Published in final edited form as:

Int Rev Cell Mol Biol. 2010 ; 281: 91–128. doi:10.1016/S1937-6448(10)81003-2.

New Insights into the Regulation of the Actin Cytoskeleton by Tropomyosin

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Abstract

The actin cytoskeleton is regulated by a variety of actin-binding proteins including those constituting the tropomyosin family. Tropomyosins are coiled-coil dimers that bind along the length of actin filaments. In muscles, tropomyosin regulates the interaction of actin-containing thin filaments with myosin-containing thick filaments to allow contraction. In nonmuscle cells where multiple tropomyosin isoforms are expressed, tropomyosins participate in a number of cellular events involving the cytoskeleton. This chapter reviews the current state of the literature regarding tropomyosin structure and function and discusses the evidence that tropomyosins play a role in regulating actin assembly.

Keywords

Tropomyosin; Actin dynamics; Cytoskeleton; Muscle; Caldesmon

1. Introduction

Tropomyosins (Tms) constitute a family of actin-binding proteins that are important in both muscle and nonmuscle cells. In skeletal muscles where it was first identified (Bailey, 1948), Tm plays a pivotal role in conjunction with troponin in regulating the interaction of actin-containing thin filaments with myosin-containing thick filaments to effect muscle contraction. Tm was subsequently identified as a component of nonmuscle cells (Cohen and Cohen, 1972; Lazarides, 1975). In fact, there are >40 mammalian Tm isoforms, 18 of which are expressed in nonmuscle cells (Gunning et al., 2008). Why so many isoforms exist, how they differ in structure and function, and what roles they play in regulating the actin cytoskeleton, are a few of the most important outstanding questions in the biology of Tms. The generally accepted role of Tms in nonmuscle cells is to stabilize actin filaments particularly against severing proteins; however, Tms have also been identified in lamellipodia of migrating cells where actin is thought to be highly dynamic, suggesting that this notion must be reevaluated. Indeed, it is possible that Tms have multiple effects on the dynamics of actin assembly and this idea will be discussed here. Tms have been the subject of many studies and recent reviews exist (Gunning et al., 2008; O'Neill et al., 2008; Perry, 2001). In particular, chapters devoted to Tms constitute a recent volume (644) in *Advances in Experimental Medicine and Biology*. In this review, we summarize the current status of Tm research with an emphasis on the interaction between Tm and other actin-binding proteins with respect to the actin cytoskeleton.

2. Biochemical and Biophysical Properties of Tropomyosin

2.1. Gene structure

In mammals there are four genes that code for Tm: α , β , γ , and δ , also referred to as *TPM1*, *TPM2*, *TPM3*, and *TPM4*, respectively (Lin et al., 2008; Pittenger et al., 1994; Vrhovski et

al., 2008) (Fig. 3.1). Alternative splicing of these four genes, the use of multiple promoters, and the choice of polyadenylation site (Helfman et al., 1986; Ruiz-Opazo and Nadal-Ginard, 1987) lead to expression of at least 22 different Tms in humans (Lin et al., 2008). As a result of alternative 5'-end exons, two types of Tms are generated by different promoters: high molecular weight (HMW) isoforms, which are 284 amino acids in length, and contain seven actin-binding regions; and low molecular weight (LMW) isoforms, which are ~248 amino acids in length, and contain six actin-binding motifs. In *TPM1-3*, there are alternative 3' exons that result in different C-termini and 3'-untranslated regions. And, there is mutual exclusive splicing of exons leading to different sequences at the N-termini (*TPM1*) and within the molecule (*TPM1-3*).

α -*Tm* is perhaps the best studied Tm gene. It is ~28 kb in size and contains 15 exons; and has two alternative promoters, two mutually exclusive exons (2a/2b and 6a/6b), and four alternatively spliced 3' exons (9a/9b/9c/9d) resulting in different C-termini. It codes for skeletal (Tmsk α), smooth (Tmsm α), and several nonmuscle Tms (Tm2, Tm3, Tm5a, and Tm5b) including three brain-specific isoforms (TmBr1, TmBr2, TmBr3) (Lees-Miller et al., 1990a; Lewis and Smillie, 1980; Lin et al., 2008; Vrhovski et al., 2008). Curiously, the sequences of the alternative exons, such as 1a versus 1b, are quite different; however, the exons in one gene are similar to the corresponding exons in the different genes. β -*Tm* is 8–10 kb depending on the source, has 11 exons and a single promoter, a mutually exclusive internal exon (6a/6b), and two different C-terminal exons (9a/9d). It produces skeletal muscle β -Tm; and smooth muscle β -Tm, which is the same as cytoskeletal Tm1, all of which have 284 amino acids. In humans a second cytoskeletal form is found, hTm1-1 (Lin et al., 2008). γ -*Tm* spans 42 kb of DNA, and has at least 13 exons, two alternative exons (6a/6b), and four carboxyl-terminal exons (9a–d) (Clayton et al., 1988; Dufour et al., 1998). It codes for the slow twitch isoform of skeletal muscle α -Tm (Tmsk α 2) as well as at least 11 nonmuscle forms in mouse: TmNM-1, TmNM-2, TmNM-5, TmNM-6, TmNM-7, TmNM-4, TmNM-8, TmNM-9, TmNM-11, TmNM-3, and TmNM-10 (Beisel and Kennedy, 1994; Dufour et al., 1998; Vrhovski et al., 2008). δ -*Tm*, which codes for nonmuscle TM4, spans 16–18 kb and unlike the other Tm genes is not alternatively spliced in rat and mouse (Lees-Miller et al., 1990b). Interestingly, the molecule contains two case sequences similar to exons from the other Tm genes; however, they contain mutations that make these regions nonfunctional as coding exons (Lees-Miller et al., 1990b). The human γ -*Tm* gene spans 35 kb and in addition to LMW Tm4, codes for a HMW isoform similar to smooth muscle Tm (hTm4HMW) (Lin et al., 2008; Vrhovski et al., 2008).

In striated muscle, the main isoforms are α -fast tropomyosin (α f-Tm or Tmsk α 1) from α -*Tm*, β -tropomyosin (β -Tm or hTmsk β) from the β -*Tm* gene, and α -slow tropomyosin (α s-Tm) from the γ -*Tm* gene. Heart contains hTmsk α 1 and hTmsk α 1-1 from the α -*Tm* gene. Smooth muscle contains hTmsm α and hTmsm α -1 from the α -*Tm* gene, hTm1 from β -*Tm*, and hTm5 from γ -*Tm*.

2.2. Protein structure

Tms consist of heptapeptide repeats of the form *a-b-c-d-e-f-g* characteristic of a coiled-coil structural motif, where *a* and *d* are generally apolar residues. Two right-handed helices wind around each other to form a left-handed coiled coil. The stability of the coiled coil depends on hydrophobic interactions within the core and ionic bonds between the side chains. In some places alanines are at these positions, which serve to destabilize the coiled coil (Nitanai et al., 2007; Sumida et al., 2008; Whitby and Phillips, 2000). These variations and others including acidic residues, Asp¹³⁷ and Glu²¹⁸, at positions *d* and *a*, respectively, are believed to account for its flexibility (Sumida et al., 2008), which is observed in both low-resolution crystal structures of full-length cardiac Tm (Whitby and Phillips, 2000) and higher resolution images of the N- (Brown et al., 2001) and C-ends (Li et al., 2002). In

particular, the fact that Tm is preferentially cleaved at Arg¹³³ as a consequence of Asp¹³⁷, which destabilizes the middle of the molecule (Sumida et al., 2008), and that substitution of Asp with Leu at this position results in an increase in myosin ATPase activity (Sumida et al., 2008) indicate that differences in flexibility of Tms might be responsible for the different cellular functions of various Tm isoforms.

