, that is associated with nemaline myopathy in skeletal muscle (Laing *et al.*, 1995).

The N-terminus of TM is particularly highly conserved in the muscle isoforms. Eight of the first nine residues are identical in the muscle isoforms from man to *Drosophila* (Basi and Storti, 1986) and 18 of the first 20 N-terminal residues are conserved in all vertebrate muscle TMs.

Little is known about the distribution of the $\alpha\alpha$, the $\alpha\beta$ and the other minor isoforms of TM in the myofibril or indeed in a single thin filament. There is evidence that TM immediately adjacent to the Z line is immunologically different to that in the rest of the I filament. A monoclonal antibody to cardiac TM stained frog semitendinosus with the 24 stripes at a 38 nm periodicity as expected from the filament length. In contrast a polyclonal antibody to the same antigen only stained 23 stripes, leaving the 24th adjacent to the Z line unstained (Trombitas *et al.*, 1990). The significance of this finding is not clear but it does imply a difference in accessibility of antibody to TM, possibly as a result of structural differences in this region of the I filament.

Cardiac muscle

TM from cardiac muscle of the smaller animals such as rabbit, guinea pig and rat migrates as one band on electrophoresis (Cummins and Perry, 1973). In the case of the rabbit, at least, cardiac TM is identical in sequence to the α -TM of fast skeletal muscle (Lewis and Smillie, 1980). In this respect TM differs from the other major protein components of the striated myofibril for which the cardiac isoform is specific for that tissue e.g. actin, troponin I (TN-I) and T, or as is the case with troponin C, identical with the slow muscle isoform. As β -TM tends to be associated with slow skeletal muscles, by analogy with the situation as it relates to troponin C, this isoform would be expected to be present in a relatively slow muscle such as the heart. Although not present in significant amounts in the faster beating hearts of small animals, the TM from the hearts of larger animals such as the pig, sheep and human, which beat more slowly, contain up to 20% of the β -subunit. In this respect it is of interest that TM3 which is present in slow twitch skeletal muscle is also found in the heart of humans but not of smaller mammals such as the rat and rabbit (Pieples and Wiczorek, 2000). As is the case in skeletal muscle, when β -TM is present in the heart it occurs as the $\alpha\beta$ dimer (Leger *et al.*, 1976).

Analysis of the rates of relaxation following activation by flash photolysis of skinned cardiac muscle fibres from a transgenic mouse model in which β -TM is overexpressed suggests that the presence of $\alpha\beta$ TM increases

calcium sensitivity and decreases the rate of crossbridge detachment from actin (Buck *et al.*, 1999).

Smooth muscle

Early studies indicated that a protein with very similar physical properties to striated muscle TM could be isolated from vertebrate smooth muscles such as uterus or gizzard (see Smillie, 1996; Hodgkinson, 2000, for reviews). Nevertheless the band pattern of tactoids obtained with the chicken gizzard protein in the presence of divalent cation (Cohen and Longley, 1966) and amino acid compositions of human and sheep uterus TMs (Carsten, 1968) are different from those of the skeletal muscle proteins. On electrophoresis in dissociating conditions chicken gizzard TM migrates as two bands immunochemically different and of slightly different mobility to striated muscle α - and β -TM subunits (Cummins and Perry, 1974). As the smooth muscle isoforms have been derived from the genes expressed in skeletal muscle the same nomenclature has been retained, α for the faster and β for the slower component on electrophoresis (Helfman *et al.*, 1984). In some of the earlier papers smooth muscle α - and β -TM have been designated β and γ respectively (e.g. Sanders and Smillie, 1985; Lau *et al.*, 1985; Jansco and Graceffa, 1991). Although different in sequence from skeletal TMs the smooth isoforms are of identical chain length (284 residues). The smooth muscle α -TM is produced by splicing the RNA transcript so that exons 2a and 9d replace respectively the exons 2b, 9a and 9b present in the skeletal isoform. Similar alternative splicing occurs in smooth β -TM where exons 6a and 9d replace respectively exons 6b and 9a of the skeletal muscle β -isoform (Figure 2).

Chicken gizzard muscle contains the roughly equal amounts of α - and β -chains present as more than 90% heterodimer (Jansco and Graceffa, 1991). Two TM isoforms are present in approximately equal amounts in all types of smooth muscles, unlike striated muscle where the relative amounts of isoforms present may vary widely. It would appear that variants of smooth muscle and non-smooth muscle actin isoforms rather than of TM isoforms are associated with functional diversity in this tissue (Fatigati and Murphy, 1984). Upregulation of a non-smooth muscle TM isoform can occur in transition of rat aortic cells from the quiescent to the proliferative state (Hirano *et al.*, 2000).

Structure of tropomyosin

Crystals of TM are unique in being made up of a mesh-like structure of polymerised TM filaments and in containing about 90% water under normal conditions for protein crystallisation. Although they have been available since 1946, because of their low protein content it has not yet been possible to use them to obtain a high resolution structure of the molecule by

X-ray crystallography, as has been the case with other myofibrillar proteins such as actin, subfragment 1 and troponin C. Much of the information that we have about the molecular structure of TM has been deduced from the amino acid sequence data provided by Smillie and collaborators (see Smillie, 1979). From this it can be concluded that the rabbit skeletal TM molecule consists of two α -helical polypeptide chains 284 residues long, each with a M_r of about 33,000, to form a stable coiled-coil structure. Evidence of a periodicity along TM fibres, the so-called α -diagram, characteristic of the k-m-e-f group of proteins, was first provided by the X-ray diffraction studies of Astbury *et al.* (1948). Crick (1953) pointed out that the diffraction patterns of these proteins could be explained by a left-handed coiled-coil structure involving two right-handed α -helices inclined at an angle of about 20° to each other. The model requires a strip of non-polar residues to run down the side of each of the two α -helices. A slight deformation of the α -helices enables them to remain in contact winding round each other to form the coiled-coil, stabilised by the so-called 'knobs and holes' packing arrangement. For this to occur a non-polar amino acid must occupy approximately every fourth position in the polypeptide chain. The sequence of TM (see Appendix) can be considered as a repeating heptapeptide which largely satisfies these demands. Residues a and d are non-polar whereas residues b, c, e, f and g of the repeat are usually polar or ionic (Figure 3). Salt bridges between residues e and g appear to add further stability and specificity to the coiled-coil structure (McLachlan and Stewart, 1975).

There is no doubt that the polypeptide chain of each TM subunit consists of seven quasiequivalent regions which presumably contain actin interaction sites. It is much less certain, however, that in the intact thin filament there are seven corresponding half turns of the coiled-coil per subunit as postulated by Stewart and McLachlan (1975). Indeed the plot of the TM molecule obtained from X-ray crystallographic analysis at 7 Å indicates that there are approximately three full turns of the α -helix per molecule (Whitby and Phillips, 2000; Figure 4). If this structure corresponds to that of TM in the thin filament and all seven actin-binding sites are occupied they will not each correspond to a half turn of the TM molecule. It is possible that in association with the actin filament the relatively flexible TM molecule becomes distorted into three and one half turns. See Censullo and Cheung (1994) for some discussion on possible variations in the actin-binding sites on TM.

The composition of the dimer is determined by thermodynamic factors. At physiological temperatures unfolded dissociated mixtures of the α - and β -isoforms from *Rana esculenta* skeletal and duck gizzard TM reassemble as the heterodimer. On the other hand, if the $\alpha\beta$ dimer of *R. esculenta* TM is dissociated and allowed to refold at lower temperatures e.g. 20°C, α_2 and β_2 homodimers are favoured (Lehrer *et al.*, 1989; Lehrer and Stafford, 1991). Heterodimers present some advantages for filament assembly and actin binding (Jansco

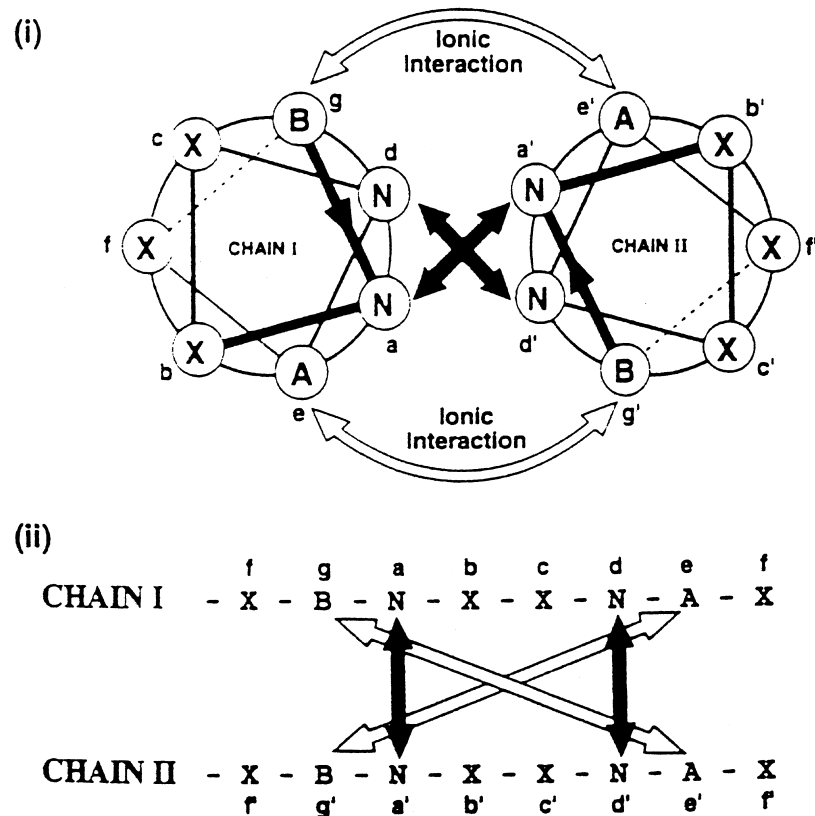


Fig. 3. Representation of the coiled-coil structure of TM. (I) Cross section. (II) Linear amino acid sequence. Letters a to g represent the amino acid positions in the pseudoheptapeptide repeat. The a and a' and d and d' are non-polar (N) and form the core of the two stranded structure. Acidic (A) and basic (B) amino acids occur frequently in e, e' and g, g' positions. These can form ionic links. The outer b, c, f and b', c', f' positions are usually but not always ionic or polar. From Smillie (1996) with permission.

and Graceffa, 1991). For a discussion on the stability of the TM dimer coiled-coil in various isoforms and its relation to sequence see Smillie (1996). The ability to form a heterodimer or a homodimer depends on the alternatively splice exons expressed in a TM isoform (Gimona *et al.*, 1995) and the difference in properties between the dimers may represent a means of modulating the function of a TM isoform. Smooth muscle TM heterodimers have stronger end-to-end aggregation than the homodimers and bind more strongly to actin with the result that thermal unfolding of the TM occurs at a higher temperature with the heterodimer than with the homodimer (Jansco and Graceffa, 1991; Levitsky *et al.*, 2000). It has been suggested that TM heterodimers also represent a more flexible structure than their homodimeric counterparts (Censullo and Cheung, 1994).

The two polypeptide chains in the dimer are in register for an intramolecular disulphide crosslink involving Cys190 can be formed with native TM (Woods, 1967; Johnson and Smillie, 1975; Lehrer, 1975; Stewart, 1975; Phillips *et al.*, 1979). At lower ionic strengths corresponding to the physiological range TM becomes highly polymerised into long filaments and forms viscous solutions. It has been proposed from model studies on the TM structure that filaments are formed by head to tail aggregation of the TM dimers involving an 8–9 amino acid residue overlap between the N- and

C-terminals (McLachlan and Stewart, 1975). This model postulates that favourable ionic and non-polar interactions can occur between side chains in the N- and C-terminal regions on adjacent molecules and explains satisfactorily the depolymerising action of high ionic strength. It also explains the fact that the effective length of the TM molecule in the muscle thin filament is $406 \pm 1.4 \text{ \AA}$ (Philips *et al.*, 1986). If the TM was arranged without such an overlap the coiled-coil structure consisting of two polypeptide chains of 284 amino acid residues would result in a molecular length of $423 \text{ \AA}$ (Smillie, 1979). Confirmation of the importance of the overlap region for filament formation is provided by the fact that acetylation of Lys7 (Johnson and Smillie, 1977) and removal of up to 11 C-terminal residues by carboxypeptidase destroy the ability of TM to polymerise (Tawada *et al.*, 1975; Johnson and Smillie, 1977). In support of the head to tail arrangement, X-ray crystallography indicates that the filaments are continuous with dense regions identified as the ends of the molecules (Phillips *et al.*, 1979). Here the filaments appear to swell slightly which might be expected if the supercoils overlap. Although the nature of the N- and C-terminals are clearly important for TM polymerisation, sequences in internal regions can also affect the polymerisability of the molecule. This is strikingly shown by the fast and slow isoforms of lobster skeletal

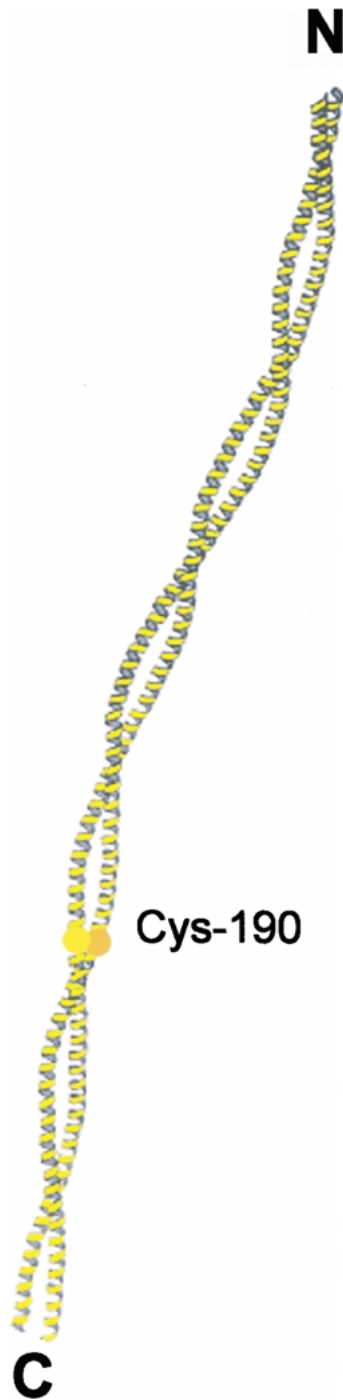


Fig. 4. The α -TM molecule. Plot at 7 Å resolution of the asymmetric unit in the spermine-induced crystals of α_2 -TM isolated from porcine ventricles. The gentle bending is similar to that seen in Bailey type TM but the analysis yields local variation in bending angles and radius. From Whitby and Phillips (2000) with permission.

muscle TM. The slow muscle isoform is much more viscous, and presumably more highly polymerised, at low ionic strength than the fast muscle isoform from which it differs only by 15 amino acid residues in the region coded by exon 2, residues 39–80. The slow muscle isoform has a higher actin affinity and inhibits the actomyosin S1 ATPase to a greater extent than the fast muscle isoform (Sano *et al.*, 2000b).

TM forms at least 15 different crystalline or paracrystalline forms, a number of which are produced by precipitation with the polycation, spermine. One form produced by stoichiometric titration with spermine (Phillips *et al.*, 1987) is well ordered with a unit cell containing 68% water by volume enabling the coiled-coil structure to be studied by X-ray diffraction to 9 Å resolution (Whitby *et al.*, 1992). In these crystals the α -helices are coiled over most of the length with an average pitch of 140 ± 10 Å. It is of interest that the precise value of the pitch varies along the length of the TM molecule, probably in response to local variations in the amino acid sequence. The data from crystals of porcine cardiac α -TM, rabbit skeletal α -TM and rabbit cardiac TM are indistinguishable at 9 Å resolution. Using a mercury derivative of the spermine-induced crystals resolution has been extended to 7 Å (Whitby and Phillips, 2000; Figure 4). This study confirms the results obtained at lower resolution and indicates that despite the regularity of the molecule implied by the heptad repeats of hydrophobic side chains, irregularities in the coiled-coils could be detected in the packing arrangements within the crystals.

The overlapping N- and C-terminal ends of the TM molecules appear on the electron density map as a thickened, more or less compact globular domain. It has, however, not so far been possible to resolve by X-ray crystallography the conformation of the terminal nine amino acid residues involved (Phillips *et al.*, 1986; Whitby *et al.*, 1992), although McLachlan and Stewart (1975) modelled them in the α -helical configuration. From the NMR analysis of a chimeric peptide Greenfield *et al.* (1998) conclude that the first 14 N-terminal amino acid residues of skeletal muscle α -TM can form an α -helical coiled-coil. The chimeric peptide, TMZip, consisting of the first 14 residues of rabbit skeletal muscle α -TM and the 18 C-terminal residues of the yeast transcription factor GCN4 (Landschultz *et al.*, 1988), exists as a coiled-coil over the whole of its length with the exception of the three C-terminal residues. This study, the first atomic resolution of any part of the TM molecule, indicates that the charged residues Asp2, Lys6 and Lys7 are highly exposed in the b, f and g positions of the heptad repeat (see Figure 3). It is likely that they provide part of a charged surface for interaction with C-terminal residues involved in the head to tail polymerisation of TM.

Localisation of tropomyosin in the myofibril and its role in the regulation of contraction

Skeletal muscle

The fact that actin and TM passed into solution when the I band was selectively extracted suggested that they were associated together in the myofibril (Corsi and Perry, 1958; Perry and Corsi, 1958). Hanson and Lowy (1963) speculated on this possibility when they proposed

their model for the structure of the F-actin filament. They were, however, unable to distinguish any differences between negatively stained I filaments obtained from glycerol extracted myofibrils and F-actin filaments obtained from the purified protein. The model, however, did not account for the 400 Å periodicity observed in the I substance of fibrils in the electron microscope or the 400 Å reflection observed in the axial X-ray diffraction pattern of intact muscle. This was ascribed to the combination of actin with other material, possibly TM. Although it had not been clearly demonstrated, there was a presumption at this time that TM was associated with the actin filament possibly forming a 'backbone' that could assist the integrity of the actin filament (Huxley, 1960), or as suggested by Hanson and Lowy (1963) it was located as a filament lying in each of the two grooves between the actin filaments. In such a location it was considered that TM might exert some control over the contractile function of actin by masking certain monomers of the filament (Hanson and Lowy, 1964). The suggestion that additional material representing the regulatory proteins, troponin and TM, lay in the long pitch grooves of F-actin was confirmed by Moore *et al.* (1970) using three-dimensional (3D) reconstruction techniques. A particularly significant observation was the increase in the actin second layer line reflection of optical diffraction patterns when impure TM was added to F-actin paracrystals (O'Brien *et al.*, 1971). This result was consistent with the presence of additional material in the grooves of the F-actin structure. Spudich *et al.* (1972) confirmed that the presence of the regulatory proteins in the grooves gave rise to the second and third actin layer lines of the diffraction pattern. The results of these optical diffraction studies and 3D reconstruction from electron micrographs of F-actin paracrystals in the absence and presence of TM and troponin, suggested that TM strands lay off the centre line in each of the two long pitch grooves of the actin helix in such a manner that the TM molecule only contacts one of the two actin helical chains that define these grooves (Figure 5). Because of the overlap involving its C- and N-terminals TM forms a continuous polymer lying in each of the two grooves of F-actin in the thin filament. Due to the overlap and its helical conformation in the actin groove one molecule of TM occupies a length of 385 Å along the filament axis and is associated with seven actin monomers.

On activation of vertebrate skeletal muscle the actin second layer line in X-ray diffraction patterns increases in intensity relative to the third layer line (Huxley, 1970, 1971, 1972). From analysis of the data it was concluded that the changes in intensity were a consequence of the movement of TM into a more central position in the groove. It was suggested that in the resting muscle the TM was positioned on the actin monomers in a manner that blocked the interaction with myosin that was required to activate the MgATPase. On activation the TM moved into the centre of the groove leaving the site on actin exposed for interaction with myosin that leads

to activation of the ATPase: the so-called 'steric blocking mechanism' for the regulation of contraction (Haselgrove, 1972; Huxley, 1972; Parry and Squire, 1973). Due to an incorrect assumption about the polarity of the actin filament in the original studies, the TM is now considered to be located in resting muscle on the other side of the actin groove from that originally proposed (Taylor and Amos, 1981).

The evidence is now strong that TM moves on activation and that this movement is a prerequisite for contraction as it occurs 12–17 ms before the mechanical response (Kress *et al.*, 1986). Neither the sites on actin nor those on myosin that interact to produce activation of the MgATPase have been precisely defined in terms of the amino acid residues involved. In consequence there are still uncertainties in deciding, as demanded by the theory, whether the interaction of actin and the myosin head that results in activation taking place is a direct consequence of the postulated movement of TM. Certainly under defined ionic conditions *in vitro* (see below) TM can be shown to inhibit the actomyosin MgATPase. Nevertheless in view of the many factors that influence the binding of TM to actin that results in inhibition of the MgATPase (see section on actin–TM interaction), it is difficult to decide precisely if in resting muscle TM is associated with actin, in the manner indicated by the *in vitro* solution experiments to produce inhibition.

