Tropomyosin-Based Regulation of the Actin Cytoskeleton in Time and Space

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I. Introduction and Historical Perspective 2
   A. Discovery and structure 2
   B. Actin filament and muscle contraction 3
   C. Isoforms and the cytoskeleton 3
   D. Genome evolution and the role of isoforms 3
   E. Diversity of actin filament function 4

II. Gene Structure and Isoform Generation 4
   A. Mammalian gene structure 4
   B. Alternative splicing 4

III. Regulation of Gene Expression 4
   A. Regulation during development 4
   B. Isoform shifts in cancer 6
   C. Regulators of Tm expression 7
   D. Epigenetic regulation 8
   E. Regulation of alternative splicing 8
   F. Posttranslational modifications 10

IV. Tropomyosins Are Essential 10
   A. Tms have essential functions 10
   B. Tm genes are not redundant 10

V. Tropomyosin Isoforms Are Functionally Distinct 11
   A. Yeast Tm isoforms are functionally distinct 11
   B. Muscle isoforms are not equivalent 11
   C. Cytoskeletal isoforms differentially revert the cancer phenotype 11
   D. Cytoskeletal isoforms contain different structural information 12

VI. Involvement of Tropomyosin in Human Disease 12
   A. Familial hypertrophic cardiomyopathy 12
   B. Nemaline myopathy 13
   C. Anti-Tm antibodies in human disease 14

VII. Protein Structure and Function 14
   A. Dimer formation and composition 15
   B. Cooperative binding of Tm to actin 15
   C. Emerging view of Tm’s role in muscle contraction 16

VIII. Spatial Segregation of Tropomyosin Isoforms 16
   A. Isoform sorting is intrinsic to Tms 16
   B. Regulation of sorting 18
   C. Mechanisms of sorting 19

IX. Tropomyosin-Directed Regulation of Actin Filament Dynamics, Organization, and Mechanochemistry 20
   A. Tm regulates actin dynamics 20
   B. Isoform differences in Tm binding to actin 21
   C. Tms regulate actin interactions with binding proteins 22

X. Conclusion: Tropomyosins As Spatial Regulators of Actin Filament Function 23
   A. What we think we know 23
   B. What we need to know 24
   C. What Tms offer cell physiology 25
   D. Conclusion 25
I. INTRODUCTION
AND HISTORICAL PERSPECTIVE

The tropomyosin (Tm) family of actin-binding proteins plays a pivotal role in regulating the function of actin filaments in both muscle and nonmuscle cells. Historically, it was thought that actin and its binding proteins only existed in muscle. With the discovery of the actin cytoskeleton in nonmuscle cells, it became popular to talk of muscle and nonmuscle isoforms of these contractile proteins. Subsequently, it became clear that muscle cells also contain a cytoskeleton that is physically distinct from the contractile system and composed of so-called nonmuscle contractile proteins. For clarity in this review, we will therefore use the terms muscle isoforms when referring to the proteins found in the contractile apparatus of striated and smooth muscle and cytoskeletal isoforms when referring to the components of the cytoskeleton found in all cells.

Muscle Tm is responsible for regulating the interaction between actin and myosin and is therefore a regulator of muscle contraction, which involves the sliding of myosin filaments (thick filaments) across actin filaments (thin filaments). For a long time it has been unclear what Tm is doing in the actin cytoskeleton because there is not a simple structural reproduction of muscle contraction in the cytoskeleton. Recent studies in a variety of systems have shown that Tm contributes to a number of functional properties of actin filaments and that the diversity of actin cytoskeletal function is paralleled by a diversity of Tm isoforms. The increasing appreciation of this role, particularly in the actin cytoskeleton, has been driven by the use of genetic manipulation in a variety of biological systems.

It is therefore timely to review the field in an attempt to synthesize our understanding of this emerging role of Tm function. This review explores the emerging view of Tms as spatial regulators of the actin cytoskeleton. The initial focus of this review is on the genes, their regulation, and evidence for essential and unique functions of Tms. Following this information, we consider the molecular basis for unique function in terms of protein chemistry, spatial sorting, and the regulation of actin filament biochemistry and mechanochemistry.

A. Discovery and Structure

Tm was initially isolated as a major myofibrillar protein from skeletal muscle (9). It is an asymmetric protein (9, 10) that is capable of forming highly viscous solutions at low ionic strength (7). Because of similarity to both the amino acid (aa) composition and physical properties of muscle myosin, it was named “tropomyosin” with the expectation that it may be a precursor to myosin (9). Subsequent work demonstrated this not to be the case and that Tm is a dimer of identically sized subunits of approximate size 33 kDa (351, 352).

The structure of Tm was initially deduced from the primary aa sequences determined by the Smillie group (see Ref. 299 for review). Muscle Tm was shown to consist of two α-helical polypeptide chains, 284 aa in length, which would form a stable coiled coil structure (303).

Whitby and Phillips (345) have resolved the structure of Tm to 7 Å. Their results indicate approximately three full turns of the α-helix per molecule. More recently, Brown et al. (36) have resolved the crystal structure of an 81 aa NH₂-terminal fragment of muscle α-Tm at 2 Å. They have detected specific bends in the molecular axis of Tm as a result of alternating areas of axial register and axial staggering of the two α-helices. This suggests that α-Tm may be able to adopt and be stabilized in up to 128 bent conformations, each of similar energy. Tm molecules could therefore move in a “jointed” or “segmented” manner.

At low ionic strength, Tm polymerizes into long filaments that appear to result from head-to-tail aggregation of dimers with 8–9 aa overlap in the NH₂ and COOH termini (220). The overlap model is consistent with the effective length of Tm molecules in the muscle thin filament, 406 Å (266), compared with the length of nonover-
lapping single molecules at 423 Å (299). The model is further consistent with the findings that acetylation of lysine-7 and removal of the last 11 COOH-terminal amino acids eliminates Tm polymerization (160). Finally, X-ray data reveal Tm filaments as continuous with dense regions apparent at the ends of individual molecules (267). The overlap complex between Tms has recently been examined using solution NMR of model peptides (112). The resulting structure reveals that the NH2-terminal coiled coil is inserted into a cleft generated by spreading of the COOH-terminal chains. In the cellular environment, Tm has only been detected as a dimer and is primarily, if not entirely, found associated with actin filaments.

B. Actin Filament and Muscle Contraction

The original observation that selective extraction of the I band of the striated muscle sarcomere released both actin and Tm suggested that they were associated with each other in the myofibril (56). The actin filament itself consists of a two-stranded polymer of actin with the strands wound around each other to create helical grooves along each side of the filament. It was suggested that Tm could form a “backbone” along the length of the actin filament (153) and further that Tm may exert a regulatory function over the contractile properties of actin (130). Subsequently, Moore et al. (229) confirmed that the regulatory proteins Tm and troponin lay in the long pitch grooves of actin filaments. Because the NH2 and COOH terminals overlap, Tm therefore forms a continuous polymer along each of the two major grooves in the actin thin filament.

The regulation of muscle contraction requires that the interaction of myosin heads in the thick filament with actin in the thin filament be coordinated via a signal originating at the neuromuscular junction. The finding that electrical stimulation of skeletal muscle increases the intensity of the second layer line in X-ray diffraction patterns relative to the third line implicated a movement in the position of Tm (154). This was consistent with a model in which the movement of Tm into the center of the filament groove facilitated the engagement of the myosin head with actin. This is known as the “steric blocking mechanism” in which Tm usually covers the myosin interaction site on actin (131, 154, 255).

The movement of Tm upon activation of muscle contraction is now unambiguously established (263). Furthermore, Tm undergoes this movement 12–17 ms before the mechanical response to muscle activation can be detected (166). While consistent with the “steric blocking model,” this does not establish whether the movement of Tm causes the myosin head to interact with actin. The molecular interpretation of this and other data was that Ca2+ influx following nerve stimulation resulted in binding of Ca2+ to troponin C, which in turn removed troponin I binding to Tm and allowed Tm to move, uncovering myosin interaction sites on actin [see comprehensive review by Gordon et al. (108)]. This view has largely dominated thinking on the function of Tms for the last 30 years.

C. Isoforms and the Cytoskeleton

In comparison with the specialized contractile systems found in striated and smooth muscle, the actin filaments which form an essential part of the cytoskeleton in all cells are remarkably more complex in terms of both composition and function. Whereas the contractile systems use 4 actins (2 striated and 2 smooth muscle isoforms) and 5 Tms (3 striated and 2 smooth muscle isoforms) (141, 268, 285), the cytoskeleton utilizes 2 actins (β- and γ-isoforms) (141, 285) and over 40 Tm isoforms (119, 268).

The diversity of cytoskeletal actin and Tm isoforms presents the potential to diversify or specialize actin filament function. It has been historically difficult, however, to demonstrate that isoforms are functionally distinct, particularly when considering structural molecules (118). The first demonstration that the actin isoforms are functionally distinct came from gene transfection studies which demonstrated that β-actin promotes cell spreading and stress fiber formation, whereas γ-actin antagonizes this process (293). Similar conclusions were drawn by von Arx et al. (330) and Mounier et al. (233). Most compelling, however, was the demonstration by the Lessard laboratory that replacement of α-cardiac actin by γ-enteric actin could not maintain normal function of the mouse heart (169). These experiments served to emphasize the potential significance of protein isoform diversity.

D. Genome Evolution and the Role of Isoforms

Perhaps the most extraordinary finding of the human genome project was the finding that biodiversity is driven much more by the generation of isoforms than it is by the formation of novel genes (173, 327). On the one hand, this has mechanistic appeal because it is much easier to expand a gene/protein family than it is to create entirely new proteins. On the other hand, it presents a mechanistic challenge to understand how isoforms can drive functional diversity. For a recent discussion, see Reference 118. The essential conclusion is that the basic biochemical building blocks are largely unchanged between most organisms, with a number of notable exceptions, but variation in the function of these molecules is sufficient to fuel biodiversity. The variable functions of isoforms is decided by the timing and tissue specificity of gene expression, mRNA, and protein localization and subtle dif-
ferences in protein function. For example, isoforms expressed in different lineages or at different developmental times provide the opportunity to enhance protein function for a more specialized context. Thus time and space are the variables that provide isoforms with a specialized functional context and in turn promote diversified function of isoforms (118). Tms are an excellent example of this challenge to link isoforms to the diversity of function of biological systems, in this case, the cytoskeleton.