Tms are long α -helical dimers that polymerize head-to-tail. In skeletal muscle Tm is present predominantly as a mixture of $\alpha\beta$ heterodimers and $\alpha\alpha$ homodimers, which are more thermodynamically stable than $\beta\beta$ homodimers (Bronson and Schachat, 1982). Correct dimer formation is critical as evidenced by the finding that mutation in Tm leading to the formation of $\alpha\alpha$ dimers rather than $\alpha\beta$ dimers results in nemaline myopathy (Corbett et al., 2005), a human genetic disease characterized by muscle weakening (Kee and Hardeman, 2008). In mice both α - and β -Tm are expressed in cardiac muscle during embryogenesis and fetal development; however, soon after birth the expression of β -Tm decreases leaving the $\alpha\alpha$ chain as the predominant Tm (Muthuchamy et al., 1993). Substitution of α -Tm for β -Tm in the mouse heart has a significant effect on diastolic function (Muthuchamy et al., 1995).

Seven nonmuscle Tm isoforms are expressed at the same time in rat liver fibroblasts, which suggests that a mechanism must be in place to ensure that proper pairing occurs. Studies of epitope-tagged Tms in living cells showed that the HMW nonmuscle isoforms, Tm1, Tm2, and Tm3, form homodimers; whereas the LMW Tms, Tm4, Tm5NM1, Tm5a, and Tm5b, form both homo- and heterodimers (Gimona, 2008; Gimona et al., 1995; Pittenger and Helfman, 1992; Temm-Grove et al., 1998). HMW and LMW Tms do not form stable dimers together. Studies indicate that specificity of dimer formation is contained within the amino acid sequence of the Tms themselves and is influenced by alternatively spliced exons (Gimona et al., 1995). Curiously, Tm5a and Tm5b, which differ only in exon 6, are not able to form heterodimers *in vitro*; whereas Tm2 and Tm3, which also differ in their use of exon 6, form heterodimers. Studies *in vitro* have indicated that for frog skeletal and gizzard smooth muscle Tm, heterodimers are preferentially formed to minimize overall thermodynamic dissociation (Lehrer and Qian, 1990; Lehrer et al., 1989).

Tm dimers bind along the length of actin filaments and wind around the actin helix (Hanson and Lowy, 1963; Lin et al., 1997; Moore et al., 1970; Phillips et al., 1986). In skeletal muscle each Tm binds to seven successive actin subunits (38 nm). Although binding of monomeric Tm to actin is weak (Wegner, 1979), the head-to-tail interactions of multiple Tms increases binding. There is an overlap of ~4–18 amino acids in the N- and C-termini of adjacent Tms depending on the isoform (Heeley et al., 1989; Tobacman, 2008). One would think that the length of the overlap has implications for Tm binding to actin with a long overlap correlating with more cooperative binding to actin, but yeast Tms have an overlap of only four amino acids and bind to actin with the same cooperativity as seen for muscle Tms, suggesting that other factors are at work (Strand et al., 2001; Tobacman, 2008). The overlap regions share similar axial positions on actin (Tobacman, 2008).

Structural studies have suggested that Tm is a highly flexible molecule in which regions of destabilizing residues are interspersed with more stable coiled-coil regions (Brown et al., 2001, 2005; Phillips and Chacko, 1996; Smith and Geeves, 2003; Smith et al., 2003). The joint between successive Tm molecules has been considered flexible allowing Tm to act like a flexible cable along the length of the actin filament and adjust its position in response to myosins and troponin (Greenfield et al., 2006). A recent report using electron microscopy and molecular dynamic simulations suggests that Tm has a curved conformation that matches the helical shape of F-actin and rather than being flexible is actually semirigid (Li et al., 2009). This would allow Tm to move more easily as a cooperative unit as previously suggested (Geeves and Lehrer, 1994). Alternatively, local destabilization rather than

segmental bending allows Tm to conform to the actin filament (Singh and Hitchcock-DeGregori, 2003, 2009).

The polypeptide chains contain multiple quasi-equivalent domains of approximately 40 amino acids each of which interacts with an actin subunit when bound to an actin filament. There is, however, no repeated consensus sequence for actin binding. Deletion analyses showed that an uninterrupted coiled coil is required for actin binding (Hitchcock-DeGregori and Varnell, 1990), and that the seven periodic repeats are not equivalent. For example, deletion of period 2 has little effect on actin binding or actomyosin ATPase activity, whereas deletion of period 5 severely reduces actin affinity and myosin binding to actin (Hitchcock-DeGregori et al., 2002). One idea based on mutagenesis studies is that the Tm coiled coil is locally destabilized where it binds actin and that these regions are able to reorient to maximize their interactions (Singh and Hitchcock-DeGregori, 2009). In addition to actin-binding sites, there are two troponin T-binding sites on skeletal muscle Tm, one near the C-terminus and the other near Cys-190 (Lamkin et al., 1983), a region in which the sequences of fibroblast and skeletal muscle Tm differ (Helfman et al., 1986).

Skeletal and smooth muscle Tms require acetylation of the N-terminal methionine for strong binding to actin. Lack of an acetyl group destabilizes the N-terminal coiled coil and reduces the affinity of striated muscle Tm for actin (Greenfield et al., 1994; Palm et al., 2003). The likely role of acetylation is to facilitate end-to-end binding of Tms. Structural data showed that the region of Tm containing the C-terminal 11 amino acids is splayed whereas the N-terminus is not, provided that it is acetylated (Brown et al., 2001). This allows the N-terminus to fit inside the C-terminus facilitating assembly of Tms into filaments; the N-terminus is rotated $\sim 90^\circ$ relative to the C-terminus (Greenfield et al., 2006). The addition of a di- or tripeptide at the N-terminus of expressed Tm substitutes for the *N*-acetyl group of muscle Tms and allows binding to actin (Monteiro et al., 1994).

Crystal structures of native Tm are available, although at low resolution, whereas the structures of several fragments have been solved at high resolution. The structure of the N-terminal 81 amino acids of chicken skeletal α -Tm, solved to 2.0 Å (Brown et al., 2001), is almost entirely α -helical with the exception of the first two residues, which are in an extended conformation. Because the fragment used was expressed in bacteria the amino terminus was unacetylated, which might account for the extended conformation of the first two residues. A previous NMR study of the acetylated first 14 amino acids of Tm showed the structure to be completely α -helical (Greenfield et al., 1998). The crystal structure revealed that the core has an unusually high content of alanines that might be responsible for a small axial shift that breaks the symmetry and causes bending of the molecule, which supports the winding of Tm around the actin filament (Brown et al., 2001). The mid-region also exhibits specific bends of the coiled coil and apolar patches representing actin-binding sites (Brown et al., 2005). Other crystallographic studies showed that rather than forming a coiled coil, the α -helices that comprise the C-terminal 22 amino acids of skeletal muscle α -Tm splay apart; this region is critical to troponin binding (Li et al., 2002). Studies of a C-terminal fragment comprising more than 40% of α -Tm suggest that the structure of Tm best resembles a “rubber rod with ... flexible regions” because they observed that the hydrophobic core has holes that contain water molecules (Minakata et al., 2008).

Three-dimensional reconstructions of both native and reconstituted thin filaments show densities attributable to Tm in one of two equilibrium positions: on the inner edge of the outer domain of the actin filament in the absence of Ca^{2+} and on the outer edge of the inner domain of the actin filament when Ca^{2+} is present (Lehman et al., 2000; Vibert et al., 1997; Xu et al., 1999). These studies demonstrate the Ca^{2+} -sensitive movement of Tm on actin filaments that is critical to muscle contraction when troponin is present. Similar studies

performed with various Tm isoforms, although in the absence of troponin, show that the position that Tm adopts on actin filaments is a function of the specific actin or Tm isoform being studied (Lehman et al., 2000). For example, although skeletal ($\alpha\alpha$ and $\alpha\beta$) Tm and nonmuscle Tm5a prefer to bind to the outer edge of the inner domain of actin (the same so-called C-state that Tm adopts on reconstituted filaments in the presence of Ca^{2+} ; Section 3.1), cardiac ($\alpha\alpha$) Tm and smooth ($\alpha\beta$) Tm under the same conditions bind to the inner part of the outer domain. The studies support the notion that troponin acts as an off switch that keeps Tm at the outer domain in the absence of Ca^{2+} . These Tms bind at one of two possible sites on the inner domain of skeletal muscle F-actin, but, on the outer domain of actin isolated from yeast (Lehman et al., 2000) emphasizes the importance of investigating the interaction of nonmuscle Tms with nonmuscle actin.