Unambiguous interpretation of the X-ray data to determine the exact position and role of TM in relaxed and contracting muscle is not yet possible without detailed structural knowledge of its relation to the components of the troponin complex, and to nebulin which is also considered to lie along the thin filament in the actin cleft, information which so far does not exist. 3D reconstruction of thin filament electron micrographs have been used to visualise the movement of TM that occurs when the filament changes from the 'on' (high calcium, activated MgATPase) to the 'off' (low calcium, low MgATPase) state (Figures 5 and 6). In earlier reconstructions with vertebrate filaments the TM was only resolved in the 'on' position, possibly because in the 'off' state the TM is disordered and may not be making regular contacts with the actin (Phillips *et al.*, 1986). In the 'on' state the TM molecule is considered to bind close to the inner domain of actin just in front of the myosin binding site (Milligan and Flicker, 1987). Values for the binding radius of TM to actin of 38–40 Å have been reported (Milligan and Flicker, 1987; Milligan *et al.*, 1990; Bivin *et al.*, 1991). A similar binding radius and location which corresponds to the 'on' state is given by the model obtained from X-ray diffraction data from unregulated thin filaments in actin–TM gels (Lorenz *et al.*, 1995). In this model it is concluded that there are strong electrostatic attractions between charged side chains on TM and domains 3 and 4 of actin.

Although there is reasonable agreement that the average radial value of attachment remains constant on addition of calcium there is some disagreement about

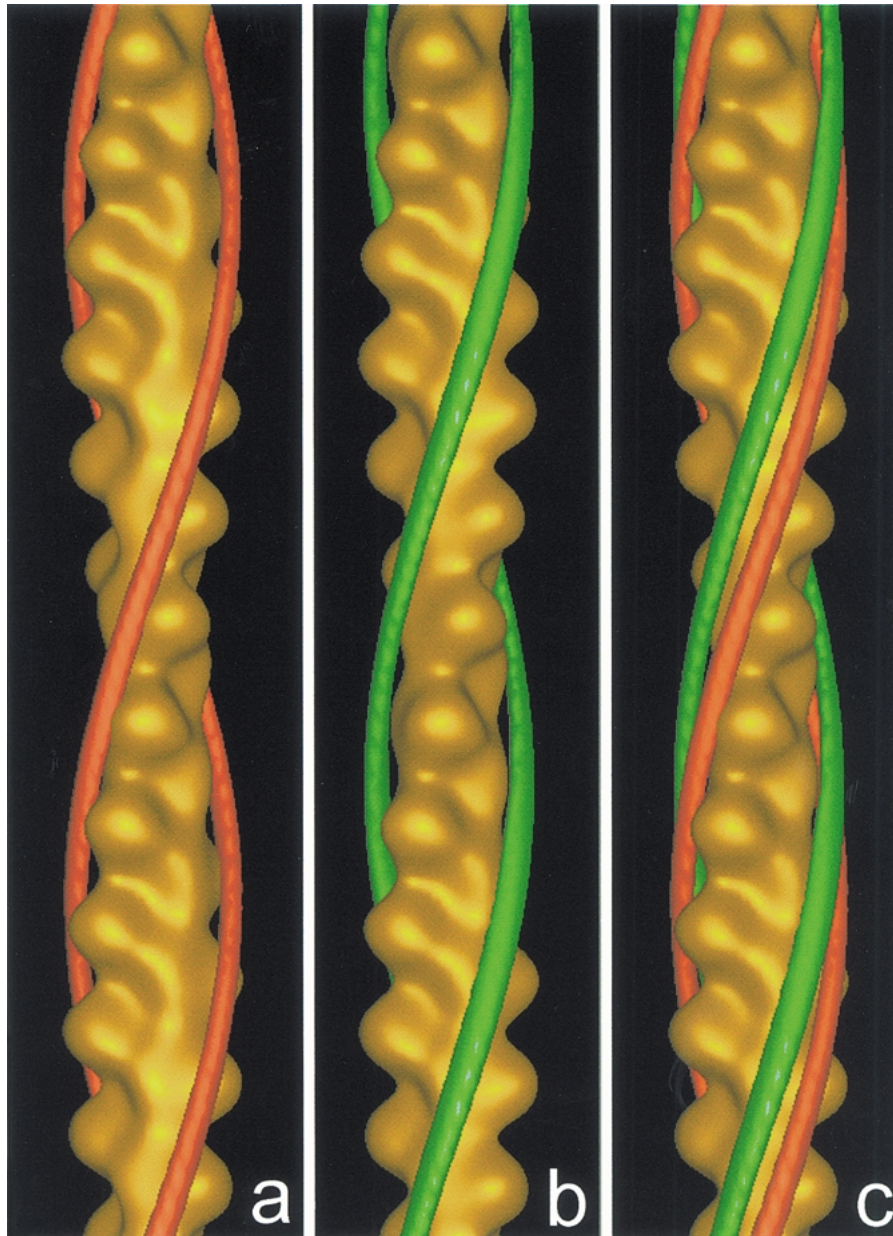


Fig. 5. Reconstructions of thin filaments showing positions of TM in the absence and presence of calcium. Surface views of reconstructions showing TM superimposed on actin in (a) EGTA, (b) Ca^{2+} . In (c) TM strands associated with both EGTA and Ca^{2+} are superimposed on actin for comparison. Reconstructions show characteristic bilobed actin in gold. In EGTA the TM (red) occupies a position on the inner edge of the outer domain of actin whereas in Ca^{2+} the TM (green) lies along the outer edge of the inner domain. Surface rendering was carried out by superimposing TM strand densities obtained by difference analysis on the maps of pure F-actin. Reproduced with permission from Xu *et al.* (1999).

the azimuthal shift in position of TM in the ‘off’ and ‘on’ states. The ‘off’ state position of TM has been observed in striated muscle filaments from the arthropod *Limulus* which retain their structure in EGTA and calcium to enable well-resolved 3D reconstructions of electron micrographs to be made (Lehman *et al.*, 1994). They conclude that the TM strand moves azimuthally by $\sim 25^\circ$ about the actin helix axis when the filament changes from the calcium bound to the calcium-free state. This figure should be compared with the values for this shift provided by other workers, ranging from no shift at all (Borovikov *et al.*, 1993), $15\text{--}20^\circ$ (Milligan and Flicker, 1987) and $50\text{--}60^\circ$ (Yagi and Matsubara, 1989; Kabsch and Vanderkerckhove, 1992). The results

of recent fluorescent energy transfer studies on reconstituted filaments containing labelled TM are compatible with an azimuthal movement of TM of 20° and 46° rotation around its own axis due to calcium binding (Bacchiocchi and Lehrer, 1999, 2000). The best fit of this data positions TM at low calcium concentrations between the inner and outer domains of actin covering the postulated myosin binding sites. Neutron scattering experiments suggest that TM does not move as a rigid rod as there is no apparent change in the average radial position of TM on the thin filament (Bivin *et al.*, 1991).

Using negatively stained thin filaments isolated from frog thigh muscle isolated in the relaxed state, Lehman *et al.* (1995) have been able to make further conclusions

about the relation of TM to the presumed regions of actin involved in the interaction with the S1 region of myosin in the 'off' position. The TM strands closely approach, or contact, actin subdomain 1 near its junction with subdomain 3 then bridge over without touching subdomain 2 to neighbouring subdomains 1 and 3 on the next actin monomer. In this position actin residues 24–28 and 340–346 in subdomain 1 and residues 144–148 and 332–336 at the junction of subdomain 1 and 3 would also appear to be blocked by TM. This group of residues is presumed to be involved in myosin binding. On the other hand, the group of charged residues also thought to be involved in binding, residues 1–4 and 93–95, appear to be accessible to myosin. These findings are supported by the earlier conclusions of Milligan and Flicker (1987) and Milligan *et al.* (1990) that in the 'on' position TM is bound in the inner domain of actin with the result that the small change in azimuthal position of TM suggested to occur on relaxation would not be adequate to eclipse totally the sites of interaction between actin and myosin. The possibility that even in the 'off' position one of the myosin binding regions of actin is free to interact with myosin can be accommodated by assuming that this interaction corresponds to the weak binding state of actomyosin interaction considered to be a feature of the relaxed state of muscle. On activation the other myosin binding site postulated to be in the region of the junction of subdomains 1 and 3 becomes accessible and myosin forms the strong interaction with actin characteristic of the contracted state. Taken at face value the result of 3D reconstruction carried out by Vibert *et al.* (1997) appears compelling evidence for the steric blocking mechanism. Similar conclusions regarding the position of the TM on the F-actin filament in relaxing and activating conditions were obtained by the application of cryo-electron microscopy and helical image reconstruction to reconstituted thin filaments (Xu *et al.*, 1999). In these studies it is concluded that the TM adopts a position at a radius of 38 Å, slightly less than that obtained in studies on negatively stained filaments. In the latter preparations some uncertainty exists as to the effect of staining and drying on the preservation of protein interactions and positions.

Smooth muscle

Changes in the actin layer line diffraction patterns have been observed during contraction of vertebrate and invertebrate smooth muscles (Lowy and Small, 1970; Lowy and Vibert, 1972; Vibert *et al.*, 1972). Although the changes are similar to those observed in skeletal muscle and are interpreted as indicating movement of TM they have been reported as less marked. Significant differences in the TM filament position were observed in filaments in calcium-free buffer compared to those treated with Ca^{2+} -calmodulin. In the absence of calcium when the MgATPase would be expected to be inhibited, the TM is in contact with the inner actin domain leaving

the outer actin domain, the putative site of myosin interaction, exposed. In the activating conditions with Ca^{2+} -calmodulin the TM filament moves closer to the outer actin domain, an azimuthal rotation of $\sim 20\text{--}25^\circ$ corresponding to a distance of $\sim 15\text{--}20$ Å (Vibert *et al.*, 1993). Thus activation of the smooth muscle actomyosin MgATPase by the addition of calcium to the caldesmon-calmodulin system results in a change of the TM filament position in the actin groove which is almost the opposite to that observed on activation striated muscle actomyosin via the troponin system (Lehman *et al.*, 1996). Some attempts to answer this problem have been made (see Marston *et al.*, 1998) but the differences in the TM movement in the two muscle systems become less significant if the movement of TM in the actin groove is considered to be the result of conformational changes imposed on actin by caldesmon in smooth and the troponin complex in striated muscle.

Actin is a strongly conserved protein and it would be expected that the myosin binding site on the skeletal and smooth muscle isoforms of actin to be very similarly located. This suggests that a simple steric mechanism of the type postulated for the role of TM in skeletal muscle does not apply in smooth muscle. In striated muscle the well-documented binding of TM to the troponin complex via troponin T provides a possible mechanism through which conformational changes following from the binding of calcium by the troponin complex could lead to TM movement (see Phillips *et al.*, 1986; Heeley *et al.*, 1987; Kobayashi *et al.*, 2000 for possible schemes). Such a mechanism cannot apply in smooth muscle from which troponin is absent. In solution systems movement of smooth muscle TM on actin filaments that is induced by the presence of skeletal myosin S1, skeletal heavy meromyosin and gizzard smooth myosin S1 (enzymically active) can be demonstrated by fluorescent resonance energy transfer. This occurs in the absence or presence of ADP but is reversed by ATP (Graceffa, 1999). In the absence of nucleotide and at low myosin head/actin ratios only phosphorylated heavy meromyosin produced TM movement in this system (Graceffa, 2000). These results could be explained by the assumption that when the myosin head binds to actin and initiates events at the enzymic centre which result in ATP hydrolysis, the actin monomer undergoes conformational change. To accommodate the consequent changes at the binding site for TM on the actin, energetic considerations require the TM to move. Both caldesmon (see section on caldesmon) and calponin are considered to have roles in smooth muscle regulation and both are present in actin filaments closely associated with TM. Their precise roles are still unclear but in binding to actin they would be expected to induce conformational changes which would also result in TM movement.

Three state model

It has become apparent that the two state model as originally proposed, with TM acting as a simple steric in-

hibitor, does not satisfactorily fit with the experimental evidence available. It does not explain the cooperative effects of TM on thin filament function nor the existence of two separate 'off' states suggested from equilibrium and kinetic studies. From analysis of the kinetics of activation in reconstituted filament systems (Lehrer and Morris, 1982; McKillop and Geeves, 1993; Lehrer, 1994; Lehrer and Geeves, 1998) a three state model has been proposed. Structural evidence (Holmes, 1985; Poole *et al.*, 1995; Vibert *et al.*, 1997) also suggests that TM moves between three, and not two, distinct states on full activation of the thin filament. These correspond to the 'blocked B' state in the absence of calcium in which myosin does not bind to actin, followed by the 'closed C' state when calcium binds to troponin which is then converted to the 'open M' state by myosin binding when full activation occurs (McKillop and Geeves, 1993). Image reconstruction of electron micrographs of thin filaments partially saturated with myosin S1 provides evidence for the two stages of activation. When activated by calcium a 25° azimuthal movement of TM occurs that uncovers most of the presumptive myosin binding sites on actin. On addition of saturating amounts of S1 a further 10° movement leads to uncovering the entire myosin binding sites and leads to full activation of the system (Vibert *et al.*, 1997). From extensive image reconstruction studies the position of the TM molecule on the actin filament of striated muscle as it moves from the blocked B, position characteristic of relaxed muscle to the fully activated, M, state has been determined (see Figure 6). A puzzling feature of these analyses is that it is not yet possible to visualize the troponin complex in

relation to the TM strands although in total mass its contribution is at least equal that of the TM in each structural unit. It is considered that it makes no contribution to the studies summarised by Lehmann *et al.* (2000a) since TM strands can be visualised in the absence and presence of troponin in both B and C states depending on the source of TM used.

Some evidence of a 'closed', C, state has been obtained by the use of mutant forms of TM. Deletion of the actin-binding pseudo repeats 2, 3 and 4 of α -TM (residues 49–167) weakens the binding of full-length TM to F-actin decorated with myosin S1. In consequence the mutation suppresses the actomyosin MgATPase rate and the *in vitro* sliding rate of filaments over a heavy meromyosin coated surface (Landis *et al.*, 1997). This inhibition was not reversed by calcium and in the presence and absence of calcium the position of the mutant TM is unchanged and identical with that obtained with α -TM in the presence of calcium (Rosol *et al.*, 2000). On the addition of rigor-bonded *N*-ethylmaleimide-treated myosin S1 to the mutant thin filaments inhibition was overcome and full activation obtained. It is considered that these results support the three state model in that the mutant TM is trapped in the calcium induced, 'closed', position which is converted to the 'open' position by binding the maleimide treated myosin S1 (Rosol *et al.*, 2000).

Is the role of tropomyosin active or passive?

In current models of regulation of the contractile cycle the role of TM is largely active in that in resting muscle

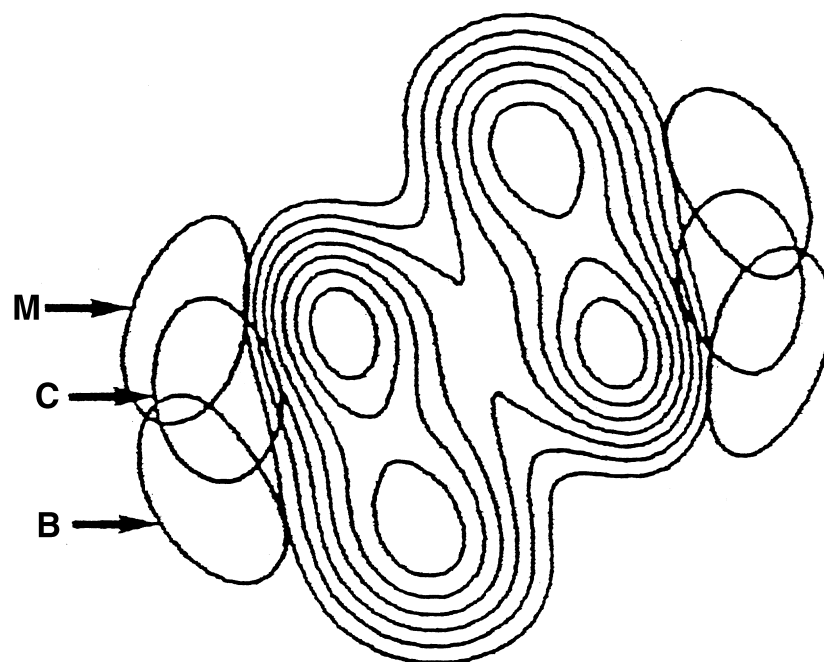


Fig. 6. Relative positions of TM on the actin filament in the B, C, and M states. F-actin and B- and C-state TM densities (depicted in Figure 4, Lehman *et al.*, 2000) have been merged and plotted as helical projections for comparison with myosin S1 decorated thin filaments generated by Vibert *et al.* (1997). TM densities labelled B, C and M, respectively. Note the three distinct TM positions and apparent binding contact points on the surface of actin; only the outermost contours of TM densities are displayed to help demarcate their boundaries. From Lehman *et al.* (2000) with permission.

its position in the filament is considered to block the myosin interaction sites on actin. The possibility, however, exists that TM's role is principally passive, that it does not exert a direct blocking role and that the movement observed is the consequence of conformational changes in the actin monomers with which it is associated (see Perry, 1996, 1999). The large increase in affinity of TM for actin on binding myosin can be explained as a consequence of conformational changes in the actin monomers produced by the interaction.

Direct evidence exists for conformational change in actin on binding TN-I, the actin-binding component of the troponin complex (Levine *et al.*, 1999). This occurs in the absence of calcium when TN-I interacts with actin to block the interaction of myosin. On activation in intact muscle this change will be reversed as the TN-I will bind preferentially to troponin C in the presence of calcium. Subsequent attachment of the S1 region of the myosin molecule leads to another conformational change in the actin. As a consequence of each of these conformational changes in the actin molecule binding energy considerations would be expected to cause the TM molecule to adjust its position on the surface of the actin filament. There is now increasing evidence involving TM mutants that indicate the movement of TM is the results of conformational changes in actin when it binds myosin and troponin (Rosol *et al.*, 2000; Lehman *et al.*, 2000b).

The known properties of TN-I indicate that in the intact thin filament of the relaxed myofibril only one (possibly two) of every seven actin monomers has a myosin binding site blocked by TN-I. It is difficult to visualise the role of TM in extending the inhibitory activity of one molecule of TN-I to up to six adjacent actin monomers. For TM to achieve this by steric blocking whilst the interaction site of one actin monomer of the structural unit of seven is blocked by TN-I, suggests the location of TM in the thin filament is less regular than that observed. It is more likely that the role of TM is to facilitate cooperativity in the thin filament so that the conformational changes in the actin monomer that binds the molecule of TN-I are transmitted to neighbouring monomers. In this way, in the 'off' position, actin monomers to which TN-I is not directly bound will adopt conformations that do not bind to myosin. In the presence of calcium TN-I binds to troponin C and leaves its binding sites on actin free. All the actin monomers will then revert to the myosin binding conformation. By a similar effect the interaction of myosin S1 with actin would induce a conformational change that is transmitted to neighbouring monomers enhancing their ability to interact with myosin S1, explaining the cooperative effect described by Bremel *et al.* (1972).

The myosin effect however can occur under conditions in which TN-I does not bind to actin, e.g. *in vitro* in the absence of troponin or *in vivo* in the presence of the troponin complex and calcium. TM is essential for cooperative effects and it is reasonable to postulate that

its role is to stabilise the actin filament and maintain the actin-to-actin contacts despite the conformational changes induced by interaction both with myosin and troponin. This enables the conformational changes to be transmitted through actin-to-actin contacts to neighbouring monomers that do not interact directly with the ligands. As a consequence of this TM will alter its position on the actin filament to accommodate the resulting changes in the relative positions of residues in the actin monomers caused by the conformational changes. This provides a more plausible mechanism for the movement of TM on contraction than the schemes involving an active unblocking role for the movement of TM from the 'off' to the 'on' position. Such schemes postulate that changes resulting from the binding of calcium at the troponin complex are transmitted, probably through the binding site for troponin T, to cause the TM, which increasing evidence indicates is not a rigid molecule, to move sideways along the whole of its length.