E. Diversity of Actin Filament Function

Actin filaments are involved in an extraordinary array of cellular functions. Beyond their classic role in muscle contraction, actin filaments are required for or participate in cell shape, cell adhesion, cell motility, vesicle transport, endocytosis and exocytosis, Golgi function, cytokinesis, and membrane function. The actin filaments involved in these different processes display differences in filament organization, intracellular location, motor protein interactions, and dynamics. Based on these considerations, the Tms are well suited to contribute to the diversity of function of the actin cytoskeleton. This review focuses on the evidence which supports such a role for the Tms.

II. GENE STRUCTURE AND ISOFORM GENERATION

A. Mammalian Gene Structure

Mammals utilize four genes to generate the more than 40 Tm isoforms. The structure of each of the human genes is shown in Figure 1. The genes have very similar organization, although it is interesting to note that the δ-gene shows some variation in functional exons between some mammals. The similarity in structure between genes is consistent with their generation by duplication of an ancestral gene. The genes are no longer linked, however, and in humans the genes are widely dispersed. In humans, the α-, β-, γ-, and δ-genes are formally known as TPM1, TPM2, TPM3, and TPM4, and their locations are 15q22 (80), 9p13 (152), 1q22 (348), and 19p13 (349) respectively.

Transcription is initiated at the start of either exon 1a or 1b. The α- and γ-genes are the most highly related to each other, utilizing two promoters and differing only in the presence of the unique 2a exon in the α-gene (Fig. 1; Refs. 287, 288). The δ-gene also has both 1a and 1b exons which appear to be functional in humans (Genbank accession no. AK023385.1), but it is unclear if the 1a and 2b exons are functional in rodents. The β-gene has only one transcription initiation site, exon 1a (Fig. 1).

Historically, Tms have been classified as high molecular weight (HMW) or low molecular weight (LMW) corresponding to ~248 aa and 284 aa in length (268). They differ by the use of exons 1a plus 2 (for HMW) or exon 1b (for LMW) at their amino termini. All known Tms contain exons 3–9. Exon 6 is provided by mutually exclusive splicing of exon 6a or 6b, and the COOH terminus is provided by a choice between the different exon 9s.

Sequence comparisons reveal substantial differences between alternative exons in the same gene. Thus 1α differs from 1b, 6a from 6b, and similarly for exon 9s (177, 178). Most exons are, however, highly conserved between genes such that exons 1a, 1b, 6a, 6b, 9α, 9c, and 9d are quite similar when each is compared between the different genes (18, 177, 178, 268).

B. Alternative Splicing

The diversity of Tm isoform generation is primarily driven by alternative splicing (178). Alternative splicing of Tms was initially discovered in Drosophila melanogaster where different products from the TmI gene were detected in the embryo and the thorax (15, 16). Subsequent studies demonstrated that alternative splicing from the α-Tm gene generates both the striated muscle and smooth muscle α-Tms (287). This was extended to the cytoskeleton with the demonstration that human Tm genes can produce both muscle and cytoskeletal Tms by alternative splicing (205, 206). Subsequent studies in birds and mammals confirmed that the genes are capable of generating multiple isoforms and that splicing patterns are usually conserved between species (69, 86–88, 177, 179, 185, 186, 191, 198, 288, 346; see Refs. 119 and 178 for reviews). The major splice products from the mammalian Tm genes are shown in Figure 1. Additional isoforms from the mammalian α- and β-Tm genes have been reported (50, 334, 335). It is not clear if these are significant products because neither corresponding mRNAs nor proteins have been quantitated in any mammal at this time.

III. REGULATION OF GENE EXPRESSION

A. Regulation During Development

The original observations that different muscle-specific Tms and in some cases cytoskeletal Tms could be generated by alternative splicing demonstrated widespread tissue-specific regulation of these proteins. The breadth of organisms in which this occurs, Drosophila melanogaster (15, 16), rat (287, 288, 346), human (205, 206), Xenopus (96), and chicken (86, 87), serves to emphasize that tissue-specific regulation via alternative splicing is an ancient mechanism in this gene family.

Subsequent comparative studies have shown that the Tm isoform profile varies both qualitatively and quantita-
FIG. 1. Tropomyosin (Tm) isoforms are generated using a combination of different genes and alternative splicing. The organization of the four mammalian genes is remarkably similar. The α-, β-, γ-, and δ-Tm genes correspond to TPM 1, 2, 3, and 4 in humans. All the major mammalian products that have been verified by Northern and Western blots are shown, and old nomenclature for isoforms is included, where appropriate. There are a number of mRNAs which thus far have only been detected by RT-PCR and are not shown.
tively between tissues. Levels of different Tm mRNAs were found to differ between fibroblasts, smooth muscle, and intestinal epithelia (246). More recently, Schevzov et al. (295) have measured the levels of over 10 isoforms in a range of mouse tissues. They found that no two tissues had identical levels of the same set of Tms. This suggests that the Tms are subject to extensive qualitative and quantitative regulation between different tissues. It is noteworthy that very few Tms are restricted in expression to only one tissue type (295).

Regulation of isoform expression has been observed in many developmental lineages and most frequently accompanies morphogenesis or changes in cell function. Muscle differentiation in cell culture is accompanied by extensive changes in Tm isoform expression (116). In general, three muscle isoforms are induced and cytoskeletal Tms are repressed. However, both myotubes in vitro and muscle in vivo retain expression of LMW cytoskeletal Tms, one of which is localized to a specialized compartment in adult muscle (116, 161). Changes in Tm isoform expression are also seen during maturation of the chicken intestine (148, 353, 354). Similarly, morphogenetic changes in granulosa cells are paralleled by changes in Tm isoform composition and organization of the actin cytoskeleton (17, 19, 113). Morphogenetic changes in smooth muscle cells shifting from a contractile to a synthetic phenotype are also accompanied by elevated expression of Tm4 (1) and altered expression of Tm1 (105). Surprisingly, Tm6 (α-sm Tm) is not constitutively expressed in smooth muscle cells. It is a relatively late marker for smooth muscle maturation in both mice and humans (332). Neuronal morphogenesis and maturation are paralleled by induction of the brain-specific isoforms TmBr1 and -3, and their expression requires maintenance of the neuronal phenotype (81, 343). Finally, very early embryogenesis is also accompanied by changes in isoform expression which correlate with organ and tissue differentiation (49, 239).

The consistent pattern of change in Tm isoform expression during differentiation and morphogenesis is consistent with a role in regulating the participation of actin filaments in these processes. It also predicts that a deregulation of this process, such as found in cancer, might be expected to produce analogous changes in expression of Tms.

B. Isoform Shifts in Cancer

Highly reproducible changes in Tm isoform expression accompany cell transformation (Fig. 2). Rous sarcoma virus transformation of chicken embryo fibroblasts was found to result in decreased synthesis of HMW Tms (139, 140, 195). Similarly, transformation of the rat embryo fibroblast cell line REF-52 and of normal rat kidney cells with DNA and RNA viruses, respectively, leads to decreased synthesis of HMW Tms (187, 215, 218, 323). In both systems, decreased synthesis was mediated by a decrease in mRNA levels (140, 215). The parallel changes in actin filament organization and cell shape were consistent with a direct role for Tm expression in this process (195).

The association of changes in Tm expression with transformation was subsequently strengthened by three additional studies. Cooper et al. (52) analyzed NIH3T3 cells transformed by six retroviral oncogenes. They found that of seven proteins whose synthesis was repressed, two were HMW Tms. The repression was specifically associated with oncogenic transformation rather than viral transformation (52). Leavitt et al. (174) demonstrated that carcinogen-transformed HUT-14 human fibroblasts also undergo a switch from a predominance of HMW to LMW Tms that correlates with tumorigenicity. The direct association of Tm with transformation was tested by transfecting a mutated form of β-actin from HUT-14 cells into normal and partially transformed human fibroblasts (175, 176). The mutant β-actin promoted decreased expression of HMW Tms, changes in cell morphology, and growth of the partially transformed cells as tumors in nude mice (175). Finally, Boyd et al. (30) demonstrated that the levels of the HMW Tm1 tracked with tumor suppressor activity in Syrian hamster embryo cells, and more recent data suggest that the NH2 terminus of Tm1 is responsible for tumor suppressor activity (22).

Changes in Tm expression have also been associated with the acquisition of metastatic properties. Comparison of low- and highly-metastatic Lewis lung carcinoma cells revealed a decrease in HMW Tm2 protein and mRNA levels associated with metastasis (313, 315). The same result was also obtained in v-Ha-Ras-transformed NIH3T3 cells (314). Altered Tm levels have also been associated with resistance of human leukemia cells to the chemotherapy agent vincristine (329).

The association of altered Tm expression with cancer has been confirmed with studies of primary tumors and human models. Galloway et al. (99) reported an increase in HMW Tms in more anaplastic human astrocytomas compared with those that were well differentiated. This apparent contradiction, induction of HMW Tms correlating with increased malignancy, was subsequently resolved by Hughes et al. (151). They found that mature astrocytes only produce HMW Tms when dividing, and these Tms localize to the contractile ring. Thus low-grade astrocytomas broadly express HMW Tms, as do dividing normal glia. However, as the malignancy of astrocytomas increases, there is a parallel decrease in the levels of HMW Tms (Fig. 2) (151). Stress fiber structure was also found to track with Tm1 levels in glioblastoma (286).

Franzen et al. (90) found that all HMW Tms were substantially reduced in primary human breast tumors compared with noncancerous breast lesions. The loss of
Tm1 in breast cancer cells confers resistance to anoikis (23). Evaluation of HMW Tm1 revealed that its expression is virtually abolished in primary breast tumors compared with normal surrounding tissue (279) and is decreased in hepatocellular carcinoma (359). Similarly, increasing tumorgenicity of neuroblastoma is associated with reduced Tm1 (356), and Tm1 and Tm2 are reduced in human transitional cell carcinoma of the urinary bladder (259). Interestingly, colon and bladder cancer show elevated expression of a LMW Tm5NM isoform containing the 9c exon (197, 259). The Tm5NM1 isoform is also elevated in transformed rat fibroblasts (225) and is required for motility of highly metastatic melanoma (224). Elevated expression of Tm4 is associated with lymph node metastasis in breast cancer (188). It is therefore apparent that changes in Tm isoform expression are intrinsic to cancer and that cancer cells in general become more reliant on LMW Tms as HMW Tms disappear with increasing malignancy (308) (Fig. 2).