2.3. Biochemical properties

The LMW Tms Tm4, Tm5NM1, Tm5a, and Tm5b and the HMW Tms Tm1, Tm2, Tm3, and Tm6 are expressed in nonmuscle cells. Tm1, Tm2, and Tm3 along with Tm4 are the major isoforms expressed in untransformed cells (Helfman et al., 2008). The large number of Tm isoforms expressed in cells might represent how Tms play a variety of functions in different cell types. These different Tm isoforms display different biochemical properties, and are not redundant in function, support this notion. For example, both HMW and LMW Tms bind actin simultaneously *in vitro*; however, the LMW Tm, Tm5b, readily displaces other Tms from actin, including Tm5a which differs from Tm5b only in the use of exon 6a (Tm5b) versus 6b (Tm5a), a difference of some 24 amino acids (Pittenger and Helfman, 1992). Other studies showed that although γ -gene product, Tm5, binds F-actin more strongly than α -gene product, Tm3, the latter has a higher cooperativity of binding than Tm5; and Tm5/Tm3 chimeras have even stronger actin binding than Tm3 (Novy et al., 1993). A study using bacterially expressed chimeras representing α -Tm isoforms with different N- and C-termini indicated that the ends of Tm determine Tm's affinity for actin, which has a direct effect on the cooperativity of myosin in inducing Tm binding to actin. When actin affinity is high, fewer myosin molecules are necessary to activate the filament (Moraczewska et al., 1999).

Tms are not equal in their interactions with myosin. Rabbit skeletal Tm activates or inhibits the myosin II S1 ATPase activity depending on the S1 concentration (Lehrer and Morris, 1982); whereas nonmuscle cytoskeletal Tms and smooth muscle Tm stimulate the myosin II ATPase activity, although not equivalently. This can now be understood in terms of the different cooperativities due to end-to-end interactions of different Tm isoforms. Tm5, product of the human γ gene, stimulates the actin-activated myosin ATPase activity threefold greater than α -gene product, Tm3 (Novy et al., 1993).

Tms bind along the length of actin filaments and protect them from actin-severing proteins such as gelsolin, villin, and ADF/cofilin (Bernstein and Bamburg, 1982; Burgess et al., 1987; DesMarais et al., 2002) and depolymerization of actin from the pointed end (Broschat, 1990). There is evidence from cellular studies that some LMW Tms protect actin filaments from severing better than HMW Tms (Creed et al., 2008). Tms inhibit branching and nucleation of actin in the leading edge of motile cells by the Arp2/3 complex activated by the C-terminus of the Wiskott–Aldrich syndrome protein, WASp-WA (Blanchoin et al., 2001). In particular, Tm5a inhibits actin assembly stimulated by the Arp2/3 complex and WASp-WA to a greater extent than Tm2 (Blanchoin et al., 2001). Tms also prevent bundling of actin filaments by villin (Burgess et al., 1987) and in the case of skeletal muscle Tm alone, but not nonmuscle Tm (Section 5.2.3), bundling by fascin (Bryan et al., 1993; Matsumura and Yamashiro-Matsumura, 1986). Tms interact with tropomodulin, which binds at the pointed ends of actin filaments, to prevent the disassembly of actin subunits (Fowler, 1996; Kostyukova, 2008).

3. Roles of Tropomyosin in Muscle and Nonmuscle Cells

3.1. Striated muscle contraction

Tm was first identified in skeletal muscle (Bailey, 1948) where it was found to extract from the I bands along with actin (Corsi and Perry, 1958). In concert with the Ca^{2+} -binding protein, troponin, Tm mediates the interaction of thin filaments with myosin-containing thick filaments (Greaser and Gergely, 1971). The association of mutations in both Tm and troponin with cardiac myopathies and hypertension as well as respiratory and other diseases testifies to their importance (Ochala, 2008).

Troponin, which consists of a globular region and an extended tail (Flicker et al., 1982), is a complex of three subunits: troponin-I (TnI), which inhibits the myosin ATPase activity; troponin-T (TnT), which binds the troponin complex to Tm; and troponin-C (TnC), which binds calcium ions (Greaser and Gergely, 1971; Potter and Gergely, 1974). Because troponin is positioned at every seventh actin, there has been little information regarding its structure on actin because it is averaged out with available reconstruction methods. New single-particle analyses suggest that in low Ca^{2+} troponin is tapered with the widest point toward the barbed end of the thin filament (Paul et al., 2009). This is opposite to that proposed in an earlier and more generally accepted model (Ohtsuki, 1979), and calls for a reevaluation of how TnT and Tm interact. Further structural studies done in the presence of Ca^{2+} are likely to shed more light on exactly where Tn binds actin and its relationship to Tm.

Muscle contraction involves the sliding of actin-containing thin filaments past myosin-containing thick filaments in the presence of ATP. Myosin heads, which form crossbridges that extend out from the thick filament, interact with actin subunits in the thin filaments and through a conformational change associated with ATP hydrolysis push the actin filament relative to the thick filament effecting contraction. It is generally believed that in striated muscle filaments, Tm and troponin lie on the outer domain of the long grooves of F-actin obscuring the myosin-binding site on actin (Hanson and Lowy, 1964; Moore et al., 1970). This has been referred to as the *blocked* (no myosin binding, or the B-) state, one of three states that Tm assumes on actin; the others are referred to as the *closed* (Ca^{2+} -induced; or the C-) and *open* (myosin-induced; or the O-) states (Geeves and Lehrer, 1994; Maytum et al., 2001; McKillop and Geeves, 1993), both of which are more toward the inner domain of actin. The presence of three Tm states is well supported by kinetic evidence. As a consequence of Ca^{2+} signaling through troponin, Tm is shifted to the closed state by moving laterally and uncovering myosin-binding sites on actin to allow myosin to isomerize to produce the force-generating, or *open*, state. This permits the interaction of myosin heads with actin allowing sliding of the thick filaments relative to the thin filaments and muscle contraction. Ca^{2+} -dependent shifts in position of Tm on thin filaments isolated from vertebrate muscle or actin filaments reconstituted with actin and Tm are observable in three-dimensional reconstructions of electron micrographs (Lehman and Craig, 2008; Lehman et al., 1995, 2000).

α - and β - striated muscle Tms are the predominant forms in mammalian striated muscle. α Tm is found predominantly in fast twitch skeletal muscle, whereas β and γ Tms predominate in slow twitch skeletal muscle (Lees-Miller and Helfman, 1991; Schevzov and O'Neill, 2008). Each Tm gene has its own pattern of transcript accumulation in adult muscle and during myogenesis (Gunning et al., 1990). In mouse cardiac muscle only a single α isoform exists (Muthuchamy et al., 1993; Schevzov and O'Neill, 2008). Knockout of α -Tm by homologous recombination is lethal leading to death at embryonic day 10–14 in mice, which corresponds to development of the myocardium (Blanchard et al., 1997; Rethinasamy et al., 1998). New studies show that substituting the α isoform with β - or γ -Tm leads to

functional differences in the heart in the rates of relaxation and contraction (Jagatheesan et al., 2009). Mutations in two Tm genes, β -Tm and γ -Tm, are responsible for congenital muscle diseases and disorders including nemaline myopathy (β -Tm and γ -Tm), distal arthrogryposis (β -Tm), cap disease (β -Tm), and congenital fiber-type disproportion (γ -Tm) (Kee and Hardeman, 2008).

Cytoskeletal Tms are also expressed in muscle tissues. Tm5NM1 is associated with the sarcolemma and is found adjacent to the Z-line consistent with the localization of T-tubules (Kee et al., 2004). Myofibers from mice in which Tm5NM1 expression is ablated display an altered excitation–contraction coupling (Vlahovich et al., 2009). The cytoskeletal Tm, Tm4, also associates with a Z-line-associated cytoskeleton and during myofiber growth and repair with longitudinal filaments that are oriented parallel to the sarcoplasmic reticulum (Vlahovich et al., 2008). When Tm3 is expressed in mice, rather than associating with the thin filaments in the sarcomere of skeletal muscle, it becomes associated with regions where endogenous cytoplasmic Tms are found, that is, adjacent to the Z-lines in muscle. Expression of this Tm, which is not normally found in skeletal muscle, leads to late onset muscular dystrophy probably by compromising the structural integrity of the muscle (Kee et al., 2004, 2009). During myogenesis, muscle-specific isoforms are induced and cytoskeletal forms are repressed. The function of these nonmuscle Tms in muscle tissues remains to be elucidated.