The interaction of actin with tropomyosin

Some time before the steric hypothesis was proposed biochemical evidence existed for a possible functional relationship between actin and TM. An early indication of possible complex formation was the observation that conventional actin preparations often contained up to 30% TM (Laki and Cairns, 1959). Ultracentrifuge studies on these impure actin preparations indicated that in certain ionic conditions, the TM present migrated with F-actin (Laki *et al.*, 1962; Martonosi, 1962). TM does not bind to G-actin but complex formation with F-actin roughly corresponding to a 1:7 molar ratio can be demonstrated by cosedimentation (Eaton *et al.*, 1975), and flow birefringence (Maruyama, 1964). Binding of rabbit skeletal TM to skeletal actin cannot be demonstrated by cosedimentation at low ionic conditions e.g. 20 mM KCl and 1 mM MgCl₂ concentrations. Binding takes place at 5 mM MgCl₂ and 100 mM KCl (Martonosi, 1962; Maruyama, 1964; Kominz, 1966; Tanaka and Oosawa, 1971; Eaton *et al.*, 1975). Although the binding of TM to actin appears to be a property of all isoforms and very cooperative when it is observed (Yang *et al.*, 1979; Hill *et al.*, 1992: see section on the cooperative effects of TM), the precise ionic conditions for binding *in vitro* depend on the species and muscle type from which the proteins are derived. For example at pH 7.0 bovine cardiac and rat skeletal α -TM, unlike the corresponding rabbit isoform, bind little actin in 5 mM MgCl₂ and 0.15 M KCl. Further, *Acanthamoeba* actin binds almost no TM in 5 mM MgCl₂ but the binding is much strengthened in the presence of heavy meromyosin (Yang *et al.*, 1977). Reported values for the apparent binding constant of rabbit skeletal muscle TM range from $2 \times 10^7 \text{ M}^{-1}$ (Lehrer and Morris, 1982) to $1\text{--}4 \times 10^6 \text{ M}^{-1}$, depending on the type of actin (Yang *et al.*, 1979). A value within

this range ($4.6 \times 10^6 \text{ M}^{-1}$) has been reported for rat smooth muscle TM (Hammell and Hitchcock-Degregori, 1997).

The alternatively spliced exon 6 of the rat α -TM gene that encodes residues 189–213 is important for cooperative actin binding. Mutants in which this region is deleted and possessing the exon 9d encoded C-terminus, which is characteristic of smooth muscle TMs, lost the ability to bind actin. Surprisingly the actin binding property of the same deletion mutant but with exon 9a encoded C-terminus, was little changed (Hammell and Hitchcock-Degregori, 1997). This observation is compatible with a large number of earlier findings that have established that modifications at the N- and C-terminals of TM can have marked effects on the cooperative binding of actin (Mak and Smillie, 1981; Dabrowska *et al.*, 1983; Heeley *et al.*, 1987; Hitchcock-DeGregori and Heald, 1987; Heald and Hitchcock-DeGregori, 1988; Cho *et al.*, 1990; Cho and Hitchcock-DeGregori, 1991; Willasden *et al.*, 1992; Butters *et al.*, 1993; Novy *et al.*, 1993a, b; Ubancikova and Hitchcock-DeGregori, 1994; Pittenger *et al.*, 1995).

Tropomyosin and actin filament formation

There is early evidence that skeletal TM can affect the rate of conversion of G- to F-actin. Using birefringence of flow to follow polymerisation of ATP G-actin it was reported that TM slows down the rate (Maruyama, 1964) a conclusion supported by the later careful study of Wegner (1982). On the other hand, it was concluded from viscosity measurements that TM speeded up the polymerisation of both ATP and ADP G-actins, particularly the latter at 0°C (Pragay and Gergely, 1968). The somewhat conflicting nature of these reports may reflect the presence of indeterminate amounts of the various actin-binding proteins in the early preparations of actin and TM and which were not recognised at the time.

Studies employing the quasielastic scattering of laser light indicating that TM stiffens the F-actin filament (Fujime and Ishiwata, 1971) have been confirmed by direct measurements on single actin filaments (Kojima *et al.*, 1994). Further evidence that TM appears to stabilise the structure is given by observations in the electron microscope. In the presence of TM, F-actin filaments appear 'straighter' (Kawamura and Maruyama, 1970; Takebayashi *et al.*, 1977) and are stabilised during fixation (Maupin-Szamier and Pollard, 1978). These results imply that TM is the major contributor to the increased bending stiffness observed when TM-troponin is bound to actin filaments (Goldmann, 2000). Further evidence of the role of TM in stabilising F-actin by binding longitudinally along the filament is provided by studies with a polymerisation-defective actin mutant (V266G, L267G). Polymerisability at 25°C is restored by cardiac TM and yeast TPM1 and TPM2, with rescue efficiency proportional to the TM length (Kuo-Kang *et al.*, 2000).

In addition to the ionic conditions described above, a number of protein factors will also modulate the

interaction of TM with actin and its effect on filament formation. The affinity of TM for F-actin is increased 80–130-fold by troponin but cooperativity is slightly decreased (Wegner and Walsh, 1981; Hill *et al.*, 1992). These effects could be due to a number of factors. TN-I binds strongly to actin and induces a conformational change which affects its interaction with myosin, an effect that is modulated by TM (Levine *et al.*, 1999). The observation that at low ATP concentrations TM can enhance the interaction between myosin heads and F-actin (Bremel *et al.*, 1972) implies that the binding of the myosin head in some way modulates the interaction of TM with actin. This interaction was later shown by Eaton (1976) to be much strengthened in the presence of heavy meromyosin. The internal region of the TM molecule, consisting of the quasiequivalent actin binding sites 3, 4, and 5 (residues 81–188 in α -TM) is particularly important for the myosin S1 effect on actin (Landis *et al.*, 1999). A TM mutant with this region deleted possesses a similar affinity for actin as the full length TM but the affinity for actin decorated with myosin S1 was 100 times less (Landis *et al.*, 1997). A consequence of the binding of myosin (myosin S1) to actin is a change in the position of the TM molecule in its relation to the actin filament (Lorenz *et al.*, 1995; Lehman *et al.*, 1996; Vibert *et al.*, 1997).

The affinity of actin for shorter TM molecules, the wild type yeast isoforms TM1, five actin-binding sites, and TM2, four actin-binding sites, is increased much less by myosin S1 than is the case with full-length skeletal α -TM (Strand *et al.*, 2000).

Role of tropomodulin

With the elucidation of the dynamics of actin polymerisation and the availability of actin capping proteins has been possible to define more clearly the role of TM in F-actin filament assembly both in muscle and non-muscle tissues. In the myofibril the pointed end of the actin polymer is located at the end of the thin filament in the A band. Although association and dissociation of actin monomers is much slower at the pointed than at the barbed end of the F-actin filament, it is not considered slow enough to maintain the actin filaments at a fixed length. Muscle and non-muscle TM have been reported to slow down the rate of assembly of actin filaments in which the barbed end is blocked with gelsolin or villin, but only by a relatively small factor (Keiser and Wegner, 1985; Lal and Korn, 1986; Hitchcock-DeGregori *et al.*, 1988b; Wegner and Ruhnau, 1988; cf Broschat *et al.*, 1989). In the presence of TM and troponin the rate of association and dissociation at the pointed end is reduced 30-fold (Weigt *et al.*, 1990).

TM stabilises the short actin filaments in non-muscle cells (Broschat *et al.*, 1989) and the longer filaments of muscle cells (Broschat, 1990) by lowering the off rate constant for actin polymerisation at the pointed end of the filament. The effect of TM on the on rate is much less significant but to inhibit filament elongation at the pointed end completely the capping protein tropomod-

ulin is required (see reviews, Coluccio, 1994; Fowler, 1996). Tropomodulin was first isolated as a 40.6 kDa TM binding protein attached to the pointed ends of the short actin filaments of the erythrocyte membrane (Fowler, 1987, 1990). It is also present in muscle tissue and has been shown to be located in rat skeletal muscle at the pointed ends of the thin filaments by antibody staining of two bands in the A band of the myofibril (Fowler *et al.*, 1993). Similar location is shown by antibody staining of chicken skeletal muscle but in addition strong staining of the Z line is also seen in this tissue (Sussman *et al.*, 1996). This observation suggests there may be a difference between the relation of TM and tropomodulin in the Z line structure in rat and chicken muscle. It could, however, be explained by the report that under some conditions tropomodulin can also cap the barbed end of F-actin filaments (Kimura *et al.*, 1999). Two isoforms of tropomodulin, which differ in their tissue distribution, have been identified, E- and Sk-Tmod (Almenar-Queralt *et al.*, 1999), which are 62% identical in amino acid sequence. Sk-Tmod predominates in fast skeletal muscle fibres, lens and erythrocytes whereas E-Tmod is present in cardiac and slow skeletal muscle. When both isoforms are present in skeletal muscle they are associated with different actin filaments. Little is known about the specific association between TM and tropomodulin isoforms but the major TM isoforms in the erythrocyte are TM5 and TM5b, products of the TM3 gene and both of which bind strongly to the E-Tmod isoform (Sung *et al.*, 2000).

The binding of tropomodulin alone to the pointed end of the filament acts as a leaky cap for it does not completely inhibit chain elongation. Complete blocking of elongation and depolymerisation requires that TM must also be present (Weber *et al.*, 1994; Kimura *et al.*, 1999). The short erythrocyte actin filament has a similar length to the erythrocyte TM molecule, 33–34 nm. In view of this, and the fact that approximately equimolar amounts of tropomodulin to TM (1.4:1) are present, it has been proposed that TM specifies the actin filament length in the erythrocyte membrane (Fowler and Bennett, 1984; Shen *et al.*, 1986). The major TMs present in the erythrocyte, the LMW isoforms TM5 and TM5b expressed by the α -gene, have a structural role in the formation of hexagonal lattices in the erythrocyte membrane skeletal network (Sung *et al.*, 2000; see Figure 7).

In the case of the skeletal muscle myofibril between 1.2–1.6 copies of tropomodulin are present per thin filament (Fowler *et al.*, 1993). The current view is that TM is required for actin filament assembly and for binding of tropomodulin at the pointed end to cap the filament. At the capped end there is probably a tropomodulin molecule bound both to the N-terminus of each TM and to a terminal actin monomer (Gregorio *et al.*, 1995; see Figure 7). If the tropomodulin is rendered ineffective by injecting antibody into embryonic chicken cardiac myocytes marked elongation of the actin filaments occurs (Gregorio *et al.*, 1995). The

importance of the molecular ratio of tropomodulin to the other myofibrillar proteins is also indicated by the effect of over or underexpressing the tropomodulin gene by transfecting neonatal rat cardiomyocytes with sense and antisense tropomodulin mRNA respectively. In both cases myofibrillar disorganisation resulted with over expression producing shortened actin filaments and dilated cardiomyopathy (Sussman *et al.*, 1999). In contrast under expression of tropomodulin resulted in abnormally long actin filaments (Sussman *et al.*, 1998). The fact that nebulin also extends along the length of the thin filament of striated muscle implies that this protein also may have a role in maintaining the filament length. A recent report that the N-terminal of nebulin interacts with tropomodulin at the pointed end of the thin filament suggests that these two proteins together with TM function as a mechanism to control thin filament lengths in skeletal muscle (McElhinny *et al.*, 2001).

The interaction of tropomodulin with TM appears to be unusual in that the N-terminus of TM binds to sites on tropomodulin than are different on each tropomodulin isoform, i.e. the TM binding site is isoform specific (Babcock and Fowler, 1994). From a comparison of the affinities of various TM isoforms and deletion and missense mutants, the erythrocyte tropomodulin binding site has been mapped to residues 7–14 of hTM5, at the beginning of the long heptad repeats. This isoform from which the N-terminal 18 residues have been deleted no longer binds tropomodulin (Sung and Lin, 1994; Vera *et al.*, 2000a, b). As the N-terminus of TM is located at the pointed end of the actin filament, the binding of tropomodulin at this region presumably prevents a further TM molecule from forming the N–C overlap required to polymerise further. The tropomodulins are highly conserved proteins, nevertheless residues 6–94 are required to bind skeletal TM but do not bind erythrocyte TM. Likewise residues 90–184 are adequate to bind erythrocyte TM but a fragment incorporating this region, residues 90–359 will not bind muscle TM (Babcock and Fowler, 1994). Bovine heart TM appears to bind somewhat closer to the N-terminus of chicken erythrocyte tropomodulin, i.e. in the N-terminal 11 kDa region (Kostyukova *et al.*, 2000). The binding site on human tropomodulin for erythrocyte hTM5 has been mapped to lie in the region bounded by residues 104–127 (Vera *et al.*, 2000b). Despite the difference in interaction sites the dissociation constants for erythrocyte and muscle TM binding by erythrocyte and muscle tropomodulins respectively, are very similar ($\sim 0.2 \mu\text{M}$). This unusual isoform specificity of the interaction sites for TM may be of functional importance.

Tropomyosin and actomyosin ATPase

The rationale of the steric hypothesis in its original form is that in resting conditions TM prevents the interaction of actin with the enzymically active myosin head. Support for this came from the observations that under defined conditions ($>1 \text{ mM MgCl}_2$ and higher KCl

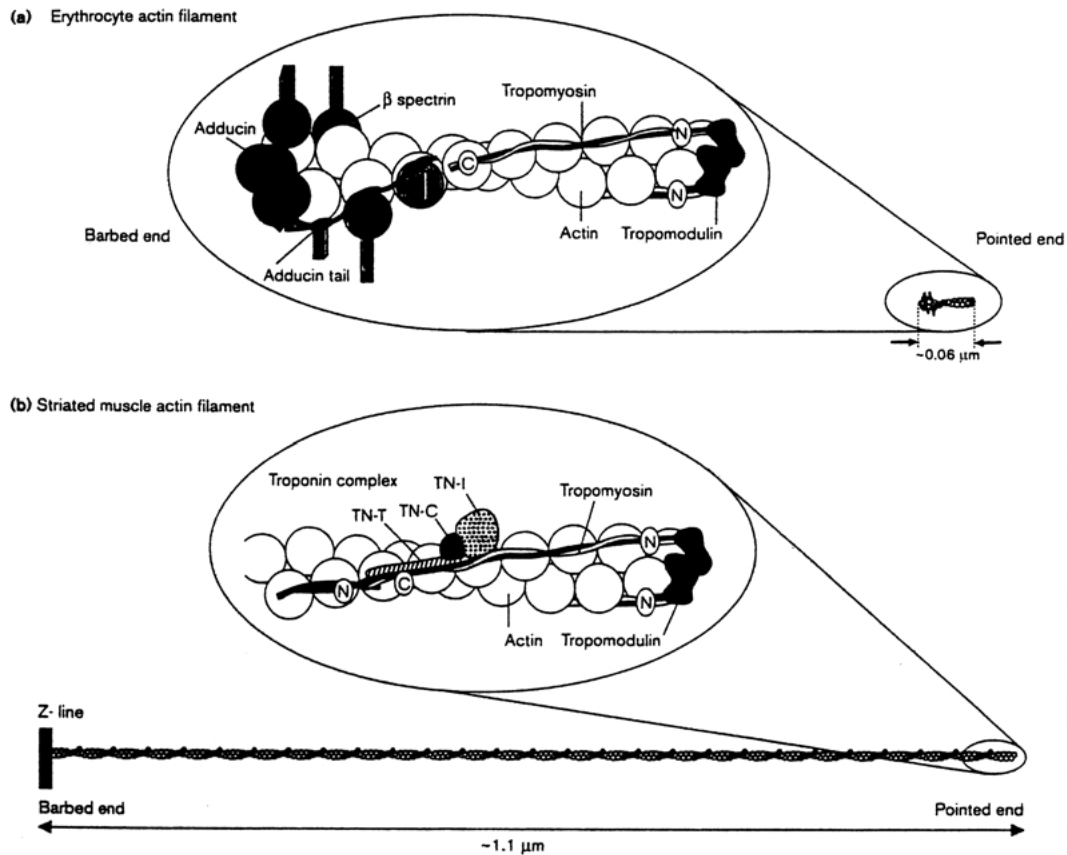


Fig. 7. Schematic models of erythrocyte and striated muscle actin filaments. (a) Model for the short erythrocyte actin filament whose length is determined by the length of the LMW TM (5/5b) capped by tropomodulin. Two tropomodulin molecules are shown to be associated with the N-terminal end of each TM molecule and with the actin subunits at the pointed end of the filament. (b) Model for the pointed end of the striated muscle thin filament. Two tropomodulin molecules are shown to be associated with the N-terminal end of each TM molecule and with the actin subunits at the pointed end of the filament. For simplicity the two nebulin filaments have been omitted from this model but each is presumed to form an extended α -helix in the central groove on each side of the actin filament. N and C indicate amino and carboxyl terminals respectively. From Fowler (1996) with permission.

concentrations) rabbit skeletal TM inhibits the actomyosin MgATPase (Eaton *et al.*, 1975). With acto-heavy meromyosin the maximum inhibition obtained in these *in vitro* experiments with TM is about 60%, much less than occurs in resting muscle. At low myosin S1 concentrations, however, inhibitions approaching 100% have been reported (Lehrer and Morris, 1982). In these studies there is correlation between the degree of inhibition of the acto-subfragment 1 MgATPase and the extent of TM bound to actin (Eaton *et al.*, 1975; Lehrer and Morris, 1982). The maximal inhibition by TN-I in the presence of TM can occur at low MgCl_2 and KCl concentrations, conditions in which TM alone, does not inhibit the MgATPase (Schaub *et al.*, 1967; Shigekawa and Tonomura, 1973; Eaton *et al.*, 1975) and does not cosediment with actin (Martonosi, 1962).

Although there is no doubt that *in situ* the TM is associated close to the actin filament there are problems in determining the nature of this relationship from *in vitro* solution studies. Under these conditions TM can either inhibit, activate or have no effect on the MgATPase of acto-heavy meromyosin, the effect depending on the ATP and TM concentrations, the molar ratio of

actin to myosin heads and the presence of other myofibrillar proteins (Schaub *et al.*, 1967; Eaton *et al.*, 1975; Lehrer and Morris, 1982). The factors that determine the binding of actin to TM are clearly complex, and as the interaction is temperature sensitive it would be expected to be reduced at physiological temperatures (Tanaka and Oosawa, 1971). The physical state of the actomyosin complex is also important. In an early study on the superprecipitation of actomyosin, Katz (1964) showed that TM inhibited the MgATPase during the clearing (sol phase) and enhanced it during superprecipitation (gel phase).

The effect of ionic conditions on the inhibitory activity of TM differs between isoforms. For example smooth muscle TM, which is expressed by differential RNA splicing of the α -gene that is also expressed in striated muscle, acts on the MgATPase of skeletal actomyosin S1 in a different way to the striated isoform of TM. At low ionic strength smooth muscle TM inhibits the MgATPase and activates at higher ionic strengths (Williams *et al.*, 1985). Skeletal and smooth muscle TM activate smooth muscle actomyosin MgATPase but only if the myosin is phosphorylated

(Chacko *et al.*, 1977; see Chacko and Eisenberg, 1990, for other references). The nature of the actin is also important. Under conditions in which rabbit muscle TM binds to both muscle and *Acanthamoeba* actin, it inhibits the MgATPase of muscle acto-heavy meromyosin and stimulates *Acanthamoeba* acto-heavy meromyosin MgATPase (Yang *et al.*, 1977).

The inhibitory activity of troponin I

TN-I is a specific inhibitor of the MgATPase of actomyosin (Schaub and Perry, 1971; see Perry, 1999) that in the absence of TM has a maximum effect at a molar ratio of TN-I to actin of about 1:1. In the presence of TM this effect is much accentuated and the maximum effect, up to 95% inhibition, can be obtained *in vitro* at much lower molar ratios, approaching 1:7 (Perry *et al.*, 1972; Eaton *et al.*, 1975; Lehrer *et al.*, 1997a; Geeves *et al.*, 2000). These effects are obtained over a range of ionic conditions and particularly at low free magnesium and KCl concentrations (Perry *et al.*, 1972) conditions under which in the absence of TN-I, TM would not be expected to bind to actin. This effect has been explained by the postulate that TN-I induces the binding of TM to actin (Eaton *et al.*, 1975). The potentiating effect on the inhibitory action of TN-I is not restricted to the muscle TMs for it has also been reported with the platelet isoform (Cote and Smillie, 1981a, b). The effect of TM on the inhibition does not require the intact TN-I molecule, for a 21 residue peptide obtained by cyanogen bromide cleavage (residue 96–116 of rabbit fast muscle TN-I, the inhibitory peptide) which is about 50% as effective as intact TN-I, has its inhibitory activity accentuated in a manner similar to that obtained with the whole molecule (Syska *et al.*, 1976). The minimum length peptide fragment to exhibit inhibition accentuated by TM corresponds to residues 104–115 (Talbot and Hodges, 1981).

There is evidence that intact TN-I may interact weakly with TM (Pearlstone and Smillie, 1983) but it does not interact in 0.2 M KCl in the ultracentrifuge (Potter and Gergely, 1974). Any interaction that may occur does not involve the functionally active site for NMR studies indicate that the inhibitory peptide does not bind to TM. On binding to actin, however, the inhibitory peptide does induce conformational changes (Levine *et al.*, 1999). The latter results suggest that the potentiating effect is not obtained by direct interaction with TM but must be mediated in some way through TN-I interaction with actin.