C. Regulators of Tm Expression

Tm levels in striated muscle display haplo-insufficiency in Drosophila melanogaster. In Drosophila, lack of one functional Tm allele results in reduced numbers of myofilaments in myofibrils (226). This suggests that synthesis of Tm is reduced, which in turn limits the assembly of muscle actin filaments. In contrast, mammals do not display haplo-insufficiency of muscle Tms. Knockout of one α-Tm allele in mice does not impact on the level of α-Tm protein, although the mRNA level is reduced by 50%
(27, 282, 283). Similarly, elevated expression of β-Tm in the heart leads to reduced levels of α-Tm, thereby maintaining a fixed level of muscle Tm (238). This suggests that the fixed stoichiometry of all the different structural proteins in the cardiac muscle sarcomere can determine the level of Tm protein accumulation independent of mRNA level.

A relationship between Tm levels and the composition of actin filaments has also been observed in non-muscle cells. Partial replacement of β-actin with either γ-actin or a mutated form of β-actin results in reduced levels of Tm2 but not Tm5 (294). This suggests that the composition or structure of actin filaments can influence the stable accumulation of Tms. Similarly, treatment of cells with the actin filament capping drug cytochalasin D leads to increased levels of Tm mRNAs (84). This is compatible with a model in which elevation of Tm expression is a response to reduced stability of the actin cytoskeleton. Thus the composition and stability of actin filaments may itself be a regulator of the steady-state levels of Tm.

A number of signaling pathways have been shown to impact on Tm expression. Mitogen deprivation reduces Tm levels, and exposure to mitogen restores expression (84, 144, 262). During the G1 phase of the cell cycle, the levels of a number of Tms are increased (144, 262), although the accumulation kinetics differ between isoforms (262). The induction of HMW Tm in smooth muscle cells by mitogen (144) is mediated via serum response factor and Barx1b-directed transcription of the β-Tm gene (242). α-Tocopherol also induces HMW Tm levels in vascular smooth muscle cells (6).

Tm expression is repressed by several mechanisms, and this is a subject of great interest because of the important role that Tms play in cancer (see above). It is notable that most pathways that repress Tm expression primarily regulate HMW, but not LMW, Tms. Transforming growth factor (TGF)-α initiates breakdown of stress fibers in NRF cells and suppresses the synthesis of HMW, but not LMW, Tms (51). This is accomplished by accelerating the rate of Tm degradation (337). Similarly, basic fibroblast growth factor (FGF) and cAMP also suppress Tm levels in a variety of systems (42, 249, 284). In contrast, TGF-β can restore HMW Tm levels and actin filament organization in tumorigenic cell lines (11, 74, 311) and increases HMW Tm in human trabecular network meshwork cells (363).

The signal transduction pathways responsible for regulation of Tm levels are still poorly understood, but recent studies have provided some insight into the mechanism. There is general agreement that the Ras/Raf pathway is directly responsible for repression of HMW Tm expression in transformed cells (12, 159, 203). Janssen and co-workers (158, 159) have concluded that in Ras-transformed fibroblasts, suppression of HMW Tms occurs by a MEK/ERK-independent pathway. They have found that KSRI (kinase suppressor of Ras) can restore HMW Tm expression, possibly via formation of a complex with MEK, which then acts independent of ERK to stimulate Tm expression (158). In contrast, Ljungdahl et al. (203) found that the MEK/ERK pathway was directly involved in repression of Tm2 in rat fibroblasts. Bakin et al. (12) have similarly reported that the Ras-ERK pathway is responsible for suppression of HMW Tms in the presence of TGF-β in metastatic breast cancer cells. The differences may reflect the culture conditions of the specific cells studied or the availability of specific partner proteins.

D. Epigenetic Regulation

The demonstration that HMW Tms are repressed in both primary cancer cells and transformed cells in culture has led to studies of the mechanism of repression. Shields et al. (297) found that azadeoxycytidine could restore Tm expression in Ras-transformed cell lines. This is consistent with a role for DNA methylation in repressing Tm gene expression at the level of transcription. Upregulation of Tm1 was also observed upon treatment of breast cancer cells with trichostatin A and azadeoxycytidine, suggesting that hypermethylation and chromatin remodeling are the primary mechanisms of Tm1 repression in these cells (21). Varga et al. (326) have extended this work to show that TGF-β induction of Tm1 in metastatic cell lines requires treatment with azadeoxycytidine and correlates with the methylation status of the promoter region of the gene. It is concluded that epigenetic regulation is used to silence the Tm1 gene in metastatic cells.

E. Regulation of Alternative Splicing

Tm genes have been extensively studied as a paradigm for understanding mechanisms of tissue-specific alternative splicing. These studies have mostly focused on the mutually exclusive splicing of exon 2a versus exon 2b in the α-gene and exon 6a versus exon 6b in the β-gene (see Fig. 1). The mutually exclusive splicing of exons 2a and 2b in the α-gene is ensured by the absolute incompatibility between the adjacent splice sites in the two exons (300) (Fig. 3). This is caused by the close proximity of the exon 2a splice donor site to the splice branch point upstream of exon 2b, which prevents formation of a lariat and active spliceosome complex. This was experimentally verified by showing that insertion of an extra 51–59 bases between the splice donor and branch point allows splicing of 2a to 2b (300). Hence, there is competition between the acceptor sites of the 2a and 2b exons for joining to exon 1a (285). In all cells except smooth muscle, exon 2b out competes exon 2a. This can be accounted for by the relative competitive strength of the polypyrimidine tract/
branch point elements upstream of both exons. The major determinant is the pyrimidine content adjacent to the branch point (235). Subsequent work has focused on the mechanism by which smooth muscle preferentially overcomes the intrinsic competitiveness of the two exons. The mechanism involves corepression of exon 2b by raver 1 and polypyrimidine tract-binding protein (75, 107, 114) and the involvement of four purine-rich enhancer elements to activate the splice acceptor site of exon 2a (73) (Fig. 3).

The mutually exclusive splicing of exons 6a and 6b is more complex than that seen with exons 2a versus 2b and has been extensively studied by the Helfman and Fiszman laboratories using the rat and chicken β-Tm genes, respectively. The use of minigene constructs initially demonstrated that the local sequences around exons 6a and 6b were sufficient to direct the use of 6a in nonmuscle cells and of 6b in muscle cells (192). Multiple elements upstream of exon 6b are required to select that exon (109, 121, 137, 193). In myoblasts, exon 6b is skipped and exon 6a is chosen as a default, whereas in myotubes exon 6b is revealed and now out competes exon 6a (190). The blocking of exon 6b involves RNA binding proteins that act to mask the exon (121). The model that emerges presents exon 6b plus the upstream intron as an autonomous unit in which the choice of this exon is largely regulated by repression by proteins including polypyrimidine tract-binding protein and hnRNP A1 (45, 46, 79, 111, 120, 193, 236, 298).

The selection of exon 6a involves polypyrimidine-rich elements both upstream and downstream of the exon (277, 298, 322). These elements recruit U1 snRNP to the exon 6a splice donor site, and this is mediated by SR proteins (78, 98). Differences in the SR family members present in nonmuscle and muscle cells also contribute to preferential exon choice (97). The overall mechanism of regulation therefore involves a combination of tissuespecific exon repression, relief of repression, positive selection, and exon competition. Similar mechanisms appear to operate in the α-genes of human (111) and Xenopus (71) with some differences (111).

Finally, only one paper has addressed the selection of alternative exon 9s (126). The repression of exon 9a91 in Xenopus nonmuscle cells was found to require polypyrimidine tract-binding (PTB) protein, and an intron element has 4 PTB protein binding sites (126).
F. Posttranslational Modifications

Tms are subject to two primary forms of posttranslational modification, NH$_2$-terminal acetylation, and phosphorylation. The acetylation of Tm is essential for normal function of muscle α-Tm (324) and strong binding of most Tms to actin (269). In contrast, neither β-Tm (269) nor α$^{\text{smooth}}$-Tm (47) requires acetylation for actin binding. Addition of the dipeptide Ala-Ser, or peptides of 3 or 17 residues, to the NH$_2$ terminus of α-Tm can restore actin binding in the absence of an acetyl group (228). The acetylation of Tms, however, is not used as a physiological mechanism to regulate Tm function, since NH$_2$-terminal acetylation is a constitutive modification of Tms.

Phosphorylation of Tm, in contrast, appears to be linked to regulation of function. α-Tm but not β-Tm isolated from adult rabbit and frog muscle is phosphorylated at Ser-282, (209). Both α- and β-Tm are phosphorylated to a higher extent in fetal skeletal and cardiac muscles, which suggests a more prominent role in early development (60, 132, 134, 227). Phosphorylated α-Tm has a greater avidity for head-to-tail polymerization (135) and promotes myosin activation to a greater extent than the unphosphorylated form (133). In Drosophila, Tm phosphorylation may be important for stretch activation in indirect flight muscle (214). More recently, phosphorylation of cytoskeletal Tms has been implicated in regulation of stress fiber formation (150), response to acetylcholine in smooth muscle (305), and endocytosis of the β-adrenergic receptor (240). Phosphorylation of cytoskeletal Tm on Ser-61 by phosphoinositide 3-kinase occurs at a late stage of endocytosis consistent with a role in actin polymerization (240). It is therefore suggested that phosphorylation is required for associated actin remodeling. The same conclusion may be drawn for the recruitment of Tm1 into stress fibers following its phosphorylation (150). Houle and Hout (149) have shown that activation of ERK pathway leads to Tm1 phosphorylation and the formation of focal adhesions, which allows anchorage of bundled actin filaments.

IV. TROPOMYOSINS ARE ESSENTIAL

A. Tms Have Essential Functions

Gene knockout studies have demonstrated that Tms perform essential functions and are required in species as diverse as yeast, worms, flies, and mammals. The Bretscher lab was the first to demonstrate the essential role of Tm. Elimination of the TPM1 gene in budding yeast results in reduced growth rate, disappearance of actin cables, heterogeneity of cell size, poor mating, and defective vesicular transport (200, 201). Deletion of the second yeast gene, TPM2, has no detectable phenotype but in combination with deletion of TPM1 is lethal (68). TPM1 disruption also shows synthetic lethality with a conditional mutation in the myosin V gene MYO2 (200). This reflects the dependence of polarized delivery of secretory vesicles on the myosin V motor plus Tm containing actin cables (278). Similarly in fission yeast, Tm is absolutely required for cytokinesis, and its deletion results in lethal arrest of the cells (13). In addition, Tm is also absolutely required for cell fusion during conjugation in fission yeast (170).

Subsequent studies in flies (76, 167, 320), worms (5, 251), amphibians (307, 360), and mammals (27, 147, 282, 283) have confirmed that tropomyosins are essential for a wide range of cellular functions. At its simplest, this suggests that tropomyosin is essential for the normal function of the actin filament system (119). What is less clear from these studies is whether the isoforms are functionally redundant.