3.2. Smooth muscle

The main proteins in thin filaments of smooth muscle are actin, Tm, caldesmon (h-CaD), and calmodulin, but no troponin (Smith and Marston, 1985). Smooth muscle Tm isoforms derive from splicing of the α -Tm gene and β -Tm gene; most of the Tm is present as α/β heterodimers. Smooth muscle β Tm is the same as nonmuscle Tm1.

Tm and h-CaD in smooth muscle act on the actomyosin ATPase activity in an opposite manner. Unlike striated muscle Tm, smooth muscle Tm by itself “potentiates” the enzymatic activity of actomyosin (except at low S1 concentration and high Mg^{2+}) (Chacko and Eisenberg, 1990; Chacko et al., 1977; Dabrowska et al., 1996; Lehrer and Morris, 1984). This is because of the stronger end-to-end interactions between smooth muscle Tms that facilitate the movement from the blocked to the open position (Lehrer et al., 1997). CaD alone, on the other hand, inhibits the actomyosin ATPase (Dabrowska et al., 1985; Sobue et al., 1985). Such inhibition is enhanced by the presence of Tm, thus from the viewpoint of CaD, the two proteins have a synergistic effect (Ngai and Walsh, 1984). Notably, the fact that smooth muscle Tm can further activate myosin beyond the level of actin alone (Lehrer and Morris, 1984; Lehrer et al., 1997) suggests that Tm does not simply remove some inhibitory factors, but may also change the structure of actin filaments so that myosin binding is enhanced or the myosin ATPase activity is more effectively activated.

The precise mechanism of the inhibitory action of CaD–Tm has been under extensive study, but still remains controversial. In one model (*cooperative allosteric model*) smooth muscle CaD–Tm is thought to work in a mechanism similar to that of the troponin–Tm system in striated muscles. Unlike skeletal muscle, in smooth muscle there appears to be only two possible positions of Tm on the actin filament relative to the myosin-binding site(s): *open* (or activated) and *blocked* (or inhibited) positions (Lehman et al., 2000). By pushing the bound Tm from the *blocked* position to the *open* position, myosin cooperatively activates the filament upon strong binding (e.g., in the absence of ATP). In contrast, h-CaD preferentially binds to actin–Tm at its off state (Ansari et al., 2008) as evidenced by a fluorescence change (Ishii and Lehrer, 1987), thus preventing it from being activated.

Although the switching off of the filament by h-CaD does not necessarily dissociate the bound myosin (e.g., when Tm is at the *closed* position), displacement does occur when the concentration of h-CaD is sufficiently high. Under this condition the mechanism is described by the *competition model* (Yan et al., 2003). Biochemical data suggest that h-CaD directly competes with myosin for actin binding in the presence of ATP, the so-called weak binding state (Chalovich et al., 1987; Hemric et al., 1993; Horiuchi et al., 1991). The binding sites on the actin surface for myosin and h-CaD overlap (Lehman et al., 1997); however, since the typical physiological concentration of h-CaD is low compared to that of actin (i.e., less than 1/20 of the actin concentration; Haeberle et al., 1992; Lehman et al., 1993), a level at which inhibition of the actomyosin ATPase activity can still be attained but not myosin displacement (Alahyan et al., 2006), it is generally assumed that h-CaD works *in vivo* by the cooperative allosteric mechanism (Ansari et al., 2008). Nevertheless, it should be noted that h-CaD is not evenly distributed in smooth muscle cells (Mabuchi et al., 2001). Although the overall content is low, there may be regions in the cell that contain relatively high concentrations of h-CaD. Whether such local concentrations of h-CaD would allow competition with myosin remains to be investigated.

3.3. Tropomyosin in nonmuscle cells

Major cellular events ranging from cell locomotion to intracellular transport are mediated by the actin cytoskeleton. Actin behavior in turn is regulated by the plethora (>165) of actin-binding proteins including Tm (Dos Remedios et al., 2003; Ono, 2007). There is growing evidence that different populations of actin filaments distinguished by the composition of their actin-binding proteins are spatially organized in distinct regions within cells where they perform specific functions (Gunning et al., 2008; Stehn et al., 2006).

Although the functions of various Tm isoforms expressed in nonmuscle cells are not completely understood, there is substantial evidence that Tm isoforms are critical for cytoskeletal function (Lin et al., 2008). This is likely due as described above to the protection that Tm confers on actin filaments from actin-severing proteins such as gelsolin, villin, and ADF/cofilin (Bernstein and Bamburg, 1982; Burgess et al., 1987; DesMarais et al., 2002), branching induced by the Arp2/3 complex (Blanchoin et al., 2001), and bundling (Bryan et al., 1993; Burgess et al., 1987; Matsumura and Yamashiro-Matsumura, 1986). In fact, different Tms confer different properties on cells, for example, stress fibers in cells expressing LMW Tm5 (NM1) are more resistant to latrunculin A and cytochalasin D treatment than those in cells expressing HMW Tm3 (Creed et al., 2008).

Cell biological experiments indicate that Tms participate in organelle transport. Microinjection into chick embryo fibroblasts of antibodies against Tm1 and Tm3 slows vesicle transport (Hegmann et al., 1989). On the other hand, microinjection of bacterially expressed hTm3, but not hTm5, into normal rat kidney epithelial cells induces retrograde movement of mitochondria and lysosomes into the perinuclear region (Pelham et al., 1996). Apparently, only particular Tm isoforms associate with Golgi-derived vesicles: For example, Tm5NM-1 and Tm5NM-2 derived from the *TPM3* gene are found on Golgi membranes in fibroblasts, but Tms2, 3, 5a, and 5b from *TPM1* (α Tm_f), and Tm1 from *TPM2* (β -Tm) are not (Heimann et al., 1999). These data further support the notion that different Tm isoforms support different functions.

In dividing cells, Tms are found around the cell equator and near the cell poles (Eppinga et al., 2006). Studies with cells engineered to express mutant Tms show a correlation between speed of cell division and actomyosin-II ATPase activity. Tm mutations that increase the actomyosin-II ATPase activity show an increase in the rate at which they reach 50% cytokinesis (Eppinga et al., 2006). Thus, Tms also play a role in cell division.

Tms, which localize prominently to stress fibers, were originally thought to be associated in large part with stable actin filaments; however, both HMW and LMW Tms have now been found in regions of the cell where actin is dynamic (Hillberg et al., 2006) bringing into question whether the sole function of Tms in nonmuscle cells is to stabilize actin filaments. At the leading edge of migrating cells, two distinct regions in regards to actin filament organization and behavior have been described, the lamellipodium and the lamellum, using speckle microscopy, a technique in which a small amount of labeled actin microinjected into cells is tracked and analyzed (Gupton et al., 2005; Ponti et al., 2004). The lamellipodium is a 2–4 μm wide region adjacent to the cell membrane in which actin filaments rapidly assemble and disassemble in response to the regulatory proteins ADF/cofilin and the Arp2/3 complex (Svitkina and Borisy, 1999). The lamellum, on the other hand, is a region 3–15 μm from the cell membrane and is characterized by discrete foci of actin assembly and myosin II- and Tm-mediated slow retrograde flow (Ponti et al., 2004).