Nature of interaction between tropomyosin and actin

Interaction between proteins that is of functional significance such as antibody–antigen binding, actomyosin formation or that which exists in the enzyme substrate complex usually involves highly specific sites involving a unique sequence of amino acids in each protein. This does not appear to apply in the case of the actin–TM interaction. From the nature of the structural

unit of the I filament each of the seven actin monomers must interact with a different region of the TM molecule in the coiled-coil and this relationship will be repeated along the length of the filament. The fact that the TM moves with respect to each actin monomer during the contractile cycle implies that there is not a single unique site on the actin molecule with which it preferentially interacts. If, when the muscle changes from the ‘off’ to the ‘on’ state, conformational changes occur in each actin monomer there will be some changes in the relative positions of the amino acid side chains on the surface of the actin molecule. In this case the TM movement could merely represent its adjustment on the surface of the actin monomer in response to energetic considerations arising from the new side chain environment. Such adjustment between the interfaces of the two molecules can be readily visualised if the bonds involved in the interaction are electrostatic. The atomic model obtained from X-ray diffraction studies of oriented TM–actin gels (Lorenz *et al.*, 1995) supports this view in that it gives the closest distance between carbon atoms in TM and actin to be 10.5 Å. At such a distance only ionic interactions would be expected to be significant. In this respect it is perhaps significant that although crosslinking and NMR studies have suggested residues on actin that may be involved in interaction with myosin and TN-I, similar investigations have so far been less definitive in identifying a TM binding site. In so far as specific sites on each actin monomer are concerned in the interaction with myosin, the steric hypothesis in its simplest form requires that the TM interactions corresponding to the ‘off’ and ‘on’ states to be at specific locations on each actin monomer i.e. those involved and not involved respectively in interaction with myosin. This is facilitated if the pitch of the TM coiled-coil (and the actin duplex) remains constant but it should be pointed out that at least in spermine-induced TM crystals there is evidence of variation of the pitch and the potential for irregularity as one moves along the molecule (Whitby *et al.*, 1992; Whitby and Phillips, 2000). It is not known whether similar variability occurs in the intact thin filament of the myofibril. From electron microscope reconstruction studies Milligan *et al.* (1990) conclude in the presence of calcium and subfragment 1, presumably in the ‘on’ state, TM lies on an elongated site at the extreme edge of the inner domain of actin in the filament leaving most of the outer face of the monomer accessible. From their model of the ‘off’ state as represented by unregulated TM–actin gels Lorenz *et al.* (1995) conclude that TM interacts with charged side chains in actin domains 3 and 4. On the other hand from reconstruction studies of the relaxed intact vertebrate thin filament, which is more relevant to the *in vivo* situation, Lehman *et al.* (1995) conclude that a more hydrophobic group of residues on subdomain 1 and the junction between subdomains 1 and 3 are occluded by TM.

Further aspects of the interaction have been investigated using polarisation fluorescence from a triplet

probe on TM (Chandy *et al.*, 1999). Both skeletal and cardiac TM can undergo complex rotational motions on the surface of F-actin which is decreased when myosin S1 binds to actin. Somewhat surprisingly skeletal TM which contains about 20% β -TM is less mobile than cardiac TM which is 100% α -isoform.

Amino acid residues implicated in the actin–tropomyosin interaction

The fact that the sites on actin and TM involved in their interaction are not as yet well defined probably reflects the capacity of TM to take up positions on the actin surface that vary during the contractile cycle. In a recent study, however, a mutant gizzard α -TM with a cysteine located at position 100 and labelled with benzophenone maleimide was shown to cross link with actin (Golitsina and Lehrer, 2000).

The structure of the thin filament demands that actin must lie close to a given TM molecule at seven regions equidistantly along its length. Despite the evidence of the heptad repeat no precise sequence homology can be observed in the TM polypeptide chain. Stone *et al.* (1974) pointed out, however, that the sequence could be resolved into regions of non-acid residues separated by aspartic and glutamic rich regions, the repeat distance being about 40 residues. Thus there are seven quasi-equivalent regions, not identical in amino sequence, distributed along the TM molecule which are presumed to be of significance for interaction with actin. Parry (1974) also noted a similar pattern of distribution of polar and non-polar residues in the C-terminal half of the TM molecule. From a more detailed analysis of the sequence of rabbit skeletal α -TM 14 acidic acid residue repeats spaced 19.73 residues apart, potential actin-binding sites, have been identified on each molecule (Stewart and McLachlan, 1975; McLachlan and Stewart, 1976). The latter authors suggested that the 14 quasiequivalent actin-binding sites are arranged in two sets of seven zones, the α and β sites and that binding of TM to actin occurs by salt linkages to basic groups on the actin surface. In view of the importance of magnesium in actin–TM complex formation (Martonosi, 1962) it is likely that magnesium bridges between acidic groups on both molecules can occur. By lying in the groove of the F-actin filament the TM molecule supercoils to complete seven half turns per molecular length and spans seven actin monomers. The potential binding sites are bands of acidic amino acids 10–12 residues wide and situated on the outside of the TM supercoil (Figure 8). These bands occur with a highly significant periodicity of 19.73 residues. From their analysis of TM crystal structure Phillips *et al.* (1986) considered the helical positions of the amino acid residues and concluded that there were seven dominant sites roughly equivalent to Stewart and McLachlan's α sites that could correspond to actin contact sites. When calcium is present they suggested that the α sites bind to actin in the contracted or potentiated state of muscle.

On balance the evidence strongly suggests that ionic forces have an important role in the interaction that occurs between actin and TM. In this type of interaction the juxta positioning of involved side chains may not be so critical as that required by interaction involving highly specific sites. The charged amino acids have long flexible side chains which can accommodate irregularities in the distribution of the acidic amino acids in rabbit skeletal α -TM required for interaction with F-actin. Further, the differences in sequences that exist between the various isoforms of TM present in a given species and all of which can interact with the strongly conserved actin in conditions appropriate for the isoform, can also be accommodated in this way. The concept has emerged of TM 'floating' in the actin groove and held in position by ionic forces. If this is the case any conformational changes occurring in the actin monomer which result in the movement of polar amino acid side chains would be expected to lead to readjustment of the TM molecule along the whole of its length.

Modification of tropomyosin

The fact that TM does not occur in bacteria and can be expressed as a soluble stable protein in *E. coli* means that its structure–function relationships can be conveniently studied by recombinant DNA technology. Conclusions about the structural requirements of TM for actin binding obtained from the earlier recombinant work have been described by Hitchcock-Degregori (1994).

Chicken recombinant striated muscle α -TM, which is produced without the acetyl group on the N-terminal methionine, binds weakly to actin but binds well in the presence of troponin. The fusion protein in which the acetyl group is replaced with a 80 residue polypeptide binds to actin with slightly higher affinity than the wild type isoform (Hitchcock-Degregori and Heald, 1987; Heald and Hitchcock-Degregori, 1988). Neither of these recombinant forms exhibit head to tail polymerisation indicating that this is not necessary for actin binding. The dipeptide Ala–Ser, or peptides of 3 or 17 residues, can also substitute for the acetyl group and restore actin binding to deacetylated recombinant TM (Monteiro *et al.*, 1994). Recombinant deacetylated LMW yeast TM1 which possesses five actin binding sites also requires the N-terminal peptide extension to restore actin binding (Maytum *et al.*, 2000).

It is of interest that TM expressed in SF9 insect cells that are known to have the capacity to acetylate proteins, binds actin more strongly than the bacterially produced protein (Pittenger *et al.*, 1995). More remarkably rat skeletal β -TM, which differs from the α -isoform principally in exon 2a and in the C-terminal coding exon, does not require the N-acetyl group for binding (Pittenger *et al.*, 1995). Mutant studies have indicated that Gln275 and Thr276 are important for the actin affinity of smooth muscle TM which also does not require N-acetylation for actin binding (Cho, 2000). The conclusion from these studies and those of the Hitchcock-DeGregori group (Moraczewska *et al.*, 1999;

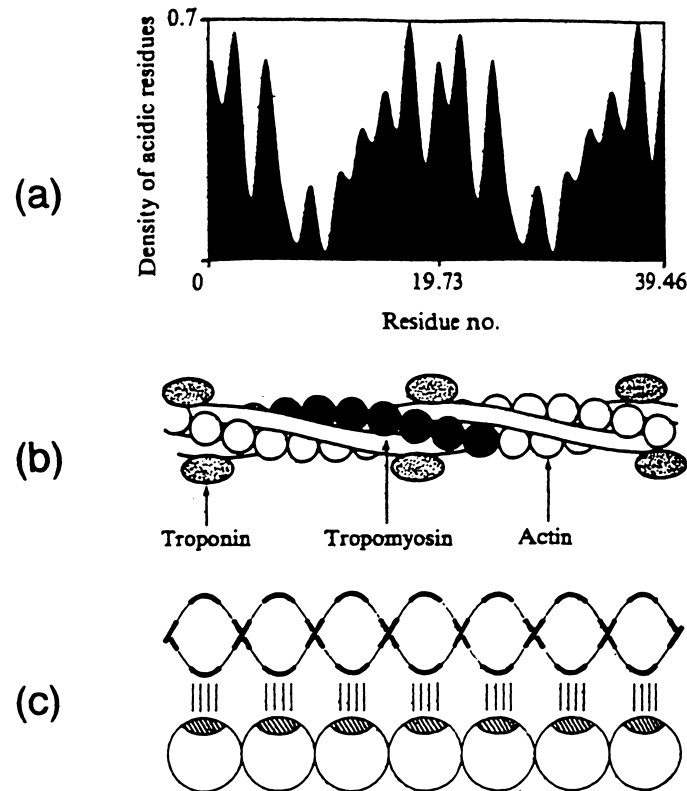


Fig. 8. Actin binding sites on TM. (a) Average composition of the acidic repeat unit of TM (two consecutive repeats shown). The origin corresponds to the N-terminus of the sequence or points multiples of 19.73 residues from it. (b) Arrangement of the proteins in the thin filament. (c) Modelling of the interaction of seven actin monomers with a TM molecule. This projection corresponds to unwinding the actin helix in (a) so that the seven shaded actin monomers lie in a straight line. This operation preserves the relative positions of the actin and TM interaction but imparts an additional half turn to the TM coiled-coil so that its apparent pitch is reduced to 114 Å as apposed to 137 Å in the thin filament. Note that the TM pitch in spermine crystals has now been shown to be 140 Å and there are approximately three full turns per molecular length of TM (Whitby and Phillips, 2000, Figure 4). The 28 negative zones on the TM molecule (indicated in the coiled-coil by dark zones) are arranged in four series of seven. One of these series is placed so that each of its zones makes an equivalent contact with an actin monomer. From Stewart and McLachlan (1975) with permission.

Moraczewska and Hitchcock-DeGregori, 2000) are that the amino acid sequences at the N- and C-terminals, in certain internal regions of TM and the interrelations of these sites are important for actin binding. Removal of nine residues of the N- and C-termini from muscle and non-muscle α -TMs greatly reduces the affinity for actin alone and in the presence of troponin + Ca^{2+} and when saturated with myosin S1. Truncation of both termini has the greatest effect and the reduction in actin affinity is greater after N- than after C-truncation. Cooperativity of actin binding was preserved but binding of actin saturated with myosin S1, unlike the case with the wild type TM, was not cooperative (Moraczewska and Hitchcock-DeGregori, 2000).

There is direct evidence from mutation studies that the sevenfold periodicity of the TM sequence (Stewart and McLachlan, 1975) is important for actin binding. The fusion protein of chicken α -TM with a complete actin-binding site, one half turn of the coiled-coil deleted (residues 47–88), retains its ability to bind actin. Mutants with half (residues 47–67) or two-thirds of a binding site (residues 47–74) deleted exhibit much weaker actin binding (Hitchcock-DeGregori and

Varnell, 1990). Deleting binding sites 2 and 3 (residues 47–123) slightly reduced the binding of fusion protein-chicken α -TM to actin (Hitchcock-DeGregori and An, 1996) but somewhat surprisingly the binding to actin by rat α -TM $\Delta(49-167)$ with sites 2, 3 and 4 deleted is very similar to that of the wild-type TM (Landis *et al.*, 1997). From analysis of the binding of mutant TMs in which internal sequences have been deleted or replaced with non-TM sequences a number of conclusions can be made about the requirements for high affinity binding to actin in the presence of troponin (Hitchcock-DeGregori and An, 1996). TM must be coiled-coil along its length although the ability to polymerise does not appear to be essential for binding to take place (Hitchcock-DeGregori and Heald, 1987). An integral number of repeats of approximately 40 residues, corresponding to the binding regions for the actin monomers, is more important than the total number. This conclusion is supported by the length of the sequences of the wild type isoforms. TM in muscle contains 284–281 residues, representing seven actin monomer binding sites, non-muscle TMs contain 247–251 residues and possess six monomer binding sites and yeast TMs containing 199 and 161 residues possess

five and four binding sites, respectively. Binding is less sensitive to the precise sequences of the sites on the TM so long as they exhibit quasiequivalence. It has been suggested that these act as weakly interacting spacers facilitating alignment of the ends of the TM molecules along the actin filament (Hitchcock-Degregori and An, 1996).

It is surprising that although single substitutions of charged residues at a number of sites on the actin molecule with uncharged residues have little effect on the affinity for TM (see below), replacing serine 283 with glutamic, lysine or alanine significantly reduces the affinity of TM for actin (Sano *et al.*, 2000a). On the other hand phosphorylation of serine 283 does not change the actin affinity (Heeley *et al.*, 1989).

Modification of actin

Lorenz *et al.* (1995) predicted that the actin residue Asp-311 had a high binding energy contribution to actin

binding. However, on replacing residues 311 and 312 of yeast actin with alanine (mutant E311A/R312A) no differences could be detected in the binding of the mutant and wild-type to TM. On the other hand increased sliding speeds and calcium sensitivity were observed in the motility assay in the presence of TM and troponin compared to the wild-type yeast actin (Gerson *et al.*, 1999). A large number of studies have been made on the effects of mutating or chemically modifying residues in the actin molecule (see Table 1). In many cases replacing a charged residue with an uncharged one, such as alanine has not produced marked effects on the TM binding when it has been measured directly. These results are perhaps not surprising as the mutated residues in these experiments only represent at most a few percent of the total charged residues in the molecule. In contrast to the majority of studies with mutant actins TM binding to actin is increased three times when the

Table 1. Effect of actin sequence modification on TM interaction

Modification	TM binding	ATPase and motility assays	Authors
<i>Mutation</i>			
G2E (<i>Dictyostelium</i>) D24A/D25A (yeast)	Little change	ATPase little changed Lower ATPase, decreased Ca sensitivity	Saeki <i>et al.</i> , 1996 Wong <i>et al.</i> , 1998
E99A/E100A (yeast)		Lower ATPase, decreased Ca sensitivity	
D56A/E57A (yeast)	3× increase in affinity	Ca does not activate regulated system	Korman <i>et al.</i> , 2000
E93K (<i>Drosophila</i>)	Not measured directly infer binds only in off state	Similar to WT in absence of TM, Decreased proportion mobile filaments with increasing TM	Bing <i>et al.</i> , 1998
K238A/E241A/E360H	Becomes Mg sensitive	Low ATPase with TM and troponin	Saeki <i>et al.</i> , 1996
E360H	Decreased binding + Mg	High ATPase and motility in presence of TM	Korman and Tobacman (2000) Saeki <i>et al.</i> , 2000
QTAAS to KAYKE (<i>Dictostelium</i>)	Decreased binding + Mg		
D222A/E224A/E226A (yeast)	3× increase in affinity		
Q228K/T229A/A230Y Q228K/T229A/L236A <i>Dictostelium/Tetrahymena</i>			
K315A/R316A (yeast)	No significant effect ± Ca	Decrease in Ca affinity of regulated filament	Korman and Tobacman, 1998
4AC actin (yeast)	No significant effect ± Ca		
E311A/R312A (yeast)	No significant effect	Speed and Ca sensitivity increase presence TN, TM	Gerson <i>et al.</i> , 1999
<i>Chemical modification</i>			
Fluorescein 5-isothiocyanate Lys-61 (muscle)	Not affected when actin polymerised with phalloidin	ATPase restored in presence of phalloidin	Miki, 1989
1,2-cyclohexanedione (muscle) Arg-95	Marked reduction	Myosin ATPase unchanged	Johnson and Blazyk 1978
Reductive methylation of lysines (muscle)	In presence of TM methylation Lys-237, -325, -327 and particularly Lys-335 reduced		Szilagyi and Lu, 1982
2,4-pentadione Lys-237 (muscle)	Failed to bind at μ = 0.12 but does in presence of Mg, Ca TN	HMM ATPase unchanged but TM does not inhibit	El-Saleh <i>et al.</i> , 1984
(<i>m</i> -maleimidobenzoyl) - <i>N</i> -hydroxysuccinimide ester Lys and Cys modified (muscle)	Reduced, but improved in the presence of troponin	ImpairsTM–troponin regulatory sytem	Miki and Hozumi, 1991

HMM, heavy meromyosin; TN, troponin complex; WT, wild type.

charged residues 56 and 57 in domain 2 are replaced with alanine (D56A/E57A). Although these residues are not considered to be directly involved in interaction with the myosin (Rayment *et al.*, 1993) calcium does not activate the MgATPase of myosin S1 in the presence of TM, troponin and D56A/E57A actin (Korman *et al.*, 2000). It is of interest, in view of the importance that the charged residues of TM are considered to have for the actin interaction, that a similar mutant of TM in which three charged residues on domain 4 are replaced with alanine (D222A/E224A/E226A) also shows an increase in affinity for actin (Korman and Tobacman, 2000).

Replacement of residues 228–230 or 228–232 of dictyostelium actin with the residues present in these positions in tetrahymena actin lead to higher calcium activation of the regulated actomyosin-S1 MgATPase activity (Saeki *et al.*, 1996). The ratio of activities with and without troponin-TM is four times higher with the mutant than with wild type actin. As X-ray crystallographic studies indicate relatively minor changes in the overall actin structure it is concluded that residues in the neighbourhood of residue 230 form part of the TM interacting site of actin in contracting muscle (Matsuura *et al.*, 2000).

More marked effects on the actin-TM interaction have been observed with chemically modified actin, no doubt because the modifications are more extreme and usually bulkier. In more complex systems such as those involving motility and ATPase assays in the presence of troponin, effects have usually been reported with the mutated and chemically modified actins, often involving changes in calcium sensitivity. These effects have usually been interpreted on the basis of the steric blocking model. For example, in experiments using a mutant of *Drosophila* actin, replacing a glutamic acid residue in domain 2 with lysine (mutant E92K) inhibited the motility of actin filaments at pCa5 from which the authors conclude that the filaments were converted from the 'on' to the 'off' state (Bing *et al.*, 1998).

The cooperative effects of tropomyosin

Cooperative effects in the actin filament system of muscle were first pointed out by Bremel *et al.* (1972) and have been investigated and discussed by a number of workers since (see Lehrer and Geeves, 1998; Squire and Morris, 1998 for reviews). It is clear that the manner in which actin interacts with several of the protein components of the I filament is modulated by TM in subtle ways not yet understood. When actin alone interacts with heavy meromyosin kinetic studies of the MgATPase indicate that the rate constants are similar under all conditions (Eisenberg and Moos, 1970). With the regulated filament in the presence of calcium at low ATP concentrations the activating effect of actin on the MgATPase is potentiated and the response is biphasic. The effect in the presence of calcium does not depend on troponin for it is obtained with TM alone (Bremel *et al.*,

1972; Lehrer and Morris, 1982; Williams and Greene, 1983). It is considered to be due to an increase in apparent affinity of actin for the myosin S1 enzyme substrate complex (Bremel *et al.*, 1972) as a result of the actin monomers acquiring the ability to act cooperatively. It has been suggested that the myosin S1 rigor complexes present at low ATP concentration induce conformational changes in one actin monomer that can be transmitted to neighbouring monomers (Bremel *et al.*, 1972). Although troponin does not affect the cooperative effect of TM on the binding of myosin S1 to actin in the presence of calcium, in the absence of calcium it markedly enhances the binding of myosin S1 to actin (Williams and Greene, 1983). Gizzard TM has cooperative effects on the actin activated MgATPase of gizzard smooth heavy meromyosin. The effect is considered to be induced by the action of myosin S1 rigor complexes on actin in a similar manner to that postulated for the skeletal actomyosin system although differences in the kinetic parameters of the two systems are reported (Chacko and Eisenberg, 1990).

Another striking cooperative effect induced in actin by TM is its potentiation of the inhibitory effect on the actomyosin MgATPase of TN-I and the inhibitory peptide derived from it (see section on interaction of actin and TM). It is unlikely that rigor complexes are involved in this effect which is obtained in 2.5 mM ATP when their concentration must be negligible (Perry *et al.*, 1972). Recently it has been shown that binding of the inhibitory peptide of TN-I to actin leads to conformational changes in actin that result in the dissociation of a peptide corresponding to a region on the myosin head known to interact with actin (Levine *et al.*, 1999). TM alone is not able to bring about dissociation from actin of the interacting myosin peptides.