B. Tm Genes Are Not Redundant

The interpretation of gene knockout experiments in terms of gene redundancy needs to be carefully evaluated. In organisms with multiple Tms, a gene product may appear essential because its deletion leads to lethality. This may reflect either a truly unique role for the isoform or an inability of other isoforms to rescue function because they are not expressed in the compromised cells. For example, in budding yeast, deletion of both Tm genes is lethal, loss of TPM2 has no phenotype, and loss of TPM1 compromises cell growth (68). It therefore appears that TPMs1 and -2 have overlapping function. Elevated expression of TPM2 cannot compensate for TPM1 deletion, indicating that some functions of TPM1 are unique (68).

In the case of deletion of α-Tm, the failure of other striated muscle Tms to compensate may simply reflect their inability to be expressed in the developing heart (27, 282). In contrast, the sensitivity of axolotl hearts to knockdown of Tm isoforms suggests some isoform-specific roles (307, 360). Similarly, the inability of three co-expressed Tm genes to compensate for elimination of the γ-Tm gene suggests isoform-specific roles in mouse embryonic stem cells (147). The observation that elimination of a subset of isoforms from the mouse γ-Tm gene has no phenotype, however, indicates that not all products of this gene are essential (331).

These experiments indicate that in some but not all situations Tm genes/isoforms perform unique functions and are not redundant. The experimental design of these studies needs to be carefully considered to identify what is really being tested. Is it the requirement for Tm per se, or does it test the functional difference between isoforms?
V. TROPOMYOSIN ISOFORMS ARE FUNCTIONALLY DISTINCT

Tm isoforms are regulated in a variety of physiological and developmental situations (see above). In particular, isoform switching accompanies morphogenesis, and isoforms display both qualitative and quantitative tissue-specific expression (295). Alterations in Tm isoform expression are also seen in cancer cells (308). This raises the question of whether changes in Tm expression are directing accompanying changes in cell structure and function. Studies in a number of systems have increasingly provided evidence that the isoforms carry distinct structural information which supports a direct role in guiding morphogenesis and cell transformation.

A. Yeast Tm Isoforms Are Functionally Distinct

Yeast expresses two Tm isoforms that have different functional characteristics. The two isoforms share 64.5% sequence identity and differ in size such that Tpm1p spans five actins in a filament, whereas Tpm2p spans four. Tpm2 is expressed at ~20% the level of Tpm1. Elevated expression of Tpm2 alters the axial budding of haploids to a bipolar pattern, and this is partially overcome by co-overexpression of Tpm1. This suggests that these two isoforms are functionally distinct and that the ratio of their levels is important for correct morphogenesis. Consistent with this conclusion, Tpm2 cannot restore function to Tpm1 minus cells (68).

B. Muscle Isoforms Are Not Equivalent

The Wieczorek laboratory (238) has performed a series of carefully constructed experiments to test the functional equivalence of two striated muscle Tms, α-Tm and β-Tm. In normal mouse heart, α-β-Tm is the predominant sarcomeric Tm with only trace levels of β-Tm detectable. Partial replacement of α-Tm with β-Tm in transgenic mice leads to a significant impact on diastolic function, indicating that α- and β-Tm are not functionally equivalent (238). The presence of β-Tm results in an increased activation of the actin filament by strongly bound cross-bridges with myosin, increased Ca^{2+} sensitivity of steady-state force, and a decrease in the rightward shift of the Ca^{2+}-force relation induced by cAMP-dependent phosphorylation (254). Mice that express β-Tm at 80% of total sarcomeric Tm die in the second postnatal week and display severe cardiac pathology (237). Cardiac function can be restored in these mice by downregulation of β-Tm (272). This quite unambiguously demonstrates the functional difference between these two striated muscle Tms. Indeed, substitution of just the last 9 aa from α-Tm with those from β-Tm changes the performance of this protein in the heart (95).

A similar conclusion has been reported in studies where α- and β-Tm have been used to rescue defective expression of cardiac Tm in axolotl hearts (361). Mouse α-Tm was able to restore myofibrillar structure, whereas β-Tm had no impact. This suggests that α-Tm, but not β-Tm, provides structural information that is essential for both cardiac myofibril assembly and function (237, 361).

C. Cytoskeletal Isoforms Differentially Revert the Cancer Phenotype

HMW Tms differ in their ability to regulate the transformed phenotype of cancer cells. Prasad et al. (275) were the first to demonstrate that Tm1 can suppress the neoplastic growth of Ras-transformed NIH3T3 cells. Subsequent studies have shown that Tm1 can reverse transformation in Syrian hamster embryo cells (30), src-transformed fibroblasts (276), and the breast cancer line MCF-7 (207) (Fig. 2). In contrast, forced expression of the very similar Tm2 isoform failed to rescue anchorage-dependent growth in Ras-transformed NIH3T3 cells (31) and Raf-transformed NRK cells (312).

Further studies have shown that the ability of HMW Tms to rescue the transformed phenotype is both isoform and cell type dependent. Gimona et al. (103) compared the ability of Tm2 and Tm3, which differ by only one alternative spliced exon, to rescue Ras-transformed NRK cells. They found that both could restore cell spreading and microfilament organization, but only Tm2 could restore contact-inhibited cell growth. This difference in impact of Tm2 on Raf-transformed NRK cells (312) may reflect the transforming gene and/or the assay used to evaluate cell growth (103) and will require further experimentation. Similarly, Yager et al. (356) reported that Tm1 could not restore actin filament organization in neuroblastoma cells. Tm1 may therefore not be able to restore actin organization in all transformed cells.

Tms may also collaborate to regulate the transformed phenotype. Tm2 can synergize with Tm1 to restore actin organization in Ras-transformed NIH3T3 cells to a level far beyond that seen with either isoform alone (296). This suggests that Tm1 and Tm2 contribute different and complementary functional properties to the actin filament system and that the changes in Tm levels observed in cancer cells directly contribute to the transformed state. For example, the level of Tm5NM1 is directly associated with the motility of metastatic mouse melanoma cells (224).

Physiol Rev • VOL 88 • JANUARY 2008 • www.prv.org
D. Cytoskeletal Isoforms Contain Different Structural Information

The first evidence that cytoskeletal Tm isoforms have distinct roles in normal cell function came from the injection of Tm3 and Tm5NM1 into NRK cells (260). Tm3 injection resulted in relocation of organelles into the perinuclear space, whereas Tm5NM1 had no impact. Because bacterially produced proteins were used, they did not contain acetylated NH₂ termini and may not have behaved in a completely normal manner (269, 324). Nevertheless, the results certainly indicate that these two isoforms are not functionally equivalent.

Bryce et al. (37) provided a convincing demonstration that Tm isoforms contain distinct structural information (37). Elevated expression of Tm5NM1 in neuroepithelial cells promoted stress fiber formation, cell spreading, and decreased motility, whereas TmBr3 induced lamellipodial formation, increased motility, and reduced stress fibers. Transient expression of TmBr3 in Tm5NM1-expressing cells resulted in relocation of actin from stress fibers to very large lamellipodia. Tm5NM1 was found to recruit specific myosin II motors and inhibit cofilin activity, whereas TmBr3 recruits active cofilin to actin filaments (37).

This observation was supported and extended in transgenic mice that overexpress Tm5NM1 and Tm3 in the nervous system (291). It was shown that these two Tms can differently regulate specific aspects of neuronal size and shape (291). Li and Gao (189) similarly found that loss-of-function mutations in the Drosophila TmII gene result in cell-autonomous expansion of neuronal dendritic fields. These studies indicate that Tms convey specific structural information in an isoform-specific manner (119).

The LMW Tms 5a and 5b have been implicated in morphogenesis and membrane function. Zucchi et al. (364) used a proteomics approach to identify proteins whose levels change during "dome" formation, a model of mammary gland differentiation, by a rat mammary adenocarcinoma cell line. Tm5b was identified as a strongly induced protein during dome formation and antisense inhibition of its synthesis abolished domes (364). Tm5a and/or Tm5b have also been shown to regulate the levels of the cystic fibrosis transmembrane conductance regulator (CFTR) at the apical surface of epithelial cells (61). This is compatible with a role for these isoforms in exocytosis.

The observation of isoform-specific roles for Tms suggests that mutations or defects in isoform function may not be easily compensated by other isoforms. This fits with the role that Tms play in a number of human diseases.

VI. INVOLVEMENT OF TROPOMYOSIN IN HUMAN DISEASE

The earliest reports of an association between Tm and human disease were made in neurodegenerative diseases. Tm has been shown to be an integral component of the neurofibrillary pathology of Alzheimer’s disease (100). It is unclear, however, what significance this plays in the disease process. Similarly, Tm antibodies also stain neurofibrillary tangles in progressive supranuclear palsy, Pick bodies, and some diffuse Lewy bodies (101). Lewy bodies of idiopathic Parkinson disease do not stain for Tm, suggesting that where pathological staining is observed, it is not nonspecific (101). Alterations in the ratio of cytoskeletal Tm isoforms in blood cells have been associated with essential hypertension (70). At this time, however, these are sporadic reports, and it is not clear whether Tm has a direct role in these human conditions. In contrast, Tm is directly implicated in four human conditions: cancer, familial hypertrophic cardiomyopathy, nemaline myopathy, and ulcerative colitis. The role of Tms in cancer has been covered in sections III B and V C.

A. Familial Hypertrophic Cardiomyopathy

Familial hypertrophic cardiomyopathy (FHC) is a disease of the sarcomere and results from mutations in a number of sarcomeric proteins including α-Tm (321). Mutations in α-Tm have been detected primarily in exons 2a and 6b. A map of mutations is shown in Figure 4. The reason for this clustering of mutations is not known. It is notable, however, that every mutation is expressed in both α-Tm and a subset of cytoskeletal Tms. All NH₂-terminal mutations are present in all HMW Tms from the α-gene, and V95A is present in all Tms from the gene. No pathology associated with the mutations in the cytoskel-

![Fig. 4. Distribution of mutations in α- Tm causing familial hypertrophic cardiomyopathy and in α- Tm causing nemaline myopathy.](http://physrev.physiology.org/)

Physiol Rev • VOL 88 • JANUARY 2008 • www.prv.org
et al. products has been reported, although this has not been specifically evaluated.