Although Tm is found primarily in the lamellum (Ponti et al., 2004), there is evidence that LMW Tm5a/b are specifically located in ruffling membranes in mouse primary fibroblasts (Schevzov et al., 2005a). Furthermore, HMW Tms reach out into the lamellipodia of migrating fibroblasts and both expressed HMW Tm1 and Tm2 and LMW Tm4 and Tm5 localize out to the edge of the lamellipodium (Hillberg et al., 2006). Experimental data support the notion that Tms play a role in lamellipodia. Microinjection of skeletal muscle α -Tm into epithelial cells results in mis-localization of Tm and myosin II to the leading edge (Gupton et al., 2005). As a result, formation of lamellipodia is inhibited while rapid cell movement persisted, suggesting that Tms are major regulators of cell migration. Similarly, in an *in vitro* reconstituted motility assay involving actin and bead-immobilized N-WASP, Arp2/3, capping protein, ADF, and profilin, the addition of skeletal muscle Tm causes changes in propulsion of the beads and morphology of the Arp2/3-branched actin filaments, presumably due to inhibition by Tm of actin capping activity by ADF and branching by Arp2/3 (Bugyi et al., 2009).

Together, these results indicate that a role for Tm must be incorporated into models of actin dynamics at the leading edge of cells. One idea is that only specific isoforms are found in lamellipodia and that Tms found in lamellipodia have distinct roles from those associated with stable actin structures such as stress fibers. Indeed, as will be discussed in a subsequent section, there is new evidence that Tms might regulate actin assembly in cells by spatially and temporally modulating actin dynamics. These exciting results indicate that Tms play an important role in actin filament dynamics and challenge the current dogma regarding the primary role of Tms as stabilizers of formed actin filaments.

Ultimately, the different cellular properties of Tms will be a consequence of their differences in length and/or N-terminal sequence. Tm2, a HMW Tm and Tm5a, a LMW Tm, interact with 7 and 6 actin subunits, respectively. In both α -gene products, Tm2 differs from Tm5a at the N-terminus. Tm5a is missing the sequence coded for by exon 2b and uses exon 1b instead of 1a. One reasonable hypothesis is that actin–Tm5a filaments and actin–Tm2 filaments have different physical properties. For example, due to their end-to-end interactions or length, one filament might be more or less flexible than the other. This would have serious implications in the cell as filaments that are more flexible, for example, would be more dynamic than those that are less flexible. Similarly, the properties of the filaments would differ depending on whether a mixture of Tms is located on one filament.

4. Intracellular Distribution of Tropomyosin Isoforms

4.1. Distribution of tropomyosin isoforms

Expression of Tm is required for embryonic development as demonstrated by the finding that mice lacking a functional α -Tm gene die between embryonic day 9.5 and 13.5 (Blanchard et al., 1997). Expression is also developmentally regulated. Changes in isoform expression correlate with organ and tissue differentiation in early embryogenesis (Gunning et al., 2005). In muscle, muscle-specific isoforms are induced and cytoskeletal forms are repressed during myogenesis. α - and β - striated muscle Tms are the predominant forms in mammalian striated muscle. α Tm is found predominantly in fast twitch skeletal muscle, whereas β and γ Tms predominate in slow twitch skeletal muscle (Lees-Miller and Helfman, 1991; Schevzov and O'Neill, 2008). Each Tm gene has its own pattern of transcript accumulation in adult muscle and during myogenesis (Gunning et al., 1990). There are widespread changes in isoform expression in brain. Expression of some isoforms, such as neuronal TmBr1 and TmBr3, is confined to specific cell types (Gunning et al., 2005; Lees-Miller and Helfman, 1991; Stamm et al., 1993). In addition, the levels of different Tms differ among different cell types. In a study of 10 different Tm isoforms, it was determined that no two tissues in mouse have the same levels of the same Tm isoforms (Schevzov et al., 2005b).

One of the most prominent features of transformed cells is an altered cytoskeleton due in part to the suppression of Tm expression (Bharadwaj et al., 2005; Pawlak and Helfman, 2001). Cell transformation is accompanied by highly reproducible changes in Tm expression. In particular, there is a decrease in the expression of HMW Tm in fibroblasts transformed by various oncogenes, chemical carcinogens, and DNA and RNA tumor viruses (Helfman et al., 2008; Pawlak and Helfman, 2001). The decrease in expression of HMW Tms correlates with a disruption in both stress fibers and focal adhesions. *In vitro* expression of HMW Tm1 and Tm2 in Ras- and Src-transformed fibroblasts reverts the tumorigenic phenotype by restoring stress fibers and reducing cell motility (Gimona et al., 1996; Helfman et al., 2008; Prasad et al., 1993, 1999). Expression of both Tm2 and Tm3 was found to rescue stress fiber organization in virally transformed cells; however, rescue was better with Tm2 even though both Tm2 and Tm3 are found in stress fibers (Gimona et al., 1996). In another study, Tm1, but not Tm2, rescued a transformed phenotype suggesting that Tm1 is a tumor suppressor (Prasad et al., 1999). Thus, transformed cells that are rescued by expression of specific isoforms indicate that Tm isoforms do not overlap in function. In fact, studies with Tm5NM1 and TmBr3 in a neuroepithelial cell line demonstrated that expression of the two different isoforms in the same cell type produces drastically different results. Tm5NM1 promoted stress fiber formation and decreased cell motility; whereas TmBr5 reduced stress fiber formation and increased cell motility (Bryce et al., 2003). These studies suggest that specific Tms are required for specific functions of the actin cytoskeleton. Such notions are supported by mammalian gene knockout experiments in which coexpressed Tm genes do not compensate for elimination of the γ -Tm gene (Hook et al., 2004). In some cases such as with RIE-1 epithelial cells (Shields et al., 2002) and neuroblastoma cells (Yager et al., 2003), ectopic expression of Tms did not reverse the transformed phenotype suggesting that the situation is more complex in these cells. In addition to studies on cells in culture, there are changes in expression of Tm isoforms in several different human tumors (Pawlak and Helfman, 2001). HMW Tms are reduced in malignant breast cancer (Franzén et al., 1996; Raval et al., 2003), prostate cancer (Pelham et al., 1996), CNS tumors (Hughes et al., 2003), and carcinoma of the urinary bladder (Pawlak et al., 2004).

There is evidence that Tms sort to different intracellular sites suggesting that specific Tms act as interpreters of the local signaling environment (Martin and Gunning, 2008; O'Neill et

al., 2008). In chick embryo fibroblasts and human bladder carcinoma cells, both HMW and LMW Tms associate with stress fibers as shown by indirect immunofluorescence microscopy with isoform-specific antibodies; however, only the LMW isoforms are also associated with membrane ruffles (Lin et al., 1988). In NIH 3T3 cells, Tm isoforms are differentially localized during the G1 phase of the cell cycle (Percival et al., 2000). For example, Tm5NM1 and Tm5NM2 are not incorporated into stress fibers, but are present in the perinuclear region, whereas Tm5NM3-11 is incorporated in stress fibers. Furthermore, Tm1, 2, 3, 6, 5a, and 5b are enriched at the cell edge when compared to Tm5aNM1 and Tm5NM2. In epithelial cells, the α -isoforms LMW Tm5a and Tm5b are found at the apical membrane and HMW Tm2 and Tm3 are found at the basolateral membrane, whereas the γ -Tm gene products are found in the cytoplasm (Dalby-Payne et al., 2003). In addition, LMW Tm5a/5b, but not HMW Tm2 from the same gene, is found associated with stress fibers at the cell periphery and in ruffling membranes (Schevzov et al., 2005a). What is responsible for Tm isoform sorting is unknown. Sorting information is not conserved in the same exon across the Tm genes. Isoforms containing exon 9c from the α - and γ -Tm genes do not colocalize in neurons; and those containing exon 9d from the α - and γ -Tm genes do not colocalize in fibroblasts (Vrhovski et al., 2003; Weinberger et al., 1996). There is evidence, however, that alternative exons play a role in sorting because Tm5b and Tm3 differ only in their N-terminus, yet Tm5b associates with the apical region of epithelial cells and Tm3 associates with the basolateral region (Dalby-Payne et al., 2003; Percival et al., 2000). Overall, the distribution of various isoforms of Tm is rather complicated, and does not appear to follow simple rules. Additional factors, for example, the presence of other protein components, must therefore be taken into consideration.