Inhibition of actomyosin S1 ATPase by caldesmon in the presence of smooth muscle TM is also highly cooperative for 90% inhibition is obtained when one molecule of caldesmon is bound per 10 actins. The effect is isoform specific for a similar degree of inhibition requires one molecule of caldesmon to be bound to four actins in the presence of skeletal TM (Marston and Redwood, 1993).

The evidence suggests that the head of the myosin molecule, subfragment 1, TN-I and caldesmon can interact with actin in such a manner that the effects are, in the presence of TM, transmitted to neighbouring actin monomers which are not in contact with the effector molecules, i.e. the effects become cooperative. Interaction with the effector molecules would be expected to produce conformational changes in the actin molecule, which because of the nature of the close packing in the actin filament will have an effect on neighbouring monomers with which the effector does not interact. The binding of myosin S1 and TN-I have markedly different effects on the enzymic activity of the system and must impose different conformational changes in the actin. The fact that TM is essential for

both cooperative effects could imply that its role is not specific for the effector but in some way is associated with its interaction with the actin filament to enable conformational changes in the monomers to be transmitted along the filament.

Quite apart from the effects described above on the interaction with myosin head and TN-I, the interaction of TM to actin alone is strongly cooperative (Yang *et al.*, 1979; Hill *et al.*, 1992) with the actin becoming saturated at a ratio of seven actin monomers to one TM molecule. Analysis of the binding data gives Hill coefficients in the range 2–5, depending on the ionic conditions and the TM isoform used (Heald and Hitchcock-Gregori, 1988; Hammell and Hitchcock-DeGregori, 1997). This implies that the binding of the first few molecules of TM to the actin filament is much weaker than the binding of additional molecules. The end to end association of the TM molecules does not appear to be essential, for cooperative binding. Fusion protein α -TMs that do not polymerise, bind to actin cooperatively with similar Hill coefficients to those obtained with the wild-type protein (Heald and Hitchcock-Gregori, 1988). The cooperative response of the acto-myosin S1 MgATPase to increasing calcium concentrations also is not decreased when TM is replaced by the non-polymerisable form with 11 C-terminal residues removed by treatment with carbonylpeptidase (Walsh *et al.*, 1984).

The results suggest that there is a common element to the mechanism of cooperative response when actin responds to binding TM alone and in the presence of the other components of the myofibrillar thin filament. From the fact that in the filament seven actin monomers are associated with one TM molecule it might be assumed that the cooperative unit consists of seven actin subunits. Such an assumption is supported by the

localisation of one molecule of the troponin complex every 385 Å, the length of this structural unit. It follows that the changes resulting in activation that occur in the troponin complex when calcium is bound must affect the actin–myosin interaction along the length of this structural unit. Further support for a cooperative unit of this size is indicated by the observations that in the presence of TM one molecule of TN-I can exert inhibitory activity on up to seven actin monomers. Indeed Greene and Eisenberg (1980) reported that a cooperative unit of seven actins fitted their data for the binding of myosin S1 to the actin–TM–troponin complex. The values of n , the number of actin monomers in the cooperative unit, obtained from kinetic and equilibrium studies on the fully regulated filament are somewhat variable and usually much higher than would be expected if it were the same as the structural unit. They range from the conclusion derived from tension studies (Brandt *et al.*, 1987) that the cooperative unit consists of the whole thin filament of the sarcomere, 1 μ m long and composed of about 26 structural units, 182 actin monomers, to values of 10–21 obtained from kinetic studies (Table 2). The movement of chicken gizzard $\alpha\beta$ -TM on skeletal actin by skeletal myosin S1 is also cooperative. Analysis of the data obtained by fluorescent energy transfer measurements indicates that the binding of each myosin S1 fragment to the actin filament affects the movement of 3–4 TM molecules, i.e. n , the cooperative unit, is 21–28 (Graceffa, 1999). The wide range in values of n reported for similar systems probably reflects the shortcomings of the models used to interpret the experimental data. Cooperative effects vary between TM isoforms indicating that a number of factors including length, flexibility, amino acid sequence and end-to-end interactions can all influence the properties of actin filaments. Gizzard TM, that binds more strongly to actin, and rat fibroblast

Table 2. Cooperative unit of actin

Method	TM isoform	Value of n		Authors
		TM	TM + troponin	
Tension, skinned fibres	Rabbit striated (284)		26×7	Brandt <i>et al.</i> , 1987
Myosin S1 binding, ATPase	Rabbit striated (284)	11	16	Nagashima and Asakura, 1982
Myosin S1 binding, fluorescence, light scattering	Rabbit striated (284)	5–6	10–12	Geeves and Lehrer, 1994 ^a
Myosin S1 binding, ATPase, fluorescence	Rabbit striated (284)	7–10		Lehrer <i>et al.</i> , 1997 ^a
	Chicken gizzard (284)	15–20		
	Fibroblast TM-5a (248)	15–20		
Myosin S1 binding, fluorescence	Yeast TMI (199)	~10		Maytum <i>et al.</i> , 2000
Myosin S1 binding, fluorescence TM movement	Chicken gizzard (284)	21–28		Graceffa, 1999
			TM + caldesmon	
Myosin S1 ATPase	Skeletal (284)		4	Marston and Redwood (1993)
	Chicken gizzard (284)		10	

^a In these studies the apparent cooperative unit size of the thin filament, n , is defined as the average number of actin monomers trapped in the open state by the binding of one myosin S1. Figures in brackets indicate the number of amino acid residues in the subunit of the TM isoform.

5aTM, with one less actin-binding site, both have n values twice that obtained with rabbit skeletal TM (Lehrer *et al.*, 1997b; Table 2).

A lower limit for the structural unit exhibiting cooperativity in binding myosin S1-ADP might be expected to be obtained with the LMW TM expressed by *S. cerevisiae*, but this is not the case (see Table 2). Filaments containing TM1 consisting of 199 residues encompassing five binding sites for actin exhibit cooperative binding of myosin S1-ADP whereas filaments containing TPM2, the isoform of 161 residues and four actin-binding sites, do not (Strand *et al.*, 2000). Data obtained with yeast TM1 (Maytum *et al.*, 2000) is compatible with an apparent cooperative unit size (n) of approximately twice the structural unit (five actin sites).

Interaction of troponin T with tropomyosin

The viscous properties of the original crude troponin preparations called 'natural tropomyosin' (Ebashi, 1963) were due to complex formation between TM and the troponin T component of the troponin complex. In the myofibril the troponin-TM molar ratio is 1:1 but in solution, a 2:1 complex can be formed (Grabarek, 2000). It is of interest that the 2:1 complex is much more viscous than the 1:1 complex indicating that the attachment of two molecules of troponin to one of TM in some way enhances its polymerisation. In the absence of actin the binding of troponin T to TM coated on a plate in the solid phase assay conditions is cooperative (Ogut and Jin, 2000). This implies that under these conditions binding troponin T induces a conformational change in adjacent TM dimers thus promoting troponin T interaction with them.

The high resolution structure of troponin T has yet to be determined but the evidence obtained from cocrystals

with TM suggests that it exists as an asymmetric molecule in contact with the C-terminal region of TM along much of its length. Skeletal troponin T extends 160 Å along the TM molecule whereas the cardiac isoform with a slightly longer polypeptide chain occupies 180 Å (Cabral-Lily *et al.*, 1997). Similar studies with fragments of skeletal troponin T molecules indicate that the N-terminal 70 residues overlap the TM head to tail overlap (White *et al.*, 1987). The longer cardiac isoform which has additional amino acid residues in the N-terminus is accommodated by spanning further into the N-terminus of the next TM molecule in the overlap region. When skeletal troponin is complexed with TM the reactivity of the lysine residues of TM is affected over a longer part of the TM molecule, from residue 135 to the C-terminus, beyond the overlap region and up to residue 30 in the N-terminus of the next TM molecule, a distance of about 250 Å (Hitchcock-Degregori *et al.*, 1988a). It is conceivable that in this study the lysine reactivities at the N-terminal end of the TM interaction site could in part reflect steric effects of the other components of the complex, troponins I and C, which are located in the region of the troponin T molecule interacting with more central parts of the TM molecule.

Studies with fragments indicate that two regions of troponin T are involved in binding to TM (see Perry, 1998 for references). Site 1 was originally shown to be located on residues 71–151, corresponding to the shortest cyanogen bromide peptide that gave an increase in viscosity on addition of TM (Jackson *et al.*, 1975). The N-terminal region of troponin T which includes site 1 (T1 fragment, residues 1–158, Otsuki, 1979) binds to fragments of TM encompassing residues 190–284 (Figure 9). This site appears to be of particular importance for TM binding, for the actin affinity of unacetylated skeletal α -TM-troponin T_{70–158} is 500× greater

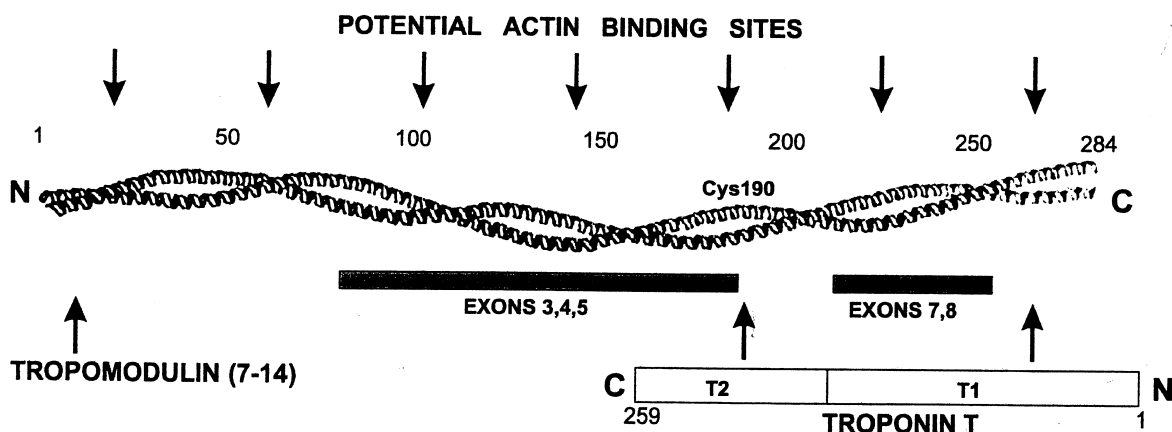


Fig. 9. Scheme illustrating interactions of striated muscle α -TM with thin filament proteins. TM molecule as determined by Whitby and Phillips (2000) illustrating irregularities in the coiled-coil observed in TM crystals (reproduced with permission). Numbers indicate positions of amino acid residues. N and C are the amino and carboxyl terminals respectively. Shaded blocks represent regions of exons that are invariant in all TM isoforms. The binding site illustrated for erythrocyte tropomodulin on human TM5 is in a region strongly conserved in TM isoforms. T1 and T2 represent the chymotryptic fragments of troponin T (Otsuki, 1979). The binding site for caldesmon is not indicated as this protein is absent from striated muscle. In smooth muscle a major site has been identified between residues 142 and 227 of TM with the suggestion of a weaker site at the N-terminus, residues 11–127 (Watson *et al.*, 1990). It appears that in striated muscle these regions are at least in part occupied by other proteins.

than that of unacetylated α -TM-troponin T_{159–259} (Willadsen *et al.*, 1992). The C-terminal residues of TM are essential for interaction with site 1.

Site 2 is on the C-terminal fragment of the troponin T molecule (T2 fragment, residues 159–259, Otsuki, 1971), a large part of which must remain intact for binding of troponin T. In the presence of TM a tryptophan residue at the C-terminus of mutant cardiac troponin T, residue 289, is shielded in static quenching by acrylamide (Robinson *et al.*, 2000). The binding region for site 2 on TM would appear to lie close to cys190 of the TM molecule (Chong and Hodges, 1982; Morris and Lehrer, 1984). Thus, the current view of the interaction is that troponin T extends along one third to one half to of the C-terminus of TM. The N-terminus of troponin T covers the overlap region of the TM polymer and extends over 10–30 residues of the N-terminal of the next TM molecule (Brisson *et al.*, 1986; White *et al.*, 1987; Hitchcock-DeGregori *et al.*, 1988a).

Troponin T is the only component of the troponin complex for which there is clear evidence of interaction with TM. This suggests that the great increase in affinity of TM for actin in the presence of troponin may be a consequence of a conformational change in the TM that occurs when it binds to troponin. As troponin also binds to actin the possibility exists that the consequent conformational changes in actin also have a role in increasing its affinity for TM. Whatever the explanation it is implicit that troponin T binds to TM in a manner that does not block the residues involved in interaction with actin. The value of the cooperative unit of the thin filament (see section on the cooperative effects of TM) is higher when measured in the presence of troponin. Troponin T is at least in part responsible for this effect for when the N-terminal chymotryptic peptide, T1, is added to the myosin S1–TM–actin system the value of n increases (Schaertl *et al.*, 1995).

The affinity of troponin–TM for actin is relatively insensitive to calcium concentration although it is twofold higher in the absence of this cation (Wegner and Walsh, 1981). An explanation of this effect of calcium can be deduced from what is known of the properties of the two TM interaction regions of troponin T. Interaction of site 1 with the C-terminus of TM is unaffected by calcium whereas that of site 2 with TM in the region of TM cys190 is stronger in the absence of this cation (Mak and Smillie, 1981). It is likely, however, that these effects with whole troponin depend on the properties of the other components of the complex, troponins I and C, the interactions of which with troponin T are also located closer to the C-terminus of troponin T where site 2 is located. Further evidence of the importance of site 2 binding is given by the fact the C-terminal T2 fragment, and not the N-terminal T1 fragment, is required for cooperative binding to TM (Ogut and Jin, 2000). In the presence of calcium, TN-I interacts strongly with troponin C but in its absence TN-I binds strongly to actin thus strengthening the binding of the troponin–TM complex to actin.

Whereas TM is usually associated with microfilaments where ever they occur, troponin T is present only as a component of the troponin complex which is restricted to striated muscle. This implies that the TM–troponin T interaction is of special significance for the mechanism of regulation of contraction in striated muscle. The significance of this interaction is emphasised by the fact that troponin T exists as a number of isoforms characteristic for the skeletal muscle type (see Perry, 1997) the binding of which for TM increases as they become more basic in nature (Ogut, 2000; Ogut and Jin, 2000).

There can be little question that the fact that troponin T binds to a specific region of TM ensures that the troponin complex is distributed with a regular periodicity along each of the F-actin filaments of the thin filament. There is less direct evidence to how the interaction of troponin T with TM is involved in the changes occurring in the troponin complex as a consequence of the binding of calcium to troponin C to initiate contraction. A number of schemes have been presented over the years postulating changes that might occur in the TM–troponin T interaction on activation of muscle (Pearlstone and Smillie, 1983; Philipps *et al.*, 1986; Heeley *et al.*, 1987; Kobayashi *et al.*, 2000).

The regions of the TM molecule, that investigations indicate as being involved in interaction with troponin T, are those encoded by exon 9, residues 258–284 and the part of the molecule close to that encoded by exon 6, residues 189–213. It is striking that these are the exons which differ in expression in α -TMs from striated muscle, controlled via the troponin system, from α -TMs of smooth muscle, which are not. Nevertheless studies using exon 6 deletion and substitution mutants, and chimeras of recombinant unacetylated skeletal α -TM indicate the interaction of troponin with TM is indifferent to the exon 6 encoded sequence (Hammell and Hitchcock-DeGregori, 1997). This sequence is, however, critical for cooperative actin binding. Although this region does not appear from the studies with recombinant mutants to interact with binding site 2 on troponin T, it must be quite close to it. The proximity of cys190, which is located in exon 6, to the C-terminal fragment of troponin T when complexed with TM is indicated by photoaffinity labelling (Chong and Hodges, 1982) and fluorescence studies which also indicate an elongated region of attachment (Morris and Lehrer, 1984).

In contrast to the role of exon 6 the entire C-terminal sequence encoded by exon 9a is essential for troponin to promote the high affinity actin binding of unacetylated skeletal α -TM. Even in the absence of calcium the first 18 residues, 258–276, of this terminal sequence of TM are critical for troponin, presumably for troponin T, to promote high affinity actin binding (Hammell and Hitchcock-DeGregori, 1996). The fact that this C-terminal exon is the most variable in the TM isoforms suggests that it may have a special role in conferring some specificity of function in the TMs occurring in different filament systems.

Although studies on the interaction of isolated troponin T with TM give insight into the nature of the interaction between these two proteins it is conceivable that this interaction and that with actin are modified in the presence of the other components of the troponin complex. This certainly would be expected to be the case with TN-I which, in the absence of calcium, binds strongly to actin and has the capacity to induce conformational changes in F-actin (Levine *et al.*, 1999). It is necessary therefore in analysing the role of TM in thin filament formation, to study the effects obtained with the troponin complex rather than troponin T. Tobacman and collaborators have attempted to study the specific interactions within the thin filament by studying TM and TM-troponin binding to actin by equilibrium analysis using the mathematical formulation for the binding of a ligand to a linear lattice (McGhee and von Hippel, 1974). From this analysis using skeletal actin and troponin with cardiac TM (and also with a homologous cardiac system) it is concluded that troponin acts by increasing the TM-actin affinity at an isolated site whilst the cooperativity effect actually decreases (Hill *et al.*, 1992). The drop in cooperativity is interpreted to imply a decrease in interaction between the TM molecules in the reconstituted filaments. This is surprising in view of the fact that the viscosity increase when solutions of TM and tropoxin are mixed in the absence of actin is usually ascribed to an increase in polymerisation of the TM. Essentially similar results were obtained with recombinant unacetylated skeletal α -TM which does not polymerise and binds weakly to actin (Hitchcock-DeGregori and Heald, 1987). The affinity of unacetylated TM is increased 1000 $\times$ by troponin even if the N-terminal 69 residues of troponin T are deleted. Further deletion of residues 70–158 much reduces the binding to the actin filament (Willasden *et al.*, 1992), indicating the importance of site 1 for TM interaction. Unlike the actin binding affinity which is much reduced in unacetylated α -TM with C-terminal residues removed by carboxypeptidase, cooperative interaction is only slightly reduced. As TM-TM interactions are likely to be very much reduced in such modified TM it is concluded that cooperative effects are likely to be induced by conformational changes along the actin filament (Butters *et al.*, 1993).

Interaction of caldesmon with tropomyosin

Caldesmon is a heat stable protein widely distributed in the thin filament systems of cells associated with actin and TM. It is expressed in two classes of isoforms of different molecular sizes. Those present in smooth muscle have molecular masses in the range 89–93 kDa with for example that from human consisting of 793 and that from chicken of 771 amino acids. Shorter isoforms, 524 amino acids in the human and 517 in the chicken, are expressed in non-muscle tissues by splicing out two exons from the primary transcript of the caldesmon

gene. (For reviews see Matsumura and Yamashiro, 1993; Marston and Huber, 1996; Marston *et al.*, 1998; Hodgkinson, 2000.) The binding of caldesmon to TM is dependent on ionic strength with affinities ranging from $2 \times 10^7 \text{ M}^{-1}$ at very low salt concentrations to 10^5 M^{-1} at $I = 0.1$ (Smith *et al.*, 1987; Horiuchi and Chacko, 1988; Redwood *et al.*, 1990; Watson *et al.*, 1990). A major site on TM for the binding of smooth muscle caldesmon has been identified between residues 142 and 227 with residues 200–227 being particularly involved in the interaction (Watson *et al.*, 1990). N-terminal (residues 1–147) and C-terminal (residues 228–284) fragments can be removed from TM without significantly affecting its binding to caldesmon but there are suggestions of a weaker site at the N-terminus, residues 11–127 (Watson *et al.*, 1990). Unlike its binding to actin the binding of TM to caldesmon is not cooperative but if the TM is already bound to actin the affinity for caldesmon is four times greater (Horiuchi and Chacko, 1988).

It is of particular interest that caldesmon, like TN-I, is able to fully inhibit the MgATPase of actomyosin when present at roughly equimolar concentrations with actin but in the presence of TM it is similarly effective at a ratio of less than 0.15 moles of caldesmon per mole of actin (Redwood and Marston, 1993). In view of these inhibitory properties and the fact that, in the presence of calcium, calmodulin combines with caldesmon and is thereby able to displace reverse inhibition (Sobue *et al.*, 1981), it has been proposed that caldesmon functions as a component of a calcium controlled thin filament regulatory system in smooth muscle (Sobue *et al.*, 1982; Smith *et al.*, 1987; Marston *et al.*, 1998). The precise way in which this system acts *in situ* is still a matter for discussion for there is good evidence that myosin phosphorylation is necessary and sufficient for the initiation of smooth muscle contraction (Itoh *et al.*, 1989). Nevertheless it is possible that caldesmon has a role in modulating the contractile response, possibly in relation to the latch state which is a feature of smooth muscle. Such a view is supported by experiments with peptide fragments of caldesmon incorporating the regions of the molecule that interact with TM and possess inhibitory activity (caldesmon domains 3 and 4). Pre-treatment with the caldesmon peptides of skinned guinea pig *Taenia coli* cells in which the myosin had been thiophosphorylated reduced the contractile speed on addition of ATP (Burton and Marston, 1998).