Watkins et al. (339) originally reported that FHC-causing mutations in α-Tm are rare, accounting for ~3% of cases in the United States. It is similarly rare in the Japanese population (241) accounting for ~5% of FHC cases (358). In both studies it is notable that the Asp175Asn mutation was detected. Genetic analysis of haplotypes has revealed that the frequency of the Asp175Asn mutation reflects an increased susceptibility to mutation of nucleotide 579 in α-Tm (57). Mutations in α-Tm result in a milder form of FHC than do mutations in myosin heavy chain β and cardiac troponin (57, 339). FHC cases with the Asp175Asn mutation can have different histopathological responses, suggesting that the hypertrophic response is modulated by additional factors (57). Nevertheless, FHC due to α-Tm mutations can lead to the more severe dilated cardiomyopathy (DCM) (280). Analysis of mutations which proceed to DCM, compared with FHC mutations which do not, suggests they have very different impact on the Ca$^{2+}$-regulated ATPase activity of myofibrils (44).

Studies of the mechanism by which α-Tm mutations lead to FHC have used both mouse models and patient material. At a structural level, FHC α-Tm mutations do not respond to the myosin head with the same conformational change as that seen with the normal protein (106). Subsequently, it was shown that the Asp175Asn mutation functions as a dominant negative rather than loss-of-function mutation (29). It was also found that this mutation changes Ca$^{2+}$ regulation (26), resulting in increased Ca$^{2+}$ sensitivity (29). This suggested that FHC does not result from contractile impairment but rather from enhanced contractile performance (29).

The dominant nature of FHC α-Tm mutations has allowed the generation of mouse models of this disease using conventional transgenesis. Transgenic mice have been created in which the Asp175Asn and Glu180Gly mutations are ectopically expressed in mouse heart (77, 271). The Asp175Asn mutation results in increased Ca$^{2+}$ sensitivity of the contractile apparatus, leading to decreased relaxation rate and a reduced response to β-adrenergic stimulation (77). Expression of the Glu180Gly mutation is more severe, leading to ventricular concentric hypertrophy, fibrosis, and atrial enlargement within 1 mo. This histopathology progressively increases leading to death between 4 and 5 mo. As with Asp175Asn, Glu180Gly also causes increased sensitivity to Ca$^{2+}$ (222, 271). These experiments confirm that the disease-causing mutations are dominant, increase Ca$^{2+}$ sensitivity, and suggest that hypertrophy arises in response to altered contractile function (271). Interestingly, transgenic rats expressing these mutations display cardiac hypertrophy, and expression of the Asp175Asn mutation, but not Glu180Gly, results in reduced Ca$^{2+}$ sensitivity of isometric force generation (344). The reason for the difference between studies is not clear.

Additional studies have found that the expression of Glu180Gly in mouse heart leads to an increased heart weight-to-body weight ratio and decreased expression of calcium-handling proteins associated with the sarcoplasmic reticulum (273). In addition, microarray analysis of transgenic versus control mouse hearts revealed 50 mRNA transcripts with altered gene expression, many of which encode proteins associated with the extracellular matrix (273).

There is general agreement that, at least for mutations studied in vitro, α-Tm mutations have only a small effect on cooperative changes in the Tm position on actin (138, 142). Rather, it appears that NH$_2$-terminal mutations influence interactions with actin and/or troponin that modulate Ca$^{2+}$ sensitivity (138). In the case of exon 6 mutations, Glu180Gly shows significantly reduced actin-induced stabilization compared with wild-type and Asp175Asn α-Tm (165). Whether this contributes to the more severe phenotype observed with Glu180Gly is unclear.

### B. Nemaline Myopathy

Nemaline myopathy is a muscle disease characterized by the presence of electron-dense rod bodies in skeletal muscle fibers. The rods are largely composed of α-actinin and actin. This is considered to be a disease of the thin, or actin, filament of skeletal muscle, and causative mutations have been detected in skeletal α-actin, Tm, nebulin, and troponin (48). Mutations in both the γ-Tm and β-Tm genes, but not the α-Tm gene, have been identified as responsible for this condition in humans.

Three mutations have been detected in the γ-gene encoding the slow α-Tm protein in patients with nemaline myopathy. The first, Met9Arg, was found to be inherited as an autosomal dominant mutation (171). This resulted in a late childhood-onset nemaline myopathy (171). Tan et al. (316) reported a much more severe case involving homozygosity for a nonsense mutation at codon 31. The patient therefore had no functioning α-Tm and, although showing no muscle weakness neonatally, died at 21 mo of age (316). In a surprising single compound heterozygous patient with intermediate severity nemaline myopathy, one γ-Tm allele carried a mutation converting the stop codon to a serine and the other allele carried a splicing mutation predicted to prevent inclusion of exon 9a (340). Finally, an atypical case of nemaline myopathy with an unusual distribution of muscle weakness was found to result from homozygosity for an Arg167His mutation in exon 5 of the γ-Tm gene (72). This is the only γ-Tm mutation detected in a constitutive exon found in all γ-Tm gene products. Two mutations have also been detected in
\(\beta\)-Tm (67). Both mutations are in exons also found in the cytoskeletal Tm1.

Comparison of the location of FHC and nemaline myopathy causing mutations (Fig. 4) shows they are spread through the length of the Tms. Because of the low number of patients, it is not clear if there is genuine clustering of mutations around specific sites in the proteins, although there is some indication this may be true for FHC mutations in \(\alpha_c\)-Tm (Fig. 4). FHC- and nemaline myopathy-causing mutations in \(\alpha_c\)-Tm and \(\alpha_s\)-Tm, respectively, have been compared in the context of gene transfer into adult cardiac myocytes. The \(\alpha_c\)-Tm mutations lead to hypersensitive \(Ca^{2+}\)-activated force production, correlating with other studies (see above), whereas the \(\alpha_s\)-Tm mutation produced a hyposensitive response (223). It is not clear, however, if the \(\alpha_s\)-Tm mutation would yield the same result in skeletal muscle.

A transgenic mouse model of nemaline myopathy has been generated by driving expression of a Met9Arg \(\alpha_s\)-Tm cDNA with the skeletal actin promoter (55). Nemaline rods were found in all muscles, but the extent of rod formation in different muscles did not correlate with the level of mutant protein in each muscle. Hypertrophy of fast glycolytic fibers was apparent at 2 mo of age, and muscle weakness was not detected until 5–6 mo, mimicking the late childhood onset observed in humans with this mutation. The onset of muscle weakness correlated with reduced muscle fiber diameter, suggesting that a failure of hypertrophy to persist reveals the intrinsic weakness in these muscle fibers (55). While in vitro protein analysis has suggested that the Met9Arg \(\alpha_s\)-Tm may compromise actin filament assembly (231), the normal assembly of thin filaments in the Met9Arg mouse suggests this may not be a major problem in vivo (55).

Analysis of skeletal muscle function in these Met9Arg mice failed to detect muscle weakness when performance was measured at optimum muscle length. At lengths below optimum, however, isometric force was reduced with greater impairment at decreasing length (63). This suggests a compromised mechanical performance of the thin filament. There was, interestingly, no evidence of altered calcium sensitivity as suggested by the cardiomyocyte experiment (63; cf. Ref. 223).

More recently, studies on the Met9Arg mouse have suggested that the mutation may compromise the formation of \(\alpha\beta\)-dimers (54). Expression of Met9Arg \(\alpha_s\)-Tm results in preferentially decreased levels of \(\beta\)-Tm in transgenic mice, and subsequent analysis of patient samples with \(\alpha_s\)-Tm mutations confirmed decreased \(\beta\)-Tm in both cases (54). In vitro Tm dimerization assays reveal preferential formation of \(\alpha\alpha\) rather than \(\alpha\beta\)-dimers in the presence of Met9Arg \(\alpha_s\)-Tm. Because of the poor actin binding capacity of \(\beta\beta\)-dimers, it is therefore proposed that the \(\alpha_s\)-Tm mutation promotes \(\alpha\alpha\)-dimer formation and more stable incorporation into the thin filaments. This would be expected to impact on thin filament performance (54).

C. Anti-Tm Antibodies in Human Disease

Autoantibodies have been implicated in the pathogenesis of ulcerative colitis, and a number of reports have detected Tm as a significant target for this activity. Das et al. (62) first reported that blood serum from 95% of patients with ulcerative colitis contains Tm-reactive antibodies. Subsequent studies have confirmed this observation and identified Tm5 and Tm1 as the primary target Tms in ulcerative colitis (24, 102). Anti-Tm antibodies have also been reported in a Japanese cohort with this condition (289). The expression of Tm5 in the ileal pouch following surgery for ulcerative colitis could also be related to the development of pouchitis (25). Das and coworkers have demonstrated that the elevated number of IgG-producing cells in the colonic mucosa of ulcerative colitis patients is largely committed to produce IgG against Tm5-related epitopes (253) and that Tm5 is capable of inducing a significant T-cell response in this condition (317).

The mechanism by which anti-Tm5 antibodies might impact on colonic cells is less clear. It has, however, been reported that Tm5 is located on the surface of colonic epithelial cells but not those of the small intestine (162). It is not clear in what state of organization the Tm5 is present nor whether it is as a result of some form of membrane shedding. There are also a number of reports of Tm on the surface of endothelial cells where it is implicated in transducing the anti-angiogenic signals of histidine-proline-rich glycoprotein (65, 115). A similar finding has been reported for the anti-angiogenic effects of HMW kininogen (362). This has led to the development of cell surface Tm as a potential target for cancer therapy (66).

Finally, Tm antibodies have also been reported in acute rheumatic fever (163) and the inflammatory disorder Behcet’s syndrome (208, 230). In both cases, the epitope appears to derive from \(\alpha_c\)-Tm (163, 208, 230). Whether these antibodies play a direct role in any of these human conditions or primarily reflect the high antigenicity of Tms released from compromised cells remains to be fully clarified.

VII. PROTEIN STRUCTURE AND FUNCTION

The increasing recognition that Tms play an essential role in a number of cellular processes and contribute to human disease has focused attention on the mechanisms of Tm function. This has involved both identification of general principles of Tm function and how function has been diversified through the generation of isoforms.
A. Dimer Formation and Composition

Muscle Tms show a marked preference for heterodimer formation. This was first established for smooth muscle Tm isolated from chicken gizzard, which is primarily composed of βα-heterodimer (157, 290). Comparison of end-to-end interactions reveals that the heterodimer forms much stronger polymers than either of the homodimers. This correlates well with enriched heterodimer association with actin filaments in vitro and suggests that this may provide the basis for preferred stable accumulation of heterodimers in vivo (157). Similarly, skeletal muscle α- and β-Tms prefer to form heterodimers (183). This appears to reflect the favored thermodynamic composition (183), and heterodimers form within minutes of mixing homodimers (184).