4.2. Interaction of actin–tropomyosin with myosin II

Myosins are molecular motors that translocate actin filaments (Coluccio, 2008a). There are >30 classes of myosins (Foth et al., 2006; Odrionitz and Kollmar, 2007); 12 phylogenetically distinct families are found in humans (Berg et al., 2001). All myosins contain an actin-binding site and an ATP-binding site in their amino terminal or “motor” domain. The motor domain is often followed by the neck or light-chain-binding region, which binds light chains or calmodulin. The C-terminus of myosins can be involved in a variety of functions including filament formation, membrane binding, and/or cargo binding. Myosins are widely expressed and in addition to a role in muscle contraction (mediated by two-headed myosin II), myosins function in various cellular activities such as organelle transport, signal transduction, cell adhesion, membrane events, and generation of tension (Mooseker and Foth, 2008).

Myosin II motors, which function in cell migration in nonmuscle cells, are differentially regulated by the Tm composition of the actin filaments. The interaction of the ends of Tm filaments with neighboring Tms can have dramatic effects on regulation. If end-to-end interactions are weak, cooperativity is low whereas if end-to-end interactions are strong then the movement of one Tm can result in activation of a large area of the actin filament. Tms that differ primarily in the amino acid sequences at their N- and C-termini have different equilibria between the *closed* and *open* states. Using a series of bacterially expressed α -Tm variants that differ in sequence at the ends, it was found that both the N- and C-termini determine actin affinity (Moraczewska et al., 1999). It is likely that regions of Tm other than the ends also contribute to thin filament activation. Although originally believed to be so, there is not a simple relationship between Tm size and actin affinity (Gunning et al., 2005). Whether different Tms by virtue of their terminal amino acid sequences confer different actin-binding and myosin II-activation properties is largely unknown.

Bryce and colleagues showed that LMW Tm5NM1 recruits myosin II into stress fibers resulting in a decrease in lamellipodia and cell migration (Bryce et al., 2003). In contrast,

expression of HMW Tm3, which differs at the N-terminus from Tm5NM1, induces lamellipodial formation, increases cell migration, and reduces stress fiber formation (Bryce et al., 2003). Neuronal cells overexpressing Tm5NM1 have significantly enlarged growth cones, which are enriched for myosin II, while overexpression of TmBr3 inhibits neurite growth (Schevzov et al., 2005a). Lehman and colleagues have shown that these two LMW Tms occupy different sites on actin, which might account for differential myosin binding (Lehman et al., 2000).

4.3. Class I myosins and tropomyosins at the cell membrane

In contrast to myosin II, class I myosins are single-headed myosins that do not form filaments. Myosins I are involved in such diverse functions as intestinal microvillar structure and function, adaptation in the specialized hair cells of the inner ear and insulin-mediated GLUT4 recycling in adipocytes (Coluccio, 2008b). Class I myosins, like mammalian Myo1b, are frequently found in association with membranes. Tm2 inhibits the actin-activated ATPase activity of Myo1b (Lieto-Trivedi et al., 2007). The molecular mechanism is not completely known, although it appears that Myo1b binds to actin-Tm, but is prevented from carrying out its power stroke. In this case, Myo1b could bind to actin-Tm filaments in cells and hold them in place at the membrane. The inhibition of Myo1b activity observed in the presence of Tm is reversed with Myo1b mutants in which a flexible loop at the actin-binding site, loop 4, is mutated (Lieto-Trivedi et al., 2007). These studies give insight into the structural relationship among actin, Tm, and Myo1b.

5. Caldesmon and Tropomyosins

5.1. Relationship between tropomyosin and caldesmon

In nonmuscle cells a shorter isoform of CaD (l-CaD) is expressed. l-CaD differs from h-CaD only by missing a central helical region through alternative splicing (Fig. 3.2). Both Tm and CaD are integral components of the contractile apparatus; each binds to actin filaments on the side and stabilizes the filamentous structure. As a result they are intimately involved in the regulation of assembly and organization of the actin cytoskeleton (Pollard and Borisy, 2003; Winder, 2003). As described in Section 3.2 above, Tm and h-CaD work together in smooth muscle cells to regulate the actomyosin ATPase activity. Although the precise function of l-CaD in nonmuscle cells has not yet been determined, a mechanism similar to that in smooth muscle cells may very well be operative.

Not only is Tm related to CaD functionally, the expression of the two proteins may also be under control of the same signaling pathways. For example, upon culturing, smooth muscle cells quickly lose their contractile phenotype and become dedifferentiated, fibroblast-like cells. At the same time, h-CaD undergoes a differentiation-dependent isoform switchover to l-CaD (Dingus et al., 1986; Owada et al., 1984). This process is accompanied by changes in several other smooth muscle-specific proteins including actin (Owens et al., 1986), myosin heavy chain (Rovner et al., 1986), Tm (Kashiwada et al., 1997), calponin (Shanahan et al., 1993), and vinculin (Volberg et al., 1986). Among all these proteins the expression of CaD and Tm may be most closely related to each other. It has been shown that the two α -Tm isoforms (Tm6 and Tm2) are expressed in a tightly coordinated fashion with the two isoforms of CaD both *in vivo* and *in vitro* (Kashiwada et al., 1997; Sobue et al., 1999). Thus, the same splicing machinery might work on both CaD and Tm. Interestingly, when cells are forced to express Tm1, there is also an upregulation of CaD expression (Shah et al., 2001). A serum response factor is necessary, albeit not sufficient, to transactivate the CaD promoter (Momiya et al., 1998). It remains to be seen whether the same or additional factors are recruited for the Tm promoter. Finally, CaD (Cerdeira-Nicolas et al., 2006; Yoshio et al., 2007) and Tm (Leonardi et al., 1982; Ryan and Higgins, 1988) are also simultaneously

downregulated in transformed cells and in certain types of cancer cells so that the two actin-binding proteins are thought to be tumor suppressors.

Like the HMW Tms 1, 2, 3, and 6 (Gunning et al., 2005), l-CaD is normally present in stress fibers of nonmuscle cells, but characteristically excluded from stable focal adhesions. On the other hand, both proteins as well as myosin II are found in nascent focal contacts and more dynamic structures such as podosomes (Tanaka et al., 1993) and neuronal growth cones (Kira et al., 1995). Since these contractile proteins are involved in the regulation of actomyosin activities, their presence, therefore, indicates loci of cellular contraction. In activated fibroblasts, l-CaD is primarily associated with short actin filaments in the core of podosomes (Tanaka et al., 1993) as well as ruffling membranes (Bretscher and Lynch, 1985). Curiously, LMW Tms (Lin et al., 1988) along with other cytoskeletal proteins including myosin I (Fukui et al., 1989; Ruppert et al., 1995; Tang and Ostap, 2001), but not HMW Tm and myosin II, are also found at the leading edge and growth cones. It has been reported that in osteoclasts, LMW Tms are present in podosomes with Tm4 in the actin core where unphosphorylated l-CaD resides, while Tm5a/5b is present in the ring that encircles the podosome core (McMichael et al., 2006), where phosphorylated l-CaD is localized (Gu et al., 2007). It appears that l-CaD and LMW Tm target two separate pools of actin filaments and regulate different types of contractile activities. This intriguing possibility remains to be investigated.