Although the thin filament regulation of smooth muscle by the caldesmon-TM system does not appear to be as important a factor in the control of contractility as the troponin-TM system of striated muscle, there are some striking similarities in the two systems. Both involve a protein that exhibits inhibitory activity by binding to actin to prevent it from activating the myosin MgATPase. In both cases the inhibitory activity is reversed in the presence of calcium by interaction with a calcium binding protein. The most striking and unique feature of both systems is the ability of TM to extend the effect of the inhibitory activity from a single actin

monomer to the complete structural unit consisting of at least seven actin monomers. This is such an unusual property, without parallel in biological systems, that it is likely a common mechanism is involved. Therefore, by analogy with the accepted view in striated muscle a regulatory role for TM in smooth muscle would also be expected to involve a steric blocking mechanism. There is, however, still some uncertainty as to the precise relationship between TM and caldesmon in the thin filament of smooth muscle. Seen in the electron microscope isolated caldesmon is a long thin molecule, 75–80 nm in length. Early models of the smooth thin filament suggested that it existed in the extended form located alongside the TM (Marston and Redwood, 1991). It has been suggested more recently, however, that the caldesmon is more localised rather than spread continuously over the TM molecule (Marston *et al.*, 1998). This could explain the transverse striations observed at 36–40 nm when native chicken gizzard filaments are stained with antibodies to C- and N-terminal fragments of caldesmon (Moody *et al.*, 1990; Lehman *et al.*, 1996). It may be significant that this periodicity is similar to that observed when striated muscle is stained with antibodies to troponin. The periodicity of caldesmon staining could also be explained on the basis of the original model if the protein was localised continuously alongside the TM but with the molecules staggered by the length of one TM molecule relative to each other (Marston and Redwood, 1991). Crosslinking studies with cysteine mutants of TM support some type of lateral association in so far as they indicate that caldesmon lies less than 10 Å from residues 56 and 100 of TM in the closed state of the actin–TM filament (Golitsina and Lehrer, 2000).

The molecular ratio of caldesmon to actin in smooth muscle thin filaments is estimated by direct determination to be 1:16 (Marston, 1990; Lehman *et al.*, 1993). In view of this, the results of *in vitro* ATPase studies and the model proposed (Marston and Redwood, 1991), it has been suggested that the molar ratios of caldesmon:TM:actin in the functional unit of the smooth muscle filament is 1:2:14. This means that whereas in striated muscle each TM is associated with a troponin molecule, in smooth muscle there is only one molecule of caldesmon for every two of TM. Caldesmon could be in contact with all the TMs if the molecule is fully extended as its length is approximately twice that of TM. The relationship of caldesmon to TM in the smooth muscle thin filament has yet to be resolved in detail. On negative staining of the thin filament of smooth muscle the actin monomers appear in the electron microscope as the characteristic double helical array with narrow asymmetric strands running helically along the filament axis (Moody *et al.*, 1990; Vibert *et al.*, 1993). These strands presumably represent TM or TM associated with caldesmon. No large mass could be observed in 3D helical reconstructions of electron micrographs of reconstituted filaments containing caldesmon replaced with a recombinant C-terminal frag-

ment (circa 20 kD) which possesses the inhibitory and TM binding sites (Marston *et al.*, 1998). From observations on TM–caldesmon cocrystals it has been concluded that the main mass is bound to residues 70–100 or 230–260 of TM (Hnath *et al.*, 1996).

The relationship between caldesmon, TM and actin in the thin filaments of non-muscle cells seems similar to that of smooth muscle. At light microscope level the distribution of caldesmon and TM is periodic and coincidental in stress fibres (Bretscher and Lynch, 1985; Bretscher, 1986; Yamashiro-Matsumura and Matsumura, 1988). In cultured rat kidney cells electron microscopy of antibody labelling of thin filaments indicates caldesmon is localised with a periodicity of 36 ± 4 nm, which is very similar to that observed with TM (Matsumura *et al.*, 1983a; Yamashiro-Matsumura and Matsumura, 1988). Non-muscle caldesmon increases the affinities for actin of the LMW non-muscle TMs (32.4 and 32 kDa) much more than the HMW non-muscle isoforms (40 and 36.5 kDa), which in the absence of caldesmon, have a higher affinity for actin (Yamashiro-Matsumura and Matsumura, 1988).

Other tropomyosin binding proteins

A number of other proteins have been reported to bind TM, particularly to the isoforms present in smooth muscle and non-muscle cells. Although the nature and function of the binding of these proteins is not so well defined as with troponin T, tropomodulin and caldesmon they appear to be associated with calcium sensitive systems. Calponin, a 34 kDa protein (see Gimona and Small, 1996; Hodgkinson, 2000, for reviews), is present in smooth muscle and its putative precursor cells, but not in cardiac or skeletal muscle. It has the property of inhibiting the MgATPase of actomyosin that can be reversed by calcium binding proteins in the presence of calcium. It is located in actin filaments of smooth muscle and cultured cells and is considered to be involved in the regulatory system in a manner not understood. Calponin binds to smooth muscle TM in a calcium-dependent manner (Takahashi *et al.*, 1986; Nakamura *et al.*, 1993) but inhibitory activity appears to be independent of TM (El-Mezgueldi and Marston, 1996).

A S100 related protein of 10 kDa, pEL98, isolated from transformed NIH 3T3 cells binds to mouse fibroblast TM2 in a calcium-dependent manner. Antibody staining in the presence of calcium indicates that pEL98 colocalises with TM associated with the actin filaments in NIH 3T3 cells. In the presence of EGTA the pEL98 antibody does not stain the filaments whereas the TM antibody does. Comparison of the binding properties of mutants indicates that the region of TM2 containing residues 39–107 is primarily responsible for binding pEL98 (Takenaga *et al.*, 1994).

Normally TM binds to structural proteins in the cell and it is therefore, of some interest that it has recently been reported that the TM of chicken liver specifically

binds glutamic dehydrogenase (Akutsu *et al.*, 2000). This is the first demonstration of the binding of an enzyme by TM but its significance and importance has yet to be assessed.

Tropomyosin phosphorylation

A single site, Ser283, present in skeletal α - and β -TMs (Mak *et al.*, 1978), is fully phosphorylated in α -TM isolated from skeletal muscle of the frog injected with ^{32}P labelled orthophosphate (Ribulow and Barany, 1977; Barany and Barany, 1980). TM isolated from striated muscle of adult rabbit, rat and the mouse is only partially phosphorylated. The dynamic studies suggest that TM phosphorylation is related in some way to the cell pool of phosphate but unlike the case with cardiac TN-I or the P light chain of myosin from skeletal muscle the extent of phosphorylation is not markedly affected by contractile activity. Despite the similarity of the α - and β -subunit C-terminal sequences (Appendix) phosphorylated β -TM could not be detected in adult rabbit and frog skeletal muscles (Mak *et al.*, 1978). Phosphorylated β -subunit can, however, be demonstrated in developing striated muscles of the rat and mouse (Heeley *et al.*, 1982, 1985) but the extent is usually less than with the α -subunit. Phosphorylated forms of the minor γ - and δ -isoforms of TM also are detectable in developing slow twitch muscle of the rat (Heeley *et al.*, 1982).

TM is not phosphorylated by phosphorylase kinase or protein kinase A (Pratje and Heilmeyer, 1972; Perry *et al.*, 1975; Stull and Buss, 1977) but an enzyme has been isolated from chicken embryo that phosphorylates TM and casein equally well. Although designated TM kinase some properties of the enzyme are similar to the casein kinases but it is not identical to the well characterised casein kinases I or II (Montgomery and Mak, 1984; deBelle and Mak, 1987). The enzyme is not detected in adult rabbit and chicken skeletal muscles. Incorporation of phosphate into α -TM can be demonstrated in adult rat heart but not skeletal muscle when tissue slices are incubated with ^{32}P labelled inorganic phosphate (Heeley *et al.*, 1982). The purified enzyme phosphorylates α -TM five times as fast as β -TM. This suggests that a possible explanation for the absence of phosphorylated β -TM in adult muscle is that the dimer $\alpha\beta$ TM, in which form most of the β -TM occurs in striated muscle, is an even poorer substrate for the phosphorylation of the β -subunit than β_2 -TM.

There are suggestions that TM phosphorylation may be of some significance in myofibrillogenesis for the extent is higher in foetal and neonatal skeletal and cardiac muscles when active development is taking place (Montarras *et al.*, 1981; Heeley *et al.*, 1982, 1985; Dabrowska *et al.*, 1983). It has been suggested that the phosphorylation has a role in stabilising the head to tail interaction involved in polymerisation, the P-ser-283 on each of the two chains forming salt linkages with ϵ -NH₂

groups of lys6 and lys12 respectively (Mak *et al.*, 1978). The observation that the viscosity at low ionic strengths is higher in the phosphorylated form of α_2 -TM gives some support for this suggestion (Heeley *et al.*, 1989). Charge, however, is not the only factor, for the mutant S283E is less viscous than phosphorylated wild type α -TM (Sano *et al.*, 2000a). Actin binding is not affected by phosphorylation but the ability of troponin to strengthen this binding is markedly reduced. In reconstituted actomyosin S1 systems significantly higher MgATPase activities were observed with phosphorylated TM but in the fully reconstituted system with troponin there was no difference in the extent of inhibition obtained in the absence of calcium (Heeley *et al.*, 1989). Whatever the role of TM phosphorylation may be, it is unique to striated muscle for TMs from smooth muscle, platelets and erythrocytes do not have a serine residue at position 283 that can be phosphorylated.

Non-muscle tropomyosins

Largely for historical reasons much of the focus of attention in the past has been on the role of TM in muscle regulation, but this is now changing since it has been recognised that the protein is widely distributed in the microfilament systems of eucaryotic cells. Soon after the first isolation and characterisation of a non-muscle TM from human platelets (Cohen and Cohen, 1972) it was shown that this protein is present in electric organ (Kaminer and Szonyi, 1972), brain, fibroblasts (Fine *et al.*, 1973; Fine and Blitz, 1975) and many other types of vertebrate cells, including, thyroid, pancreas, kidney and brush border, albeit in much smaller amounts than in muscle (see Cote, 1981 for early review on non-muscle TMs). Immunochemical staining locates TM in the microfilaments, particularly in the more stable forms such as stress fibres along which actin, usually but not always, runs continuously. In these fibres the TM is located in bands of 1–2 μm alternating with $\sim 0.5 \mu\text{m}$ bands of α -actinin, the periodicities varying with the cell type (Lazarides, 1975; Sanger *et al.*, 1983). The relationship of actin, TM and α -actinin to each other in the stress fibres is very similar to that in the thin filament of the myofibril suggesting that the association of these three proteins is common to filamentous systems concerned with tension development and movement in all cells. As a specialised contractile tissue the cytoplasm of muscle is densely packed with myofibrils containing actin filaments resulting in the relatively high content of actin, TM and other microfilament proteins in this tissue (Figure 10).

Non-muscle cells express all four of the TM genes to produce a range of isoforms that are different from those present in muscle and which exhibit some specificity for the cell type (see Lees-Miller and Helfman, 1991; Pittenger *et al.*, 1994, for reviews). This would be expected if the precise type of microfilament and its function determines the TM isoform present. For

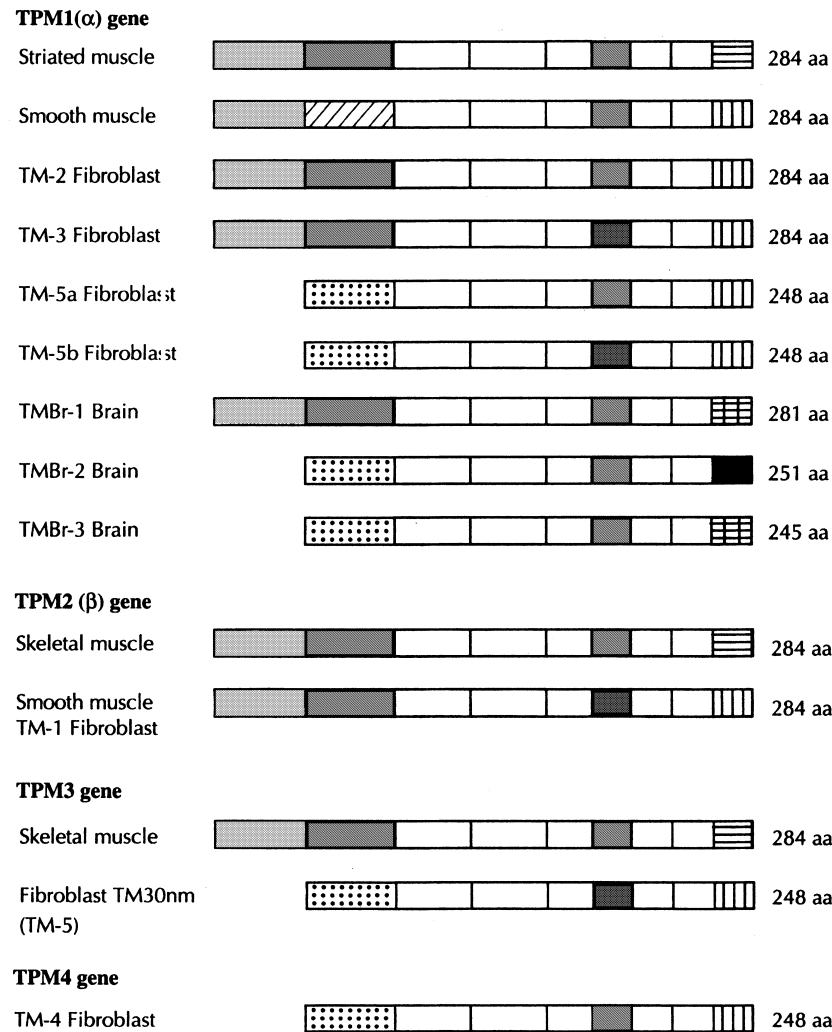


Fig. 10. Exon expression in the non-muscle TM. Schematic representation of the pattern of exon expression in the non-muscle TMs compared with those expressed in striated and smooth muscles. Exons are represented by differently shaded areas. Unshaded blocks represent exons that are invariant and expressed in all isoforms. The numbers of the exons expressed in each isoform can be obtained from Figure 1. Also see section on genes, isoforms and distribution. The skeletal muscle isoform, known as TM3, expressed by the TPM3 gene is expressed in slow twitch muscle and in the heart of the human and possibly other larger animals. Fibroblast TM4 corresponds to human platelet TM3. The human TM_{nm} isoform, also known as TM5, has been identified in the mouse (Takenaga *et al.*, 1990) and the rat (W. Guo and DM. Helfman, unpublished data). From Pittenger *et al.* (1994) with permission.

example normal rat kidney cells contain five different TM isoforms, two in low abundance, the relative amounts of which change when the cells are transformed by RNA viruses (Matsumura *et al.*, 1983a, b). At least eight isoforms are present in human fibroblasts (Talbot and McLeod, 1983; Novy *et al.*, 1993a, b) and rat fibroblasts contain seven isoforms, TMs 1, 2 and 4 in major abundance and TMs 3, 5, 5a and 5b present in smaller amounts (Pittenger *et al.*, 1994). The TM isoforms identified in fibroblasts are present in different combinations in other non-muscle tissues such as kidney, liver, blood cells and intestinal epithelium. Brain contains three isoforms, one of the HMW group, TMBr-1, and the other two, TMBr-2 and TMBr-3, of the LMW group. All three are derived from the α -TM gene by the use of alternative promoters and alternative RNA splicing and are unique to brain tissue (Lees-Miller *et al.*, 1990a, b). Characterised by the expression

of exon 9c at the C-terminus, TMBr-1 and TMBr-3 are specific for neurons and are differentially expressed, with TMBr-3 appearing first in the developing rat embryonic brain (Stamm *et al.*, 1993; Weinberger *et al.*, 1993; see Gunning *et al.*, 1998 for review on neuronal TMs).

Unlike the muscle TMs which are virtually all of the HMW group, the non-muscle TMs can occur as HMW and LMW isoforms in the same cell type. The α -helical structure is maintained in the LMW proteins. They are shorter than the HMW TMs by approximately 40 amino acid residues and thus preserves the actin-interacting regions intact.

Platelet tropomyosin

This protein is the prototype of the LMW TMs and was the first non-muscle TM to have its properties studied in detail. Platelet TM is very similar to the muscle isoform

and forms paracrystals readily but with an axial periodicity of 345 Å, significantly lower than that of the skeletal $\alpha\beta$ -TM and close to the molecular length of platelet TM (Cohen and Cohen, 1972; der Terrossian *et al.*, 1981). Isolated from horse platelets the TM subunit has a M_r of about 29,000 and consists of α - and β -forms in the ratio of about 1:2 with the β -subunit containing 247 residues. When compared with the muscle α -TM the platelet β -TM sequence is highly conserved from residues 81–260 but significantly different at the C-terminus, residues 261–284. The N-terminus is extended by five residues and there are two 21 residue deletions (residues 23–43 and 60–80 of the muscle sequence (Lewis *et al.*, 1983). Horse platelet α -TM differs in only a few residues from the β -isoform. It does not bind to troponin or troponin T (Cote *et al.*, 1978a) and as judged by the viscosity at low ionic strength polymerises much less than the muscle protein (Cote *et al.*, 1978b). Under identical conditions (1–2 mM $MgCl_2$ and 30 mM KCl) platelet TM binds much less strongly to F-actin than skeletal TM although if the $MgCl_2$ concentration is increased to 10 mM it binds cooperatively and saturates at a molar ratio of TM:actin of 1:6 (Cote and Smillie, 1981b). Porcine platelet TM has a very similar amino acid content to the horse protein but differs in that it is present as a single component and has two cysteine residues per subunit. In the porcine platelet there is twice as much actin as would be required to saturate the TM assuming a combining ratio of six actin monomers per TM molecule (der Terrossian *et al.*, 1981). The significance of this is not clear but may relate to the differences in the regulation of the actomyosin contractile system in platelets compared to that present in striated muscle.

Properties of the non-muscle tropomyosins

Cloning of the cDNAs and their expression in bacteria has enabled the preparation of amounts of the non-muscle isoforms of TM adequate to enable the study of their properties. (Pittenger and Helfman, 1992; Novy *et al.*, 1993a, b). The general properties of the proteins such as solubility, chromatographic behaviour, heat stability etc. are very similar to those of the muscle specific isoforms although some differences have been reported. For example the N-acetyl group of fibroblast TM does not seem to be essential for actin interaction as is the case with skeletal α -TM (Pittenger and Helfman, 1992). The LMW isoforms of fibroblast TM have a 2–3 times lower affinity for F-actin than the HMW isoforms (Matsumura and Yamashiro-Matsumura, 1985) and the LMW isoform of brain also has a low affinity for F-actin (Broschat *et al.*, 1986). These findings have led to the suggestion that the LMW isoforms have a low affinity for actin because of the smaller number of actin binding sites for actin per molecule and their impaired ability to polymerise by head to tail interaction. There is danger in such a generalisation for it has been shown that recombinant forms of skeletal muscle α -TM without

the N-terminal acetyl group that do not exhibit head to tail polymerisation can bind to F-actin (Hitchcock-Degregori and Heald, 1987). Also LMW isoforms from human erythrocytes (Fowler and Bennett, 1984) and chicken intestinal epithelial cells (Broschat *et al.*, 1986) have similar affinities for F-actin as the HMW isoforms. Even more striking is the fact that TM5b, the LMW TM from fibroblasts, is able to displace skeletal α -TM from F-actin (Pittenger and Helfman, 1992). It seems likely that, when compared under standard ionic conditions there is as wide a range of affinities for F-actin amongst the LMW isoforms as there is with the HMW isoforms of muscle TMs. The most important factor determining the ability to bind to F-actin is likely to be the exons that are expressed in the various isoforms of TM.

In contrast to the α - and β -TMs of skeletal and smooth muscle which invariably exist as heterodimers, the TMs present in fibroblasts occur as homodimers (Lin *et al.*, 1985; Matsumura and Yamashiro-Matsumura, 1985). On introduction of α - and β -muscle TMs into the fibroblast by transfection the muscle TMs form heterodimers with the HMW fibroblast isoforms, TM1, TM2 and TM3 but not with the LMW isoforms (Gimona *et al.*, 1995). It seems likely as with most TM properties it is the selection of the exons expressed in the isoform that determine whether the TM molecule exists as a homo or heterodimer. On the evidence of the stronger head to tail overlap exhibited by chicken gizzard $\alpha\beta$ TM compared with the α_2 isoform it has been suggested that heterodimers form a more flexible and elongated structure than homodimers. Although such a property might be appropriate for the demands made on a specialised contractile system it is not a requirement for all types of muscle. The TM of the hearts of small animals is entirely α_2 and largely so in some fast skeletal muscle fibres. It is possible that the more dynamic regulation of the actin filament system of non-muscle cells would be better served by the weaker head to tail association of some of the homodimer isoforms.