In contrast to muscle Tms, cytoskeletal Tms primarily form homodimers (195, 216). Elevated expression of Tm1 in transfected cells initially also results in homodimer formation, although over time heterodimers begin to accumulate (274). Gimona et al. (104) confirmed the preference of the HMW cytoskeletal Tms to form homodimers using overexpression of epitope tagged Tms. Cotransfection of smooth muscle α-Tm or striated muscle α- and β-Tms with HMW cytoskeletal Tms resulted in preferential formation of heterodimers. This suggests that heterodimer formation is intrinsic to muscle Tm structure and is dominant (104). Neither cytoskeletal nor muscle HMW Tms were able to heterodimerize with LMW Tms (104). The dimer status of LMW Tms is less clear. Initial analysis of rat cell lines indicated that both Tm4 and Tm5 existed as homodimers (216). However, the comigration of multiple Tm5 products from the α- and γ-genes makes it difficult to rule out heterodimerization between these products. Finally, cotransfection of tagged LMW Tms has demonstrated the potential for Tm5NM1 and Tm4 to form heterodimers with each other and with Tms 5a and 5b. Tm5a and Tm5b cannot, however, form heterodimers with each other (318). Thus, although the diversity of cytoskeletal Tms suggests the possibility of forming a very large number of different heterodimers, this does not appear to be realized in vivo.

B. Cooperative Binding of Tm to Actin

Tm binding to actin presents an interesting paradox. The intrinsic affinity of a single Tm dimer for an actin filament is very low. Indeed, X-ray diffraction studies show that the closest distance between carbon atoms in Tm and actin is 10.5 Å (204). Tm therefore appears to “float” in the major groove of the actin polymer, relying on ionic interactions to stabilize the Tm-actin interaction (see Ref. 263). But rather than individual Tm-actin interactions, it is the ability of Tm to form a polymer along the major groove in the filament which provides the high affinity and stability of the interaction (263). Indeed, evidence suggests that in the absence of sufficient available actin filaments, Tms are unstable and are degraded (337). This suggests that Tm cannot accumulate and form stable intracellular structures independent of actin (337).

In low ionic strength conditions in vitro, Tm can form long polymers involving 8–9 aa overlaps of NH2 and COOH termini (220). This polymerization of Tm explains the cooperative binding of Tm to actin filaments. NH2-terminal acetylation is crucial for cooperative binding of muscle Tm to actin (324). Similarly, it is to be expected that the choice of NH2- and COOH-terminal exons greatly influences the strength of actin binding (232). Indeed, the last 9 aa of exon 9a have been identified as determinants of the strength of actin binding of muscle Tm (124).

The cooperative effect of Tm binding to actin is not, however, simply a function of forming a continuous Tm polymer in the major groove of the actin filament. Tobacman and co-workers (347) have shown that cooperative interactions between adjacent troponin-Tm complexes remain when using nonpolymerizable Tm. Rather, this cooperativity involves troponin-Tm-induced conformational changes in the actin molecules in the actin filament (41). When the calcium concentration is varied, the interaction of a troponin-Tm actin filament with a myosin head changes in a coordinated manner, suggesting that the entire filament acts as a single conformational unit (91). X-ray studies have confirmed the intrinsic flexibility of the polymer and show that while each Tm can only mediate interactions with adjacent molecules, it is the regular repeating nature of the filament that allows the entire filament to behave cooperatively (265). This correlates well with the observation that for cooperative high-affinity binding to actin, Tm must be a coiled coil along its entire length and must contain an integral number of repeats corresponding to a whole number of actin monomers (145).

The picture that emerges for Tm binding to actin is one in which multiple interactions facilitate the coordination of the conformational state of the whole polymer. The NH2- and COOH-terminal overlaps influence Tm binding in an isoform-specific manner (124, 182, 232, 258). The actin-Tm interaction coordinates the conformational state of all actin monomers in contact with the same Tm (41, 264). Interactions between actins may also promote cooperative conformational changes, independent of Tm (4).

Virtually all studies of the actin-Tm interaction have focused on muscle isoforms and their relevance to myosin mechanochemistry. In contrast, the binding of cytoskeletal Tms to actin filaments is less well characterized and, given the diversity of isoforms, presents a substantial challenge for the future.
C. Emerging View of Tm’s Role in Muscle Contraction

The development of a more cooperative view of Tm-actin interaction has been accompanied by an evolving view of the mechanism of Tm regulation of muscle contraction. The historical view that Tm sterically blocks myosin access to the actin filament was supported by electron microscopy (181), X-ray diffraction (2), and cryo-electron microscopy (355). Although the movement of Tm associated with myosin binding has been consistent with the “steric blocking” model (8, 43, 58, 244, 270), it has not been established whether the movement of Tm directly causes the myosin head to engage the actin filament (263). An alternative view has emerged that Tm movement in the filament functions as an allosteric switch that is modulated by activating myosin binding but does not function solely by regulating myosin binding (281).

The allosteric switch role has recently been extended by an alternative interpretation of the previous literature. The model incorporates a new view of the tropinin I-Tm-actin filament interaction that has recently come from studies which map myosin-actin interactions. Patchell et al. (257) have shown that the binding of tropinin I, or an inhibitory peptide derived from it, to actin is sufficient to dissociate bound myosin peptides. Surprisingly, there is no evidence that Tm alone can inhibit the binding of myosin peptides to actin filaments (256, 257). Rather, Tm appears to enhance the binding of tropinin I and myosin (256). It is therefore proposed that tropinin I directly imposes a conformational shift in actin that is transmitted by Tm to the other actin monomers in contact with that tropinin-Tm complex. Thus Tm acts to coordinate actin monomer structure within the actin filament. In this way, Tm can coordinate the myosin-actin interaction based on regulating the conformation of actin (264). In this model, it is tropinin I that regulates an allosteric switch that is facilitated and transmitted to adjacent actins by Tm.

In the broader context of actin filament function, it is becoming more likely that Tm will in general have a role in all actin filaments of facilitating the transmission of structural changes in actin along the length of a filament in addition to its role in regulating filament stability. Thus the binding of a variety of proteins to actin and/or Tm may have the capacity to coordinateally alter actin filament structure via the Tm polymer.

VIII. SPATIAL SEGREGATION OF TROPOMYOSIN ISOFORMS

A. Isoform Sorting Is Intrinsic to Tms

The first reports suggesting that Tm isoforms are sorted to different intracellular locations were made by Burgoyne and Norman (38, 39). They first reported that adrenal chromaffin cells express three different Tms, only one of which was associated with chromaffin granule membranes. This suggested that a Tm may be specifically involved in vesicle transport or tethering (39). They also observed that a Tm antibody recognizing two isoforms in brain stained the somatodendritic compartment of neurons but not axons (38). This suggested that the composition of actin filaments may differ between dendrites and axons.

These conclusions have been confirmed and extended to other systems. Biochemical fractionation has demonstrated that one or more products from the γ-gene but not α- nor β-genes is associated with a subset of vesicles budded from liver Golgi stacks in vitro (136). This has been confirmed by confocal and electron microscopy (136, 261, 262) and visualization of transfected Tms (261).

The original direct visualization of spatial segregation of isoforms was made by Lin and co-workers (194). Using one antibody which detects only HMW Tms and a second which detects only LMW Tms from the γ-gene, they observed that while both detected stress fibers, only LMW Tms were detected in ruffling membranes (194). A similar experiment in astrocytes also concluded that Tm isoforms are associated with different structures (99). These studies have been extended to a number of cell types with an increasing realization of the very finely regulated spatial segregation of Tms.

1. Neurons

Neurons display extensive compartmentalization of Tms (for a review, see Ref. 117). Had et al. (123) compared the location of Tm4 with that of the brain-specific TmBr3. They found that Tm4 was concentrated in the growth cones of cultured neurons. In vivo, Tm4 was enriched in areas containing growing neurites and after neuronal maturation was restricted to postsynaptic sites. In contrast, the induction of TmBr3 was associated with its location in presynaptic terminals (123).

The segregation of Tms in neurons both in vivo and in vitro has been followed up in great detail. Hannan et al. (129) visualized the localization of Tm5NM1/2 protein and mRNA. They found that the mRNA encoding Tm5NM1/2 was localized to the axonal pole of differentiating embryonic rat neurons. In contrast, the mRNA encoding TmBr2 was excluded from the axonal pole and distributed throughout the remainder of the cell body. The corresponding Tm5NM1/2 protein was located in growing axons. Subsequent studies have shown that Tm5NM1 is localized to the outer region of the growth cone and filopodia, whereas Tm5NM2 is primarily located in the axonal shaft (201) (Fig. 5).

Tm5NM1/2 undergoes relocation during neuronal maturation. Weinberger et al. (342) observed that coinci-
dent with the induction of TmBr3 expression and its localization to axons was the relocation of Tm5NM1/2 to the somatodendritic compartment. This relocalization was observed to take place over a 2-day period in both chick and rat cerebral cortex, suggesting that this is a highly conserved process (342). The observation suggests that Tm5NM1/2 is chased out of axons by TmBr3. In this context, it is notable that supertransfection of TmBr3 into B35 cells overexpressing Tm5NM1 leads to breakdown of Tm5NM1-containing stress fibers and relocation of actin to giant lamellipodia (37). In adult brain, the mRNA encoding Tm5NM1/2 localizes to the dendritic side of the cell body, whereas TmBr3 mRNA localizes to a crescent across the axonal pole of the neuron (128).

The generation of additional antibodies has led to an even greater appreciation of the sorting of Tms (119, 295). Tm5a/b was observed to localize to the growth cone of cortical neurons, but upon extended culture, the growth cones diminish in size, and Tm5a/b becomes excluded from this region (292). On the basis of temporal characteristics and drug sensitivities, it was concluded that the axon and growth cone contain at least three separate populations of actin filaments based on Tm isoform composition (292). Intrinsic to this observation is the conclusion that Tm isoforms must predominantly form homo-, rather than hetero-, polymers. In support of this, immunoprecipitation of brain microfilament populations with anti-Tm5NM1/2 and anti-TmBr3 antibodies, respectively, did not bring down TmBr3 in the former nor Tm5NM1/2 in the latter precipitations (37).

More recently, antibodies have been used to discriminate between three of the four different COOH termini found in γ-Tms (333). Staining in the hippocampus, cerebellum, and cortex reveals that these different isoforms can be sorted into different neuronal compartments. While both the exon 9c containing products from the α- and γ-Tm genes are late products induced during neuronal maturation, they show quite different intracellular sorting in neurons (333). Thus the Tm composition of neuronal microfilaments is subject to extensive spatial and temporal regulation.