5.2. Phosphorylation of tropomyosin and caldesmon in cells

5.2.1. Regulation of CaD by phosphorylation—Both h- and l-CaD can be phosphorylated by a number of kinases, including MAP kinase, PAK (Van Eyk et al., 1998), PKC, CamKII, and CKII. Phosphorylation of CaD by ERK1/2, in particular, was detected in cultured smooth muscle cells upon serum stimulation (D'Angelo et al., 1999). Earlier, Matsumura and his associates had shown that l-CaD is transiently dissociated from actin filaments during mitosis (Yamashiro et al., 1990) and that the kinase p34^{cdc2} regulates this process (Yamashiro et al., 1991). It was postulated that l-CaD inhibits the activation of the contractile ring by blocking the actomyosin interaction or severing activities of gelsolin; phosphorylation alleviates the blockage during cytokinesis. On the other hand, a dynamic change in the level of l-CaD phosphorylation was observed throughout the progression of the cell cycle opposite to the change in the amount of actin stress fibers (Kordowska et al., 2006). These results indicate that phosphorylation of l-CaD is involved in cell shape changes during both cell division and postmitotic spreading. Indeed, the same residues phosphorylated in mitotic cells (Yamashiro et al., 1995) are phosphorylated when cultured smooth muscle cells are stimulated to migrate (Goncharova et al., 2002; Yamboliev and Gerthoffer, 2001).

l-CaD is also phosphorylated by PAK, which is a downstream effector in the Rac1/Cdc42 signaling pathways (Vidal et al., 2002). Like that by ERKs, PAK-mediated phosphorylation modulates CaD's action in podosome dynamics (Morita et al., 2007). The phosphorylation sites on l-CaD for both enzymes are all in the C-terminal region near the actin-binding sites (Fig. 3.3). Curiously, in both cases the two phosphorylatable serines are 30 residues apart from each other. It is thus likely that upon phosphorylation by either ERK or PAK, CaD's ability to bind actin is weakened, thus permitting severing proteins (e.g., gelsolin and ADF/cofilin) to disassemble the actin cytoskeleton, freeing l-CaD to move to the cell periphery where the cytoskeleton is reassembling. This dynamic process is essential for cells to change shape, for example, during migration. In prostate cancer cells, cotransfection of cdc2 kinase and l-CaD, but not l-CaD alone, results in a higher level of cell migration than transfection of cdc2 kinase alone (Manes et al., 2003). This would suggest that phosphorylated l-CaD

does not just passively dissociate from actin filaments, but instead, is involved in other cellular processes at the cell periphery.

It has been suggested that the function of focal adhesions involves actomyosin-based contractility (Grosheva et al., 2006). The focal adhesions in cells overexpressing l-CaD were found to be either unchanged (Jiang et al., unpublished observations), disrupted (Gabelt et al., 2006; Helfman et al., 1999), or variable, depending on the level of expression (Eves et al., 2006). The observation that phosphorylated, but not unphosphorylated, l-CaD colocalizes with vinculin (Kordowska et al., 2006) supports the notion that phospho-CaD recruits essential partners to early focal contacts. This process might be blocked by unphosphorylated l-CaD when expressed in large quantities. Thus, the apparently conflicting results in the literature might very well be consequences of differential kinase activity.

5.2.2. Tropomyosin phosphorylation—Tm is reportedly phosphorylated, although the properties of phosphorylated Tm are not yet fully characterized. Both cardiac and skeletal muscle Tms (primarily the α -form) are subjected to phosphorylation regulation at Ser²⁸³ near the C-terminus (Mak et al., 1978). Phosphorylated Tm appears to support stronger end-to-end interactions (Heeley et al., 1989) and enhanced binding to TnT (Heeley, 1994). More recently, it was shown that phosphorylated α -Tm increases the cooperative activation by myosin and generates stronger force (Rao et al., 2009). Smooth muscle Tm can also be phosphorylated, which might modify the interactions of smooth muscle Tm with h-CaD and Hsp27 (Somara et al., 2005). Several kinases have been suggested as being responsible for Tm phosphorylation *in vivo*. Protein kinase C- ζ , for example, is thought to regulate cardiac Tm (Wu and Solaro, 2007). Phosphoinositide 3-kinase, which is activated by the β -adrenergic receptor, can also lead to phosphorylation of Tm and internalization of the receptor (Praasad et al., 2005). Tm-1 in endothelial cells is phosphorylated under oxidative stress (Houle et al., 2003). Since the MEK inhibitor (PD098059) blocks this process, the phosphorylation was thought to be mediated by ERKs. Subsequently, it was found that death-associated protein kinase-1 (DAPK-1), which is downstream of ERK, directly acts on this HMW Tm and that the site of modification is also Ser²⁸³ (Houle et al., 2007). Phosphorylation of Tm-1 is accompanied by the formation of stress fibers and focal adhesions, suggesting that binding of Tm-1 to actin filaments is strengthened upon phosphorylation; however, *in vitro* experiments showed that binding of Tm to actin is not affected by phosphorylation (Heeley et al., 1989). This may indicate that additional properties of phosphorylated Tm remain to be defined. Other intriguing questions include whether some LMW Tms that contain the homologous residue of Ser²⁸³ (such as Tm5a/b and Tm4) are also under the same phosphorylation regulation, and whether other kinases act on Tm isoforms that do not contain such a residue.

5.2.3. Direct interaction between CaD and tropomyosin—The direct interaction between CaD and Tm has been shown by binding studies (Smith et al., 1987) and by the salt-dependent enhancement in viscosity of gizzard Tm (Graceffa, 1987). This direct interaction might conceivably contribute to the observed increase in the inhibitory effect of CaD by Tm, although a more indirect route through actin cannot be ruled out (Nomura et al., 1987). The affinity between CaD and Tm at physiological ionic strength is estimated to be $2.5 \times 10^{-5} \text{ M}^{-1}$ and is enhanced by actin (Horiuchi and Chacko, 1988). A model was proposed in which CaD binds Tm in an antiparallel manner at sites near Cys¹⁹⁰ (residue 201–227) (Watson et al., 1990). This region also interacts with calponin (Childs et al., 1992). CaD and Tm enhance each other's affinity for actin. They also act synergistically on other actin-binding proteins. For example, as mentioned in Section 2.3, CaD and Tm together, but not separately, inhibit the actin-binding and actin-bundling activity of fascin, a protein involved in the formation of microspikes in cultured cells (Ishikawa et al., 1998). The interaction between l-CaD and Tm is likely to play a role in this effect, but the detailed

mechanism is unclear. For example, the affinity of phosphorylated l-CaD for different Tm isoforms is unknown. Future investigations along this direction should prove to be useful. In particular, the complicated distribution of Tm isoforms may be explained by preferred interactions with respective forms of l-CaD.

5.3. How caldesmon and tropomyosins affect actin dynamics

The fact that phosphorylated CaD moves to the cell periphery where actin is undergoing rapid assembly and disassembly suggests that phosphorylated CaD plays a role in actin dynamics. It is known that CaD promotes actin nucleation, bundles actin filaments, and interacts directly with cortactin (Huang et al., 2006), a cortical actin-binding protein. Moreover, CaD competes with Arp2/3 for actin binding (Yamakita et al., 2003); however, the effects of CaD on polymerizing actin remain unclear. To address this problem, we have recently performed *in vitro* actin polymerization experiments using pyrene-labeled actin and CaD (Huang et al., 2010). We found that CaD produces different pyrene fluorescence changes depending on when it is added. If CaD or its C-terminal actin-binding fragment is added at the beginning of actin polymerization, the typical enhancement of pyrene fluorescence reflecting actin polymerization is severely suppressed. Inclusion of CaM in the presence of Ca^{2+} recovers the pyrene fluorescence intensity. As suggested by Huang et al. (2003), CaM causes the entire C-terminal region of CaD to dissociate from actin filaments. The reversibility of CaD-induced changes by CaM indicates that the lower fluorescence results from binding of CaD to actin filaments. On the other hand, when CaD is added *after* polymerization has started, it no longer inhibits pyrene fluorescence enhancement. Instead, CaD accelerates the pyrene fluorescence enhancement after an initial decrease in intensity of pyrene emission. Both the initial drop in pyrene fluorescence and the slope of recovery of pyrene fluorescence are proportional in magnitude to the level of polymerized actin at the time of addition. Thus, these observations indicate that once polymerization starts, it is actually promoted by CaD.

The same amount of sedimentable actin is observed in samples containing actin alone, actin with CaD added before initiation of polymerization, and actin with CaD added after actin polymerization. There are several important implications: (i) The observed increase in pyrene fluorescence reports a conformational change associated with actin polymerization, rather than polymerization itself. (ii) Actin polymerization is not inhibited by CaD fragment. (iii) Instead, the apparent suppression of the pyrene-actin fluorescence enhancement reflects a different conformational state of the filament. These data can be best explained by the hypothesis that actin filaments undergo a “maturation” process, and that CaD arrests actin filaments at a “young” stage if present before this process, but further stabilizes filaments once they have matured (Huang et al., 2010). This phenomenon provides evidence for a novel mechanism by which CaD regulates assembly of the actin cytoskeleton both spatially and temporally.