Function of the vertebrate non-muscle tropomyosins

In view of the similarity of the muscle and non-muscle thin filament systems it is likely that there are common features in TM's role in all cells, namely to stabilise and modulate the function of the F-actin component of the microfilaments (Payne and Rudnick, 1984). The isoforms expressed in non-muscle tissue all preserve the molecular architecture required for actin binding but with sequence differences from the muscle isoforms. These are principally in the N- and C-terminal regions presumably to accommodate the functions of the filaments with which they are associated. It is now abundantly clear that the sample of exons expressed affects the ability of the subunits and the intact molecule of TM to interact with each other and with actin and other protein components of the microfilaments.

Microinjection of labelled proteins has failed to show differential labelling in fibroblasts (Pittenger and Helfman, 1992) although antibody studies indicate that the LMW isoforms were confined to the lamella (Lin *et al.*, 1988). This observation correlates with the earlier findings of Wehland and Weber (1980) that microinjected HMW TMs stained the stress fibres but not the periphery of the cell. Evidence exists for the relatively high level of expression of TM3 and TM5b in rat kidney and intestinal brush border compared to fibroblasts (Goodwin *et al.*, 1991; Temm-Grove *et al.*, 1996), suggesting differences in localisation and function of the TM isoforms in these two types of cells. Clear evidence of this has been obtained with LLC-PK1 kidney epithelial cells in which immunofluorescence studies indicate that the HMW isoforms are confined to the stress fibres whereas the LMW TMs are located in the adhesion belts (Temm-Grove *et al.*, 1998). This implies that the LMW TMs may have a role in cell-cell adhesions. Antibody screening of La Hella cells for surface antigens, CD58 associated molecules, indicates however that these molecules are associated directly or indirectly with LMW and HMW TMs (Shishido *et al.*, 2000). It is of interest that localisation studies carried out with immunofluorescence do not always give the same results as those obtained by microinjection of the tagged isoforms. Results with the latter technique do not necessarily indicate the endogenous localisation of an isoform but merely its capacity to exchange with an injected isoform at high abundance.

There has long been evidence of the isoform composition of muscle TM changing with development and varying with muscle type (see sections on muscle TMs). In skeletal muscle fibres in which troponin T_{2f} and α -TM are predominant the calcium sensitivity falls as the proportion of β -TM and troponin T_{1f} increases (Schachat *et al.*, 1987). This suggests that TM in association with troponin T may have a role in determining the calcium sensitivity of muscle. It is unlikely that TM is implicated in a similar manner in non-muscle cells but the evidence indicates that mechanisms exist to express isoforms of TM with properties appropriate for a particular microfilament system. Further evidence of changing isoform composition has been obtained with chicken embryo fibroblasts transformed by Rous sarcoma virus (Lin *et al.*, 1985) and developing rat embryonic brain (Stamm *et al.*, 1993; Weinberger *et al.*, 1993). There are implications that the 161 residue TM of *Schizosaccharomyces pombe* encoded by the *cdc8* gene is associated with F-actin contractile ring involved in cytokinesis, which is impaired when this gene is mutated (Balasubramanian *et al.*, 1992).

Non-muscle tropomyosin and filament formation

With the increasing knowledge of the actin filament systems of non-muscle tissue it seems clear that the myofibrillar thin filament is a highly specialised form of the actin microfilament system which is widespread in all cells. In contrast to the muscle system the actin filaments

of non-muscle cells are involved in many different aspects of cellular architecture and physiology including locomotion, adhesion, membrane ruffling, localised cytoplasmic transport, cytokinesis and phagocytosis. Also the non-muscle actin filament systems are much more dynamic undergoing assembly and disassembly through the G-F-actin transformation. At any one time a number of actin filaments with different functions are present in the cell cytoplasm. Usually in the stable protein systems the proteins known to be in the muscle thin filament such as TM, α -actinin, caldesmon, tropomodulin etc. are also present and often located with a similar periodicities to those in muscle filaments. Despite the wide range of actin filament function usually only two very similar actin genes are expressed in non-muscle cells. On the other hand all four TM genes, are expressed giving rise in the case of rat fibroblasts to seven isoforms (Pittenger *et al.*, 1994). In brain two actin isoforms (β and γ) and at least 10 TM isoforms, some of which are unique to neurons (Lees-Miller *et al.*, 1990a, b), are expressed (Gunning *et al.*, 1998).

In view of the specificity of TM isoforms with certain types of filaments and cell types it is probable that TM has a role in modulating actin filament function. The observation that TM is often absent from regions where microfilaments are undergoing reorganisation, but is present in the more stable structures (Lazarides, 1975), is compatible with the various reports that TM stiffens and stabilises the actin filament. The length of the molecule, its ability to form a linear polymer by head to tail interaction of the TM isoform and the relative amounts of the capping proteins will be important factors determining the length of the actin filament (Figure 7). The LMW isoform, hTM5, determines the size of the actin protofilament in the human erythrocyte membrane (Fowler, 1996; Sung *et al.*, 2000). In comparison the actin filament of striated muscle is about 30 times as long stabilised by the overlapping HMW TM molecules lying in the actin groove.

The function of actin filaments is also modulated by a number of other proteins which bind to actin such as α -actinin, filamin, fragmin, gelsolin, spectrin villin, tropomodulin etc. and which crosslink, sever and cap the filament. Lying as it does in each of the grooves of the F-actin filament TM inhibits or competes with the effects of these proteins on the actin filament (see Payne and Rudnick, 1984, for review).

Clinical importance of tropomyosin

Myopathies

A number of myopathies have been reported with which mutations in the TM genes are associated. Missense mutations in the TPM 1 gene, coding for fast twitch skeletal α -TM, are associated with familial hypertrophic cardiomyopathy which maps to chromosome 15. The mutations reported so far are Asp175Asn; Glu180Gly,

(Thierfelder *et al.*, 1994) and for each of which a transgenic mouse model now exists, (Wernicke *et al.*, 1999), Glu180Val (Regitz-Zagrosek, 2000), Ala63Val (Nakajima-Taniguchi *et al.*, 1995), Val195Ala (Tobacman *et al.*, 1999; Karibe *et al.*, 1999), Ala63Val and Lys70Thr (Olson *et al.*, 2001). The precise role of mutant forms of TM in the onset of familial hypertrophic myopathy is not clear. The condition, an autosomal dominant disorder, is characterised by increased myocardial mass with myocyte and myofibrillar disarray. It is also associated with mutations in other myofibrillar proteins such as β -cardiac myosin heavy chain, troponin T, TN-I, myosin binding protein C, myosin regulatory light chain, myosin essential light chain (Towbin, 1998; Redwood *et al.*, 1999) and α -cardiac actin (Mogenson *et al.*, 1999). The association of the mutant forms of a number of different myofibrillar proteins with familial hypertrophic cardiomyopathy has led to the suggestion that it is a 'sarcomeric disease' (Thierfelder *et al.*, 1994). It is postulated that mutation leads to changes in gene expression which result in changes of the stoichiometry of expression of the proteins required to form the sarcomere. It is likely that this is an oversimplified explanation for the mutations occur in regions that have been associated with specific properties of TM and often produce different effects when studied in *in vitro* systems.

The presence of the Asp175Asn mutation in skeletal muscle α -TM has enabled the effect of the mutation to be studied on skinned biopsy samples of human vastus lateralis muscle. No discernible difference was observed between control and mutated fibres in cooperativity, maximum force and maximum shortening velocity but those from the FHC patients exhibited increased calcium sensitivity (Bottellini *et al.*, 1998). Thus although the mutant TM is expressed in both skeletal and cardiac muscle the clinical consequences are apparently only obvious in the heart. An increase in calcium sensitivity was also implied by the results of motility assays carried out on Asp175Asn and the Glu180Gly recombinant forms of chicken fast AlaSer α -TM and skeletal muscle filament systems. In this study it was reported that whereas the Asp175Asn mutant had a similar affinity for actin as the wild type, the affinity of the Glu180Gly mutant was threefold weaker (Bing *et al.*, 1997), but neither mutation affected the ability of TM to increase force development by actin filaments in motility assays (Bing and Marston, 1999; Table 1). Repeat of these studies with the human cardiac regulatory proteins (Bing *et al.*, 2000b) in general confirmed the results of Bottellini *et al.* (1998) but also described some differences in the behaviour of the mutant TMs from those reported in the earlier study with a heterologous system (Bing *et al.*, 1997). This work indicates the importance of drawing conclusions about the effects of mutants tested *in vitro* on reconstituted filament systems only when homologous and otherwise identical systems are used. For example the results for the comparative affinities of the Asp175Asn and Glu180Gly TM mutants

for actin obtained by Bing *et al.* (1997) using AlaSer α -TM were different from those were obtained with deacetylated recombinant human α -TM by Golitsina *et al.* (1997).

The Asp175Asn and Glu180Gly mutations are in one of the mostly highly conserved parts of the TM molecule and occur in the region of the fifth actin binding site (McLachlan and Stewart, 1976; Phillips *et al.*, 1986). The mutations have small effects on the stability of TM as judged by circular dichroism. Fluorescence measurements of TM labelled with pyrene maleimide at Cys190 indicated that both mutants bound cooperatively to actin. In the presence of myosin S1 the fluorescence changes observed were different from those obtained with wild type TM and suggest increased local flexibility. No obvious differences in interaction with troponin T or the troponin complex were noted (Golitsina *et al.*, 1997). Transgenic mice in which the Glu180Gly mutant has been expressed exhibit marked cardiac enlargement, functional changes and die after 3–4 months (Hoit *et al.*, 1999). Although the latter authors describe left ventricular hypertrophy in their mutant mice Spindler *et al.* (1999) report that the Asp175Asn mutant but not the Glu180Gly leads to altered left ventricular performance in transgenic mice. In addition to the increased calcium sensitivity transgenic mice expressing the Asp175Asn mutant TM also have impaired myocardial relaxation and blunted response to β -adrenergic stimulation (Evans *et al.*, 2000). Motility studies with the Val195Ala mutant with which the associated hypertrophic cardiomyopathy has a poor prognosis, demonstrated significant effects on calcium affinity and myosin cross bridge function (Tobacman *et al.*, 1999). The *in vitro* motility studies described were carried with α -TM produced by recombinant DNA technology in which the N-terminal acetyl group was replaced by Ala-Ser.

The differences in the results obtained with the Asp175Asn and Glu180Gly mutant forms, (Hoit *et al.*, 1999 cf Spindler *et al.*, 1999) both in the *in vitro* and the *in vivo* experiments are of some concern and indicate the caution that must be exercised in drawing conclusions from them. They may reflect differences in the recombinant forms used or the degree of expression of the mutant isoform, which, in transgenic animals is often quite low as a percentage of the total TM present.

The fact that the structural and clinical consequences associated with mutations in fast α -TM are more easily recognised in cardiac muscle must reflect the special demands of that tissue for by far the bulk of this isoform in the human body is located in skeletal muscle. As yet no clinically significant evidence of the disease has been reported in skeletal muscle (Thierfelder *et al.*, 1994). The reason for this is not clear but may be because the functional consequences of the mutation are less readily tolerated by the continuous activity pattern of the heart. Another possible explanation may be that in skeletal muscle a much higher proportion of the mutant isoform exists as a heterodimer with the wild type β -isoform in

which the deleterious effects of the mutant form are masked.

A mutation of the TPM3 gene that results in Met 9 being replaced with Arg is associated with nemaline myopathy and weakness of skeletal muscle (Laing *et al.*, 1995). As the TPM3 gene codes for the isoform of α -TM present in slow twitch muscle, which is not strongly expressed in cardiac muscle, the clinical consequences of the mutation in this case are confined to skeletal muscle. On transfection of rat cardiomyocytes with human fast α -TM cDNA modified to produce the Met9Arg mutant TM is incorporated into the thin filament at roughly similar levels to the wild type α -TM, but does not directly produce nemaline myopathy. As is the case with the Asp175Asn mutant no significant effect on maximal force generation or myofibril cooperativity as indicated by the Hill coefficient could be demonstrated on skinned fibres. In contrast to the Asp175Asn and Ala63Val mutants the calcium sensitivity decreased (Michele *et al.*, 1999; Michele and Metzger, 2000).

Met9 in TM3 corresponds to Met8 in α -TM and would be presumed to be located in the the overlap region of both protein molecules. The Met8Arg mutant of rat α -TM has severely impaired affinity for actin, being 50–100 times weaker than that of the wild type in the presence of troponin. Calcium regulatory activity is retained although activation of the actomyosin S1 ATPase by Ca^{2+} is reduced (Moraczewska *et al.*, 2000).

Antigenic significance

Analysis of the sequences of a number of proteins known to induce the formation of autoantibodies in human autoimmune diseases indicates that an unusually high proportion of them contain charge rich segments, longer than 27 residues, of α -helical coiled-coils (Dohlman *et al.*, 1993). This implies that the TM molecule, consisting as it does of 100% α -helix, has considerable antigenic potential. With its structural, and presumably antigenic, stabilities to heat and with the wide spread use of muscle for food in which it represents about 3% of the total protein, it is a likely candidate for food allergies. Nevertheless it does not appear to be an important allergen for meat sensitive subjects (Ayuso *et al.*, 1999). In contrast the TM present in arthropods, insects, arachnids and crustacea, and some other invertebrates has been widely reported as a human allergen. TM of the brown shrimp, Pen a1, has been shown to be a major allergen (Daul *et al.*, 1994, Reese *et al.*, 1999) and homologous TMs in related shrimp species have also been shown to be allergenic (Shanti *et al.*, 1993; Leung *et al.*, 1994; Witteman *et al.*, 1994). TM-like allergens have also been identified in lobster and crab (Leung *et al.*, 1996), squid and oyster (Leung *et al.*, 1996; Miyazawa *et al.*, 1996) and mollusc (Chu *et al.*, 2000).

TM is also important as an environmental antigen derived from the cockroach, *Periplaneta americana*, (Asturias *et al.*, 1999) the house dust mite, *Dermatophagoides farinae*, (Aki *et al.*, 1995) and other insects

responsible for arthropod inhalation allergy (Panzani, 1994). Sensitisation to the cockroach has been widely observed in patients with asthma, in some situations up to 60% of them exhibiting sensitisation (Kang, 1990). The sequence of *P. americana* TM is 80–89% identical with the TMs of other arthropods (Asturias *et al.*, 1999) which explains why sensitisation to cockroach often extends to insects, arachnids and crustacea.

The sequences of mammalian TMs are less similar to the arthropod isoforms, expressing 50% or more identity with that of brown shrimp Pen a 1 TM (Reese *et al.*, 1997) and are presumably less antigenic. In the human autoantigenic response to TM has been demonstrated in ulcerative colitis (Das *et al.*, 1993; Biancone *et al.*, 1995; Sakamaki *et al.*, 1996, 2000; Biancone *et al.*, 1998; Geng *et al.*, 1998). The major TM isoform in colonic epithelium is hTM5 (Geng *et al.*, 1998) the surface expression of which can be demonstrated in normal and LS colon cancer cell lines but not in small intestinal epithelial cells (Kesari *et al.*, 1999). This correlates well with reports of the localisation of TM isoforms at the cell–cell adhesion boundary (Temm-Grove *et al.*, 1998; Shishido *et al.*, 2000). The special significance of the autoimmune response to TM in ulcerative colitis is indicated by the commitment of the IgG producing cells of the colonic mucosa to hTM5. The cell surface molecule HLADPw9 is important for the surface expression of TM and is involved in the pathogenesis of ulcerative colitis (Niitsu *et al.*, 2000). The number of lamina propria B cells producing antibodies against hTM5 is very much higher in ulcerative colitis than in patients with Crohns colitis or with non-inflammatory bowel disease (Onuma *et al.*, 2000). A clone corresponding to hTM5 with the terminal exon 9 (residues 222–247) deleted has been isolated from a cDNA library prepared from T-84 cells. Preliminary staining with an antibody to a peptide specific for this isoform of hTM5 indicates a good correlation with colonic cancer cells and suggests some promise for cancer surveillance in ulcerative colitis (Geng *et al.*, 2000). The presence of circulating anti-TM antibodies may also be of clinical significance for monitoring heart diseases (Fujita *et al.*, 2000). In this respect it has been reported that antibodies in the sera of patients with acute rheumatic fever bind to cardiac tropomyosin (Khanna *et al.*, 1997).

There have been a number of reports that TM induces acquired immunity to several helminthic infections of laboratory and domesticated animals (see Jenkins *et al.*, 1998 for references). In human infection with the filarial parasite, *Onchocerciasis volvulus*, there is an inverse correlation between the anti-TM IgG levels and the density of microfilariae in the skin. Antibody to the C-terminal portion of a recombinant isoform of TM isolated from *O. volvulus* (Ov-TMY-1) reduced microfilarial infection in mice. The immunodominant B cell epitope of Ov-TMY-1 is almost identical to the IgE-binding epitope of shrimp TM that is believed to be responsible for the allergic response to shrimp and other crustacea (Jenkins *et al.*, 1998).

Concluding comments on the role of tropomyosin

Research on TM was stimulated by its discovery in muscle and until recently dominated by attempts to understand its role in the regulation of contraction in striated muscle. Indeed for almost 30 years it has been given a key role in that process. It is likely that the understanding of the basic principles involved in its function will come from study of its role in non-muscle tissues. The troponin system, unique to striated muscle, evolved later in eucaryotes to produce a system for the more rapid response of the muscles required for movement in higher animals. By this means the calcium trigger acts directly on the thin filament rather than the myosin heads that are not in direct contact with each other and offer less opportunity for cooperative effects. By acting directly on the thin filament the possibility exists for transmission of the activation effect to the more numerous actin monomers in close contact with each other along the actin filament. This close association is probably fundamental for the function of TM in specialised and primitive actin filament systems.

The issue, still unresolved, is whether contraction in muscle is regulated by the movement of TM from a position on actin that blocks interaction with myosin to one that does not or whether its movement is merely the consequence of conformational changes on the actin surface on which it lies. It is now clear that TM can occupy positions on the inner and outer domain of actin which do not correspond to those postulated by the steric mechanism for the functional state of the system. For example acto-TM filaments containing cardiac and smooth muscle TM can activate myosin ATPase *in vitro* when in the absence of calcium it would be presumed to be in the blocked conformation. This has been explained by the suggestion that in the absence of troponin TM is not fixed in a manner that obstructs myosin binding (Lehmann *et al.*, 2000a). Further it is becoming clear that the precise localisation of the TM on the actin filaments depends on the isoforms of actin and TM present (Lehman *et al.*, 2000a). Current theory for the role of TM in striated muscle regulation suffers from the current inability to describe at high resolution the location and structure of the proteins of the troponin complex in the functional thin filament. Some recent progress has been reported in attempts to solve this problem (Lehman *et al.*, 2001). In reconstituted filaments with wild type TM replaced with a mutant possessing four actin binding sites rather than seven, troponin density can be detected in the filaments. In these filaments troponin directly links TM to actin at low Ca^{2+} concentrations. This connection is broken at high Ca^{2+} concentrations and accompanied by TM movement. It is also necessary to define with precision the location of nebulin which, like TM, is considered to lie as a helical molecule in the actin grooves and extend along the length of the thin filament.

The explosion of information about the non-muscle isoforms in recent years has turned attention to a more general role for TM in cell function. In all cells TM is

usually associated with actin where its role is to modulate (Payne and Rudnick, 1984) and stabilise the filament. It is usually localised in the more stable filament systems and probably does not bind to actin in the early stages of filament formation. For example it does not bind to stress fibres newly formed when a cell attaches to a substratum until some hours later (Lazarides, 1976) and cannot be detected in filipodial extensions from growth cones (Had *et al.*, 1994; Gunning *et al.*, 1998). The robust stable coiled-coil of TM, able to lie in each of the two grooves of the F-actin filament, is particularly suitable for its role in stabilising the duplex filament of actin monomers. This has to take longitudinal stresses during tension development both in muscle and non-muscle systems. Further, the ability of the HMW TMs to polymerise by the N- and C-terminal overlaps gives continuity of structure over long lengths of filament. The fact that the cooperative effects observed on activation of actomyosin ATP hydrolysis and its inhibition by TN-I are observed in the presence of TM implies a fundamental role for this protein in the muscle system at least. There is increasing evidence for the transfer of the conformational changes induced by a ligand binding to one actin monomer to neighbouring monomers in F-actin filaments. (Bremel *et al.*, 1972; Perry *et al.*, 1972; Butters *et al.*, 1993; Levine *et al.*, 1999). Indeed such a property is fundamental to the cooperative properties of the actin filament stabilised by the associated TM molecules and indeed to a cooperative/allosteric regulatory system for contraction in muscle (Lehrer and Geeves, 1998).