FIG. 5. Tm isoforms Tm5NM1 and -2 segregate to different structures in neurons, fibroblasts, and skeletal muscle fibers. In neurons, Tm5NM1 is specifically located in the lamella of the growth cone, whereas it is restricted to stress fibers, and not the lamella, in a fibroblast. In skeletal muscle, it is located in the Z-line associated cytoskeleton. Tm5NM2, in contrast, localizes to the axon shaft in neurons and the Golgi in fibroblasts.
2. Epithelial cells

Xie et al. (353) used antibodies that recognized HMW Tms alone or both HMW and LMW Tms to localize these products in epithelial cells. They observed that only the antibody that recognized LMW Tms reacted with the terminal web of epithelial cells. Temm-Grove et al. (319) similarly observed that whereas all anti-Tm antibodies stain stress fibers in kidney epithelial cells, only antibodies that recognize LMW Tms localize to adhesion belts. This was verified using transfected, epitope-tagged Tm isoforms (319).

Subsequent studies have confirmed that HMW Tms are restricted to the basolateral region of the mature epithelium in the gut (262), whereas LMW Tms from the α- and γ-Tm genes are enriched in the apical region (61, 262). In the T84 colon cancer epithelial cell line, the HMW Tms localize to the basolateral membranes, the LMW γ-Tms localize to the central cytoplasm, and Tm5a/b are the only isoforms found at the apical surface (61).

O’Harra and Lin (248) have found that during Cryptosporidium parvum infection, Tm5NM isoforms but neither Tm1 nor Tm4 colocalize at the infection site with a novel parasite membrane protein. Furthermore, infection was enhanced with overexpression of Tm5NM1. This suggests that localized accumulation of a specific Tm at the infection site may facilitate parasite invasion (248).

3. Fibroblasts

Tm isoform sorting in fibroblasts is subject to cell cycle regulation. In early G1, the HMW Tms are sorted into newly forming stress fibers, whereas LMW γ-Tms are located in a perinuclear zone (262). As cells move through G1, the LMW γ-Tms are recruited into the stress fibers. One γ-Tm (Tm5NM2), however, is not recruited but remains associated with the Golgi. Immunoelectron microscopy confirmed that Golgi-associated vesicles are attached to actin filaments containing Tm5NM2 (261). The sorting was confirmed by transfecting tagged Tms into fibroblasts. Tm5NM1 was sorted preferentially to stress fibers and Tm5NM2 to the Golgi (261) (Fig. 5).

Recent analysis has revealed that in primary mouse embryo fibroblasts, Tm5a/b are the only Tms specifically located in ruffling membranes (295). Surprisingly, Tm5NM1 is restricted to the more central regions of stress fibers (Fig. 5), and an exon 9a containing LMW Tm from the γ-Tm gene is located in a perinuclear compartment (295). Thus the generation of new Tm-specific antibodies has added extra detail to the segregation of Tms in fibroblasts.

4. Skeletal muscle

Although original studies failed to detect sorting of Tms in newly forming skeletal muscle myotubes (127, 196), studies of mature muscle have shown sorting of cytoskeletal Tm to a nonsarcomeric compartment (161). Tm5NM1 was found to be expressed in all skeletal muscles examined and to be localized in stripes running along either side of the Z-line (161) (Fig. 5). Colocation with γ-actin implicated Tm5NM1 in the formation of a Z-line adjacent cytoskeleton. Ectopic expression of the cytoskeletal Tm3 in transgenic mouse muscle resulted in localization of Tm3 to this same compartment (161).

Rubin and co-workers (211) have demonstrated the existence of a specific Tm associated with acetylcholine receptor clusters (211) in cultured chick muscle cells. They similarly detected a cytoskeletal Tm associated with the rat neuromuscular junction (211). The precise identity of this Tm isoform has yet to be unambiguously identified.

5. Osteoclasts

Tm isoforms have been shown to segregate to functionally distinct actin filament populations in osteoclasts (221). Tm4 and Tm5a/b are specifically enriched within and around the osteoclast attachment sites, the sealing zone, and podsomes. In podsomes, Tm4 and Tm5a/b differ in their specific organization, with the former associated with the actin core and the latter in rings surrounding individual podsomes. In contrast, neither Tm2 and -3 nor Tm5NM1 are abundant in attachment structures but are rather more diffusely distributed throughout the cell. Costaining revealed, however, little colocation of the HMW Tms 2 and 3 with Tm5NM1 (221). The observed compartmentalization is entirely consistent with specific roles for these Tms in specializing the function of discrete actin filament populations.

In conclusion, there is no longer any doubt that Tm isoforms are segregated to spatially distinct actin filament populations. It is likely that all cells contain multiple distinct actin filament populations defined by their Tm isoform composition. Intrinsic to this observation is the conclusion that isoforms must primarily form both homodimers and homopolymers in the environment of a normal unmanipulated cell. It is therefore appropriate to think of the actin cytoskeleton as a multifilament system composed of a set of distinct types of actin filaments defined by their Tm composition.

B. Regulation of Sorting

The sorting of Tms to discrete intracellular locations is subject to temporal regulation. Had et al. (123) were the first to report developmental regulation of isoform sorting. They observed that Tm4 was localized to the growth cone of growing neurons but was relocated to the somatodendritic compartment in mature neurons. A similar observation was made for Tm5NM1/2 (342). Schevzov...
et al. (292) found that Tm5a/b was relocated from growth cones with time in culture.

Sorting is also subject to regulation during the cell cycle. In particular, the HMW products from the α- and β-genes and the LMW products from the γ-gene are virtually mutually exclusively segregated in early G₁ (262). As G₁ progresses, there is increasing colocation of the HMW and LMW isoforms. Treatment of cells containing colocalized LMW and HMW Tms with cytochalasin D results in selective loss of HMW Tms from stress fibers. This is most easily reconciled with colignment of different homopolymers in a stress fiber (262). Thus, even when isoforms appear colocalated, it may be that the isoforms are in different individual filaments.

C. Mechanisms of Sorting

The sorting of Tm proteins in neurons is paralleled by sorting of their mRNAs (117). It was originally observed that the mRNA encoding Tm5NM1/2 was sorted to the pole of the neuron elaborating an axon prior to morphological differentiation (129). In contrast, the mRNA encoding TmBr2 was excluded from this pole and was located throughout the remainder of the cell body (129). The localization of Tm5NM1/2 mRNA correlated with that of the corresponding proteins in the axon hillock and axon (129).

The relationship between protein and mRNA sorting is further supported by two additional observations. First, the relocation of Tm5NM1/2 from the axonal compartment to the somatodendritic compartment is accompanied by a shift in location of the mRNA from the axonal pole to the dendritic side of the neuronal cell body (128). Second, the mRNA encoding axonal TmBr3 is located to the axonal pole of the cell body (128). This provides strong support for a direct relationship between mRNA and protein localization, although it is important to note that there is not an absolute correlation between mRNA and protein location (129).

The relationship between mRNA and protein location has been tested in transgenic mice. Mice were created in which the coding regions of Tm5NM1 and Tm3 were expressed under the control of the β-actin promoter with a β-actin 3′-untranslated region lacking targeting information. Tm3 was quite broadly distributed throughout neurons, whereas Tm5NM1 was localized with absolute specificity to its normal location, the growth cone (291). This suggests that the protein itself contains targeting information independent of mRNA synthesis. It therefore seems most likely that mRNA location may facilitate sorting but is not absolutely required for appropriate sorting of the protein.

Tm isoform sorting is influenced by the actin isoform composition of microfilaments. Overexpression of γ-actin in myoblasts results in downregulation of β-actin and elimination of Tm2 but not Tm5 from stress fibers (294). This suggests that sorting is influenced by the ability to assemble into polymeric structures. Exposure of cells to cytochalasin D, which results in disorganization of the actin cytoskeleton, eliminates sorting into growth cones (292) and the apical surface of epithelial cells (61). Wash-out of cytochalasin D reestablishes sorting, which is consistent with a direct relationship between sorting and incorporation into organized arrays of actin filaments (292). Studies in epithelial cells further reveal that sorting of Tm5a/b to the apical surface does not require microtubules, nor does it require breakdown of preexisting actin filaments (61).

The mechanisms responsible for sorting therefore appear to be intrinsically flexible and dynamic. Because sorting is developmentally regulated, there is no indication that sorting is geographically programmed in the proteins (Fig. 5). Rather, isoforms appear to accumulate at specific intracellular sites that depend on the structural integrity of actin filaments at that site (61, 292). The Tms also display a capacity to differentially compete for inclusion into specific regional structures. Elevated expression of Tm5NM1 in B35 cells can chase HMW Tms out of stress fibers (37). These experiments are most compatible with a molecular sink model in which accumulation of a Tm at a specific site relies on preferred, stable association with actin at that site. Variables that may provide discrimination between isoforms at a specific intracellular site could include actin isoform type, actin filament dynamics, capping proteins, actin binding proteins, and types of myosins. The activities of a number of these proteins are in turn linked to signaling reactions at that site.

Comparison of the sorting of Tm5NM1 and -2 has demonstrated that a single exon can direct Tms to quite different destinations. Tm5NM1 preferentially localizes to stress fibers in NIH3T3 cells, whereas Tm5NM2 is found in the Golgi (261). The only difference between them is the inclusion of exon 6a for Tm5NM1 and exon 6b for Tm5NM2 (Fig. 1). It is possible that this exon choice alters actin binding. Comparison of the impact of exons 6a and 6b in the context of the α-Tm revealed that exon 6s differentially impact on actin affinity but not filament assembly (125). Such a difference may directly contribute to differential location in the cell.

A combination of mRNA location, different actin affinity and filament assembly kinetics, and combinatorial interactions with actin and actin binding proteins may provide sufficient flexibility to generate the sorting observed in vivo (see Ref. 119 and discussion in Ref. 319). The combinatorial interactions are not only likely to generate isoform sorting but also to ensure functional diversity of the resulting filament populations, and this is integrated in section xA and Figure 6.
The field has, in some ways, come full circle. The original studies of Tm isoforms were focused on protein chemistry and differential protein-protein interactions. It now seems increasingly likely that these original studies can provide insights into Tm isoform sorting and the functional differences between different actin filament populations.

IX. TROPOMYOSIN-DIRECTED REGULATION OF ACTIN FILAMENT DYNAMICS, ORGANIZATION, AND MECHANO CHEMISTRY

A. Tm Regulates Actin Dynamics

Tm inhibits both the rates of polymerization and depolymerization of actin (172). Inhibition occurs at the level of both spontaneous polymerization of ATP-actin and the rates of monomer addition and loss from actin filaments (172). The rates of elongation per filament are not altered by Tm; rather, it appears that the inhibition of polymerization arises because Tm mechanically stabilizes the filaments resulting in fewer filament ends available for polymerization (146). Tm also lowers the off rate constant from the pointed end of actin filaments (35) without preventing elongation (33).