The idea of actin filament maturation has been postulated previously based on imaging (Millonig et al., 1988; Orlova et al., 2004), biochemical (Galinska-Rakoczy et al., 2009), and kinetic studies (Kueh and Mitchison, 2009; Kueh et al., 2008). It is possible that the “ragged” morphology of nascent actin filaments represents the state before maturation, which may be the preferred configuration for interacting with accessory proteins (such as Arp2/3, cofilin, cortactin, etc.) during rapid actin assembly and disassembly. Phosphorylated l-CaD, which coexists with polymerizing actin in the cell, can therefore stabilize this configuration and promote actin dynamics. Preliminary experiments with nonmuscle Tm5a and Tm5b indicate that these LMW Tms exhibit behavior similar to l-CaD (Huang and Wang, 2009). Depending on when they bind actin relative to the initiation of actin polymerization, they, too, either arrest actin filaments at an intermediate and more dynamic

“young” state, or stabilize actin filaments at a more static, matured state. Whether this is a property unique to LMW Tms awaits further investigation.

The combined effect of CaD and Tm on actin assembly was also tested. CaD (or its C-terminal fragment) and Tm (e.g., Tm5a) were incubated with actin before polymerization was initiated. Next, CaM was added to dissociate CaD. Under this condition, F-actin is normally allowed to mature; however, since Tm5a is sufficient to inhibit the maturation process, no change in the pyrene–actin emission was observed. Thus, CaD and Tm5a could synergistically modulate the conformation of actin during polymerization and maintain actin filaments in a less static configuration by binding to nascent actin filaments as they assemble. Importantly, both phosphorylated CaD and LMW Tms, including Tm5a, are indeed found at the leading edge of cells (and similar structures such as podosomes). The possibility that Tm isoforms work with other actin-binding proteins to modulate actin dynamics undoubtedly opens up a new direction for investigation.

6. Potential Roles of Tropomyosin in Cancer Metastasis

Cell migration is critically related to cancer metastasis. Metastatic cells such as human breast cancer cell line, MDA-MB231, migrate faster than the nonmetastatic counterpart cell line MCF-7. Since the migratory activity of cells includes both a cellular extension step, which depends on dynamic assembly of the actin cytoskeleton, and a contraction step, which requires a stable actin cytoskeleton, it is conceivable that the process involves the actin-binding proteins, CaD and Tm. It was shown previously that CaD phosphorylation by cdc2 is *sufficient* to increase migration of prostate cancer cells (Manes et al., 2003). We (Jiang et al., 2009) and others (Eppinga et al., 2006) have demonstrated that phosphorylation of CaD at the ERK and PAK sites is also *necessary* for maintaining enhanced cell migration. Therefore, the ERK/PAK signaling via CaD could play a key role in controlling cell migration.

Our results further establish that compared to the nonmetastatic cell line, MCF-7, the metastatic cell line, MDA-MB231, not only has a significant higher amount of I-CaD, but the ERK-mediated CaD phosphorylation is also very extensive. This may be because MDA-MB231 cells carry the K-ras mutation (Davidson et al., 1987; Ennis et al., 1991; Kato et al., 1998; Toulany et al., 2005), and as a consequence exhibit more migration (Price et al., 1999). Interestingly, several other aggressive tumor cell lines, such as HS578T (human breast cancer; Lakka et al., 2000) and SNB-19 (human glioblastoma; Kraus et al., 1984), were also found to have high levels of CaD as well as constitutively activated ERK and/or PAK pathways. The combination of elevated CaD levels and kinase activity may be critical factors for the highly invasive and migratory behaviors of metastatic tumor cells. Phosphorylated CaD, in particular, at the leading edge of the cell stabilizes nascent actin filaments and thereby promotes actin dynamics (Huang and Wang, 2009).

The relationship between Tm and tumor metastasis has not been explored. Interestingly, we have found that the metastatic cells, MDA-MB231, contain a different form of Tm from nonmetastatic MCF-7 cells. When extracts of these two kinds of tumor cells were probed with anti-(pan)Tm, two different bands were detected. MDA-MB231 cells contain a Tm species that migrates on the gel more slowly than the species in MCF-7 cells (Fig. 3.4). Whether such a difference can be generalized to other types of tumor cells is not yet known; the nature of this difference also awaits further investigation. Nevertheless, such properties could have important bearings on the functional involvement of both CaD and Tm in tumor metastasis. For example, one possibility is that this results from a differential interaction between I-CaD and Tm depending on the phosphorylation state of I-CaD. It will also be interesting to test the level of CaD and Tm expression and the amounts of phosphorylation

in other types of malignant tumor cells. The idea that either Tm or CaD mutants might serve as therapeutic agents to battle certain metastatic cancers remains to be tested.

7. Concluding Remarks

Many questions remain regarding the structure and function of Tms beginning with the molecular basis of the interaction of Tm with actin filaments. For example, knowing what controls dimerization and what are the properties of filaments with specific Tm compositions are valuable information for predicting the cellular roles of Tms. Understanding how Tm isoforms are targeted to different pools of actin filaments in the cell is also critical. In fact, the apparent lack of simple rules to account for the localization of Tm isoforms requires consideration of additional players such as other actin-binding proteins. The ongoing studies directed at investigating the effects of Tm on actin dynamics will also spur further work designed to reveal how Tms affect actin assembly and whether the cooperation of Tms with other actin-binding proteins control the rate and extent of actin assembly. The recent recognition of the large size of the Tm family and the availability of DNA sequences coding for the various isoforms should facilitate the generation, for one, of isoform-specific antibodies and other reagents to target individual Tm gene products. Antibodies that allow visualization of specific isoforms in the cell will be of particular importance to understanding where specific isoforms localize in the cell, providing clues to cellular function. In addition, or alternatively, the identification of inhibitors specific for particular Tm isoforms will also be very useful for distinguishing the roles of specific isoforms in the cell. Understanding the role of Tms in cell migration is of particular importance given that changes in expression of Tms accompany transformation. The future holds the promise of developing biomarkers and therapeutic targets based on Tms. The roles of Tms in cardiovascular disease and skeletal muscle disease, in particular how mutations give rise to specific pathologies, also remain to be revealed.

Acknowledgments

We thank BBRI colleagues, Sam Lehrer, and Zenek Grabarek for helpful comments. Studies in the Wang laboratory are supported by NIH HL092252. Studies in the Coluccio laboratory are supported by NIH DC08793.

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Figure 3.1.

Schematic of exon organization of the four tropomyosin genes in mammals where exons are represented by boxes and introns are represented by solid lines. Isoform variation is primarily at the amino and carboxyl ends and a result, for example, of the alternative use of exon 2a versus 2b in TPM1 and one of the four exons (9a, 9b, 9c, and 9d) at the C-terminus. The figure is based on previously published figures (Gunning et al., 2008; Lin et al., 2008; Vrhovski et al., 2008).

Figure 3.2.

Domain structures of smooth muscle-specific CaD (h-CaD) and nonmuscle CaD (l-CaD). Both isoforms are derived from the same gene via alternative splicing. In l-CaD a highly charged repeating sequence, which forms a helical stretch in the middle of the h-CaD molecule, is missing, while the other functional domains are identical for the two isoforms.

Figure 3.3.

Major phosphorylation sites in the C-terminal actin-binding region of CaD. The sequence near the C-terminal end of CaD encompasses the sites for ERK (497, 527)- and PAK (452, 482)- mediated phosphorylation. The numbers for the residues in the human I-CaD sequence are shown here. The numbers in parentheses relate to the corresponding residues in the h-CaD sequence. Note that the two phosphorylation sites in each set are separated by the same number (30) of residues.

Figure 3.4.

Metastatic and nonmetastatic human breast tumor cells contain different Tms. Western blot analysis with Odyssey software showing that the Tm in the two types of tumor cells, MDA-MB231 (metastatic) and MCF-7 (nonmetastatic), exhibit different mobilities on SDS-polyacrylamide gels (lower panel). β -Actin was used as a reference for loading (upper panel).