There is some evidence relating TM directly with the physiological properties of muscle. It has been reported that TM has a role in controlling the pH dependence of isometric tension in reconstituted actin filaments (Fujita and Ishiwata, 1999). This property probably depends on the stabilisation of the binding of actin to TM that occurs on the lowering of the pH (Tanaka, 1972). The differences in this property exhibited by the various isoforms of TM may be responsible for the variation in the pH dependences of active tension observed in different muscle types. Evidence of a role for TM in tension development is provided by motility assays with immobilised heavy meromyosin and α -actinin to retard actin filament movement. Both TM and troponin increased the tension developed by the actin filaments in this system, but the effect was not additive (Bing and Marston, 1999; Bing *et al.*, 2000b). In a more direct study employing the motility assay TM had no effect on filament velocity at saturating myosin surface densities. At 50% myosin saturation, force development was enhanced two fold by TM (Vanburen *et al.*, 1999).

The TM molecule exists in a robust stable conformation that is designed to interact with actin in that all the isoforms so far identified consist of an integral number (4–7) of the quasiequivalent actin-binding sites. In some cases but not all (see Pittenger and Helfman, 1992) this can account for the different affinities of the various isoforms for actin. The marked differences in affinities

for actin shown by α -TM from striated muscle of different species but with similar physiological properties, are more difficult to explain. Indeed it is surprising, but possibly functionally significant, that the isoforms of TM which invariably occur associated with thin filaments show such a wide range of affinities for actin when determined under standard conditions.

The various isoforms of TM show some specificity for cell type, localisation within the cell and its stage of development which suggests that they may have specialised functions. Within the cytoskeleton there are a number of filamentous structures with different roles. Indeed the view is growing that the population of filaments of actin and the associated TM isoforms are spatially segregated into different cellular compartments such as growth cones, filipodia, stress fibres and lamellipodia (Gunning *et al.*, 1998). The creation of distinct microfilament compartments by differential sorting of TM isoforms is observable in primary fibroblasts, cultured 3T3 cells and epithelial cells in the G0 and G1 phases of the cell cycle (Percival *et al.*, 2000).

TM isoforms which vary in their strength of binding to actin, could also affect its polymerisation and they also have a role in determining the nature of other proteins than can bind to the filament and modulate its function. For example the binding of troponin to striated muscle α - and β -TMs ensures that these muscles are regulated by calcium binding at the thin filament. Changes in the cytoskeletal structure that occur as a result of oncogenic transformation are associated with changes in the amounts and isoforms of TM expressed (Hendricks and Weintraub, 1981; Leonardi *et al.*, 1982; Matsumura *et al.*, 1983b; Lin *et al.*, 1985). Despite the changes in TM amounts and composition observed in transformed fibroblasts, there is usually little change in the amount or isoform composition of the actin present. Reports that LMW non-muscle TMs are required for mRNA targeting (Erdelyi *et al.*, 1995) and head morphogenesis (Tetzlaff *et al.*, 1996) in *Drosophila* suggest highly specific roles for these isoforms. It is difficult to decide whether the requirement of TM for these processes implies direct or indirect involvement. It may be that the TM isoforms are essential for the specialised cytoskeletal system required for the biological processes involved.

The role of TM in the regulation of non-muscle thin filament function is uncertain. The report that injection of antibody to TM5 into chick fibroblasts alters intracellular granule movement (Hegman *et al.*, 1989) suggests that it is involved in intracellular transport as do the effects observed in yeasts when the function of the TPM1 gene is disrupted (Liu and Bretscher, 1989, 1992). A role in cytokinesis is implied by the impairment of this function in *Schizosaccharomyces pombe* in which mutations have been introduced in the *cdc8* gene that codes for a TM isoform which forms part of the F-actin contractile ring (Balasubramanian *et al.*, 1992).

It is impossible to decide with any certainty at this stage on the role of TM in the motile systems of non-muscle cells. The sequence and structural information

indicate that the molecule is strongly conserved, implying a basic similarity of function in all actin filament systems. More detailed study of the extent and nature of the conformational changes that occur in actin on binding ligands is required to understand the function of the associated TM. High resolution structural studies indicating the location of troponin C, I, T and nebulin in the muscle thin filament have so far been unsuccessful. Until this is achieved there will still be doubts about the specialised role of TM in striated muscle contraction.

This review has been written as a tribute to Kenneth Bailey who discovered and first isolated tropomyosin in a pure form in 1945 but died under very tragic circumstances in 1963 (see Perry, 1997) before the importance and widespread distribution of this protein in eucaryote organisms was appreciated.

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Appendix

Amino acid sequences of tropomyosin isoforms

Sequences are of human, rabbit and rat tropomyosin isoforms obtained from the SwissAll data base and aligned using the EBI Fasta3 facility. Sequences are arranged in descending order of identity with human skeletal muscle α -TM. Acidic residues are shown in red and basic residues in blue.

Key to isoform names on SwissAll data base list

TPM1_HUMAN

TROPOMYOSIN ALPHA CHAIN, SKELETAL MUSCLE (TROPOMYOSIN 1, SKELETAL MUSCLE). {GENE: TPM1 OR TMSA} – Homo sapiens (Human)

TPM2_HUMAN

TROPOMYOSIN BETA CHAIN, FIBROBLAST AND EPITHELIAL MUSCLE-TYPE (TROPOMYOSIN 2, FIBROBLAST AND EPITHELIAL MUSCLE-TYPE) (TM36) (TME1) (TM1). {GENE: TPM2 OR TMSB} – Homo sapiens (Human)

TPM3_HUMAN

TROPOMYOSIN ALPHA CHAIN, SKELETAL MUSCLE TYPE (TROPOMYOSIN 3, SKELETAL MUSCLE). {GENE: TPM3} – Homo sapiens (Human)

	1	80
TPM1_HUMAN	MDAIKKKMQMLKLDKENALDRAEQAEADKKAAEDRSKQLEDELVSLQKKLKGTEDELDKYSEALKDAQEKLELAEKKATD	
TPMA_RABBIT	MDAIKKKMQMLKLDKENALDRAEQAEADKKAAEDRSKQLEDELVSLQKKLKGTEDELDKYSEALKDAQEKLELAEKKATD	
TPMA_RAT	MDAIKKKMQMLKLDKENALDRAEQAEADKKAAEDRSKQLEDELVSLQKKLKGTEDELDKYSEALKDAQEKLELAEKKATD	
TPM3_HUMAN	MEAIKKKMQMLKLDKENALDRAEQAEAEQKQAEERSKQLEDELAAMQKKLKGTEDELDKYSEALKDAQEKLELAEKKAAD	
TPMX_RAT	MDAIKKKMQMLKLDKENALDRAEQAEADKKAAEDRSKQLEDELVSLQKKLKGTEDELDKYSEALKDAQEKLELAEKKATD	
TPMB_RABBIT	MDAIKKKMQMLKLDKENAIDRAEQAEADKKQAEDRCQLEEEQQALQKKLKGTEDEVEKYSESVKDAQEKLEQAEKKATD	
TPMB_HUMAN	MDAIKKKMQMLKLDKENAIDRAEQAEADKKQAEDRCQLEEEQQALQKKLKGTEDEVEKYSESVKDAQEKLEQAEKKATD	
TPMF_HUMAN	MDAIKKKMQMLKLDKENALDRAEQAEADKKAAEDRSKQLEDELVSLQKKLKGTEDELDKYSEALKDAQEKLELAEKKATD	
TPMS_RAT	MDAIKKKMQMLKLDKENALDRAEQAEADKKAAEDRSKQLEEDISAKEKLLRASEDERDRVLEELHKAEDSLAADETAAK	
TPM1_RAT	MDAIKKKMQMLKLDKENAIDRAEQAEADKKQAEDRCQLEEEQQALQKKLKGTEDEVEKYSESVKDAQEKLEQAEKKATD	
TPM2_HUMAN	MDAIKKKMQMLKLDKENAIDRAEQAEADKKQAEDRCQLEEEQQALQKKLKGTEDEVEKYSESVKDAQEKLEQAEKKATD	
TPMZ_RAT	-----MAGSSSLQAVRRKIRSLQEQADAAERAGSLQRELDQERKLR	
TPMY_RAT	-----MAGSSSLQAVRRKIRSLQEQADAAERAGSLQRELDQERKLR	
TPM4_HUMAN	-----MAGLNSLEAVKRRKIQALQQQADAEADRAQGLQRELDGERERREK	
TPM4_RAT	-----MAGLNSLEAVKRRKIQALQQQADAEADRAQGLQRELDGERERREK	
TPMS_HUMAN	-----CRLRIPLRTASSEHHLHERKLR	
TPMN_HUMAN	-----MAGITTIEAVKRRKIQVLQQQADAEERAEFLQREVEGERREAREQ	
	81	160
TPM1_HUMAN	AEADVASLNRRRIQLVEEELDRAQERLATALQKLEEAKEADESERGMKVIESRAQKDEEKMEIQEIQLKEAKHIAEDADR	
TPMA_RABBIT	AEADVASLNRRRIQLVEEELDRAQERLATALQKLEEAKEADESERGMKVIESRAQKDEEKMEIQEIQLKEAKHIAEDADR	
TPMA_RAT	AEADVASLNRRRIQLVEEELDRAQERLATALQKLEEAKEADESERGMKVIESRAQKDEEKMEIQEIQLKEAKHIAEDADR	
TPM3_HUMAN	AEAEVASLNRRRIQLVEEELDRAQERLATALQKLEEAKEADESERGMKVIENTRALKDEEKMEIQEIQLKEAKHIAEEDR	
TPMX_RAT	AEADVASLNRRRIQLVEEELDRAQERLATALQKLEEAKEADESERGMKVIESRAQKDEEKMEIQEIQLKEAKHIAEDADR	
TPMB_RABBIT	AEADVASLNRRRIQLVEEELDRAQERLATALQKLEEAKEADESERGMKVIENTRAMKDEEKMEIQEMQLKEAKHIAEDSDR	
TPMB_HUMAN	AEADVASLNRRRIQLVEEELDRAQERLATALQKLEEAKEADESERGMKVIENTRAMKDEEKMEIQEMQLKEAKHIAEDSDR	
TPMF_HUMAN	AEADVASLNRRRIQLVEEELDRAQERLATALQKLEEAKEADESERGMKVIESRAQKDEEKMEIQEIQLKEAKHIAEDADR	
TPMS_RAT	AEADVASLNRRRIQLVEEELDRAQERLATALQKLEEAKEADESERGMKVIESRAQKDEEKMEIQEIQLKEAKHIAEDADR	
TPM1_RAT	AEADVASLNRRRIQLVEEELDRAQERLATALQKLEEAKEADESERGMKVIENTRAMKDEEKMEIQEMQLKEAKHIAEDSDR	
TPM2_HUMAN	AEADVASLNRRRIQLVEEELDRAQERLATALQKLEEAKEADESERGMKVIENTRAMKDEEKMEIQEMQLKEAKHIAEDSDR	
TPMZ_RAT	AEADVASLNRRRIQLVEEELDRAQERLATALQKLEEAKEADESERGMKVIESRAQKDEEKMEIQEIQLKEAKHIAEDADR	
TPMY_RAT	AEADVASLNRRRIQLVEEELDRAQERLATALQKLEEAKEADESERGMKVIESRAQKDEEKMEIQEIQLKEAKHIAEDADR	
TPM4_HUMAN	AEGDVAALNRRRIQLVEEELDRAQERLATALQKLEEAKEADESERGMKVIENTRAMKDEEKMEIQEMQLKEAKHIAEEDR	
TPM4_RAT	AEGDVAALNRRRIQLVEEELDRAQERLATALQKLEEAKEADESERGMKVIENTRAMKDEEKMEIQEMQLKEAKHIAEEDR	
TPMS_HUMAN	AEADVASLNRRRIQLVEEELDRAQERLATVLQKLEEAKEADESERGMKVIESRAQKDEEKMEIQEIQLKEAKHIAEDADR	
TPMN_HUMAN	AEAEVASLNRRRIQLVEEELDRAQERLATALQKLEEAKEADESERGMKVIENTRALKDEEKMEIQEIQLKEAKHIAEEDR	

	161	240
TPM1_HUMAN	KYEEVARKLVIIIESDLERAEEAEELSEGKCAELEEELKTVTNNLKSLEAQAEKYSQKEDRYEEIKVLSDKLKEAETRAE	
TPMA_RABBIT	KYEEVARKLVIIIESDLERAEEAEELSEGKCAELEEELKTVTNNLKSLEAQAEKYSQKEDRYEEIKVLSDKLKEAETRAE	
TPMA_RAT	KYEEVARKLVIIIESDLERAEEAEELSEGKCAELEEELKTVTNNLKSLEAQAEKYSQKEDRYEEIKVLSDKLKEAETRAE	
TPM3_HUMAN	KYEEVARKLVIIIESDLERTEERAELAESKCELEELKNVTNNLKSLEAQAEKYSQKEDRYEEIKILTDLKEAETRAE	
TPMX_RAT	KYEEVARKLVIIIESDLERAEEAEELSEGKCAELEEELKTVTNNLKSLEAQAEKYSQKEDRYEEIKVLSDKLKEAETRAE	
TPMB_RABBIT	KYEEVARKLVILEGELERSEERAEEVAESKCGDLEELKIVTNNLKSLEAQADKYSTKEDRYEEIKLLEEKLEAETRAE	
TPMB_HUMAN	KYEEVARKLVILEGELERSEERAEEVAESKCGDLEELKIVTNNLKSLEAQADKYSTKEDRYEEIKLLEEKLEAETRAE	
TPMF_HUMAN	KYEEVARKLVIIIESDLERAEEAEELSEGQVRLQEEQLRIMDQTLKALMAAEDKYSQKEDRYEEIKVLSDKLKEAETRAE	
TPMS_RAT	KYEEVARKLVIIIESDLERAEEAEELSEGKCAELEEELKTVTNNLKSLEAQAEKYSQKEDRYEEIKVLSDKLKEAETRAE	
TPM1_RAT	KYEEVARKLVILEGELERSEERAEEVAESRARQLEELRTMDQALKSLIASEEEYSTKEDRYEEIKLLEEKLEAETRAE	
TPM2_HUMAN	KYEEVARKLVILEGELERSEERAEEVAESRARQLEELRTMDQALKSLIMASEEEYSTKEDRYEEIKLLEEKLEAETRAE	
TPM1_MOUSE	KYEEVARKLVILEGELERSEERAEEVAESRARQLEELRTMDQALKSLIASEEEYSTKEDRYEEIKLLEEKLEAETRAE	
TPM2_RAT	KYEEVARKLVIIIESDLERAEEAEELSEGKCAELEEELKTVTNNLKSLEAQAEKYSQKEDRYEEIKVLSDKLKEAETRAE	
TPMY_RAT	KYEEVARKLVIIIESDLERAEEAEELSEGKCAELEEELKTVTNNLKSLEAQAEKYSQKEDRYEEIKVLSDKLKEAETRAE	
TPM4_HUMAN	KYEEVARKLVILEGELERAEEAEVSELKCGDLEELKNVTNNLKSLEAASEKYSQKEDRYEEIKLLSDKLKEAETRAE	
TPM4_RAT	KYEEVARKLVILEGELERAEEAEVSELKSSDLEELKNVTNNLKSLEAASEKYSQKEDRYEEIKLLSDKLKEAETRAE	
TPMS_HUMAN	KYEEVARKLVIIIESDLERAEEAEELSEGQVRLQEEQLRIMDSLEINAAEDKYSQKEDRYEEIKVLSDKLKEAETRAE	
TPMN_HUMAN	KYEEVARKLVIIIESDLERTEERAELAESRCREMDQIRLMDQNLKCLSAEEKYSQKEDRYEEIKILTDLKEAETRAE	
	241	284
TPM1_HUMAN	FAERSVTLEKSIDDLEDELYAQKLKYKAISEELDHALNDMTSI	
TPMA_RABBIT	FAERSVTLEKSIDDLEDELYAQKLKYKAISEELDHALNDMTSI	
TPMA_RAT	FAERSVTLEKSIDDLEDELYAQKLKYKAISEELDHALKDMTSI	
TPM3_HUMAN	FAERSVAKLEKTIDDLLEDELYAQKLKYKAISEELDHALNDMTSI	
TPMX_RAT	FAERSVTLEKSIDDLEDELYHQLEQNRRLTNELKALNED---	
TPMB_RABBIT	FAERSVAKLEKTIDDLLEDEVYAQKMKYKAISEELDNALNDITSL	
TPMB_HUMAN	FAERSVAKLEKTIDDLLEDEVYAQKMKYKAISEELDNALNDITSL	
TPMF_HUMAN	FAERSVTLEKSIDDLEEKVAHAKEENLSMHQMLDQTLLELNNM	
TPMS_RAT	FAERSVTLEKSIDDLEEKVAHAKEENLSMHQMLHQTLLLELNNM	
TPM1_RAT	FAERSVAKLEKTIDDLLEETLASAKEENVEIHQTLQTLLELNNL	
TPM2_HUMAN	FAERSVAKLEKTIDDLLEETLASAKEENVEIHQTLQTLLELNNL	
TPM2_RAT	FAERSVTLEKSIDDLEDELYHQLEQNRRLTNELKALNED---	
TPMY_RAT	FAERSVTLEKSIDDLEDEKFLCFSPKTPSSSRMSHSELICIL	
TPM4_HUMAN	FAERTVAKLEKTIDDLLEEKLAQAKEENVGLHQTLDQTLNELNCI	
TPM4_RAT	FAERTVSKLEKTIDDLLEEKLAQAKEENVGLHQTLDQTLNELNCI	
TPMS_HUMAN	FAERSVTLEKSIDDLEEKVAHAKEENLSMHQMLDQTLLELNNM	
TPMN_HUMAN	FAERSVAKLEKTIDDLLEDEKLCCTKEEHLCTQRMQLDQTLLELNNM	

TPM4_HUMAN
TROPOMYOSIN, FIBROBLAST NON-MUSCLE
TYPE (TROPOMYOSIN 4)

(TM30-PL). {GENE: TPM4} – Homo sapiens (Human), Sus scrofa (Pig)

TPMB_HUMAN
TROPOMYOSIN BETA CHAIN, SKELETAL
MUSCLE (TROPOMYOSIN 2,

SKELETAL MUSCLE). {GENE: TPM2 OR TMSB}
– Homo sapiens (Human)

TPMF_HUMAN
TROPOMYOSIN ALPHA CHAIN, FIBROBLAST
ISOFORM TM3 (TROPOMYOSIN 1, FIBROBLAST
ISOFORM TM3). {GENE: TPM1 OR TMSA} – Homo
sapiens (Human)

TPMN_HUMAN
TROPOMYOSIN, CYTOSKELETAL TYPE
(TROPOMYOSIN 3, CYTOSKELETAL)

(TM30-NM). {GENE: TPM3} – Homo sapiens (Human)

TPMS_HUMAN
TROPOMYOSIN ALPHA CHAIN, SMOOTH
MUSCLE (TROPOMYOSIN 1, SMOOTH MUSCLE)

(FRAGMENT). {GENE: TPM1 OR TMSA} – Homo
sapiens (Human)

TPMA_RABIT
TROPOMYOSIN
ALPHA CHAIN, SKELETAL AND CARDIAC
MUSCLE. {GENE: TPM2 OR TPMA} – Oryctolagus
cuniculus (Rabbit), Mus musculus (Mouse)

TPMB_RABIT
TROPOMYOSIN BETA CHAIN, SKELETAL
MUSCLE. – Oryctolagus cuniculus (Rabbit), Mus
musculus (Mouse), Rattus norvegicus (Rat)

TPM1_RAT
TROPOMYOSIN, FIBROBLAST ISOFORM 1
(TM-1). – Rattus norvegicus (Rat)

TPM4_RAT
TROPOMYOSIN 4, EMBRYONIC FIBROBLAST
ISOFORM (TM-4). – Rattus norvegicus (Rat)

TPMA_RAT
TROPOMYOSIN ALPHA CHAIN, SKELETAL
MUSCLE. {GENE: TPMA} – Rattus
norvegicus (Rat)

TPMS_RAT
TROPOMYOSIN ALPHA CHAIN, SMOOTH MUS-
CLE. {GENE: ALPHA-TM} – Rattus norvegicus
(Rat)

TPMX_RAT
TROPOMYOSIN ALPHA CHAIN, BRAIN-1
(TMBR-1). {GENE: TPMA} – Rattus
norvegicus (Rat)

TPMY_RAT
TROPOMYOSIN ALPHA CHAIN, BRAIN-2
(TMBR-2). {GENE: TPMA} – Rattus
norvegicus (Rat)

TPMZ_RAT
TROPOMYOSIN ALPHA CHAIN, BRAIN-3
(TMBR-3). {GENE: TPMA} – Rattus
norvegicus (Rat)