The most dramatic impact of Tm on actin filament dynamics has been reported in yeast (278). When yeast containing a temperature-sensitive mutation in Tm are shifted to the restrictive temperature, actin cables disappear within 1 min. Conversely, return to the permissive temperature results in reformation of Tm containing actin cables in polarized arrays within 1 min (278). This serves to emphasize the crucial role Tm plays in the stable formation of large arrays of actin filaments.

The suggestion that Tm regulation of actin dynamics may regulate filament length (33) has been confirmed in striated muscle (199). Inhibition of pointed end dynamics of actin thin filaments in sarcomeres was achieved by overexpression of GFP-tagged tropomodulin. Under normal circumstances, tropomodulin binds to the pointed end of actin filaments.
end of actin filaments via direct interactions with both actin and Tm (85). Disruption of these interactions leads to shorter thin filaments, implicating the collaboration between Tm and tropomodulin in the regulation of filament length (199). This conclusion has subsequently been confirmed with gene knockout of tropomodulin in mice (93).

Tm plays an important role in actin filament dynamics at the leading edge of the cell. Blanchoin et al. (28) observed that Tm inhibits actin filament branching and nucleation by the Arp2/3 complex. DesMarais et al. (64) integrated these data with the observation of diminished Tm in the leading edge of migrating cells to propose that the absence of Tm allows Arp2/3-driven branching under the membrane (for review, see Ref. 53). However, the use of a wider range of Tm antibodies suggests this absence of Tms at the leading edge only applies to a subset of isoforms (37, 295). Hillberg et al. (143) have used both antibodies and tagged Tms to demonstrate that Tms are indeed present out to the edge of lamellipodia.

Gupton et al. (122) used microinjection of muscle α-Tm into epithelial cells to test the ability of Tm to disrupt actin dynamics at the leading edge. They found that α-Tm diminished Arp2/3 and ADF/cofilin levels at the leading edge and inhibited formation of functional lamellipodia. Surprisingly, the velocity of cell migration was increased, as was the persistence of leading edge protrusion (122).

In yeast, the turnover of actin cables containing Tm can be regulated by the coordinate activity of Aip1 and cofilin (250). These molecules function together to “prune” Tm-containing cables. This model is supported by the ability of Aip1 deletion to rescue loss of actin cable defects in Tm deletion mutants. Thus Aip1 and cofilin both function as antagonists of Tm stabilization of actin filaments (250).

It may therefore be concluded that Tm has the capacity to regulate actin filament dynamics both in terms of the pointed end off rate and filament branching. The stoichiometry of actin:Tm:tropomodulin is a likely regulator of average filament length. There is, however, little data concerning isoform-specific regulation of these processes.

B. Isoform Differences in Tm Binding to Actin

Tm isoforms differ in their affinity for actin, and this appears to correlate with changes in actin organization in transformed cells. Three papers published from two groups around the same time concluded that HMW Tms have a higher affinity for actin than LMW Tms. Chick embryo fibroblast LMW Tms 3a and 3b (equivalents of mammalian Tm4 and Tm5) were found to have reduced actin affinity in 100 mM KCl compared with the HMW Tms 1 and 2 (195). Because Tm1 was greatly reduced in transformed cells and actin cables are also reduced, it was concluded that the loss of high-affinity Tm1 may cause the decrease in actin cables (195).

A similar conclusion was drawn from studies of rat fibroblasts. LMW Tms were shown to have less capacity to form head-to-tail polymers and lower affinity for actin than HMW Tms (216). This correlates with reduced levels of HMW Tms and poor actin organization in transformed cells (216). Additionally, it was found that HMW Tms were more resistant than LMW Tms to dissociation from actin in the presence of an actin bundling protein (217). In both these studies, the primary Tms were most likely Tm1, Tm2, and Tm3 as HMW Tms and Tm4 and Tm5NM1 as LMW Tms. Later studies, which include other LMW Tms, revealed that a simple relationship between Tm size and actin avidity was not correct.

LMW Tms isolated from microvilli preparations from rat mammary adenocarcinoma cells display differences in actin binding (202). One of the LMW Tms displays similar actin binding properties to that of HMW muscle Tm, whereas another LMW Tm binds more weakly to actin. This indicates that size is not the sole arbiter of actin affinity and that Tms from a common intracellular location can have different affinities for actin (202). Broschat and Burgess (34) compared the physical and functional properties of LMW Tms prepared from intestinal epithelium and brain. Brain Tm had a 10-fold lower $K_a$ for binding to actin than that of epithelial Tm. However, both Tm preparations had similar reduced head-to-tail polymerization compared with muscle Tm. Erythrocyte LMW Tm has similar actin affinity but lower polymerizability than muscle Tm (210). Tm5NM1 has higher avidity for actin than Tm3 but lower cooperativity of binding, most likely reflecting a difference in head-to-tail polymerization (341). Finally, the LMW Tm5α/b has the greatest avidity for actin binding of all studied Tms (232). The determinants of these differences in actin binding are not understood at a molecular level but are likely to involve cooperativity of Tm binding, actin-Tm interactions, and head-to-tail interactions of Tms.

The intrinsic differences in actin binding of Tm isoforms need to be considered in the light of more complex interactions occurring in any cell. Pittinger et al. (269) demonstrated that whereas the isoforms differ in actin affinity, this is modulated by the presence of both other Tms and actin binding proteins. Caldesmon, for example, provides a greater enhancement of Tm5NM1 binding to actin compared with Tm3 (341). Lehman et al. (180) have shown that Tm isoforms differ in their location in the major groove of the actin filament, which may in turn influence their competitive or cooperative interactions with actin binding proteins.

The intrinsic differences in actin binding and the differences in interactions with other actin binding pro-
teins suggest a possible mechanism that could preferentially segregate the isoforms to different intracellular sites. Intrinsic to this is the corollary that by virtue of the differences in actin binding protein interactions, the different actin filaments would be expected to be functionally quite distinct.

C. Tms Regulate Actin Interactions
   With Binding Proteins

   It is becoming increasingly clear that Tms do not simply regulate the properties of actin filaments in an independent manner. Rather, the Tms collaborate with a range of actin binding proteins, in an isoform-specific manner, to form complexes with differing functional output. We consider five examples that highlight the collaborative nature of these interactions.

1. Myosin

   Given the role Tm plays in regulating the actin-myosin interaction in striated muscle (see above), it was not unexpected that a similar relationship would exist in the cytoskeleton. Fanning et al. (82) demonstrated that Tms differ in their ability to inhibit both the Mg-ATPase activity and translocation of actin filaments by muscle myosin II. Indeed, Xenopus Tm4 was found to stimulate, rather than inhibit, myosin II activity. In contrast, all Tms inhibited to a similar extent both the Mg-ATPase and translocation activity of brush-border myosin I. This provided two important insights: 1) Tm isoforms can differ in their interaction with the same myosin motor, and 2) the same Tm can stimulate one motor and inhibit another. A possible molecular explanation was provided by Lehman et al. (180), who observed that different Tms can occupy different positions in the major groove of the actin filament. This, in turn, may account for differential access of the same myosin motor to filaments containing different Tms.

   Direct visualization of isoform-specific recruitment of myosin motors was observed by Bryce et al. (37) in Tm-transfected neuroepithelial cells. Elevated expression of Tm5NM1 resulted in recruitment of myosin IIA, but not IIB, to stress fibers, but of both IIA and IIB to the edges of the cell. Coincidentally, myosin II activity levels were also substantially elevated by Tm5NM1. Recruitment of myosin IIA into dendrites was also observed in transgenic mice expressing Tm5NM1 in the dendrites of cortical neurons (37). Finally, Tm5NM1 was found to increase recruitment of myosin IIB into the growth cones of primary neurons (37, 291). In contrast, elevated expression of the neuron-specific TmBr3 in neuroepithelial cells resulted in decreased activity of myosin II (37).

   These results are most easily reconciled with a model in which collaboration between Tms and myosins creates isoform-specific higher order complexes with unique functional capacities. Differences between the affinities of actin-Tm, actin-myosin, and myosin-Tm and its regulation by myosin activity could provide sufficient specificity to favor formation of isoform-specific complexes.

2. Tropomodulin

   Tropomodulin was initially identified as the pointed end cap of erythrocyte actin filaments which also binds to Tm (89). Localization studies subsequently demonstrated that tropomodulin, Tm, and actin filaments colocalize on the plasma membrane of erythrocytes and lens fiber cells (325, 350). Tropomodulin binds to both the NH2 terminus (aa 7–14) of Tm (328) and to the pointed end of actin filaments. This is accomplished using two separated domains; the NH2 terminus binds Tm and the COOH terminus binds actin (94). The binding has two distinct effects on the actin filament. The binding to actin prevents elongation of the actin filament at the pointed end, and the binding to Tm prevents depolymerization of actin from the pointed end (234).

   The stoichiometry of tropomodulin to Tm has the potential to regulate the average length of actin filaments as originally noted in the comparison of tropomodulin levels in the erythrocyte and lens fiber cell (325, 350). Subsequent manipulation of tropomodulin levels has confirmed that the precise stoichiometry of tropomodulin and Tm is required to maintain correct organization and function of the contractile system in the heart (310). Knockout of tropomodulin in mice leads to failed cardiac development and associated embryonic lethality (93).

   Erythrocyte tropomodulin differs in its affinity for different Tm isoforms. Perhaps not surprisingly, its highest affinity is for the erythrocyte Tm5 (309). Comparison of binding to Tm5a, αTm, and Tm2 revealed that the latter two HMW Tms had the lowest affinity for erythrocyte tropomodulin (164). Similarly, neuronal tropomodulin was found to bind to the LMW TmBr3, Tm5a, and Tm5NM1 but not to the HMW Tm2 and TmBr1 (338). The preferred binding presumably reflects the similarity of the NH2 termini of LMW Tms and the substantial difference to that region of HMW isoforms (Fig. 1).

   The isoform preferences of tropomodulins and Tms provide an opportunity to tailor the dynamics of the pointed ends of actin filaments (85). In particular, the absolute levels of specific tropomodulins and Tms and the local association of these Tms with actin filaments would be expected to collaborate to regulate the average length of filaments at that site.

3. Actin depolymerizing factor/cofilin

   Actin depolymerizing factor (ADF)/cofilin was originally isolated as a factor which promotes the depolymerization of actin filaments (20, 245) and has been the
subject of a recent review (14). ADF competes with Tm for binding to the actin filament (20, 245), providing a mechanism for promoting depolymerization of actin in the presence of Tm. Ono and Ono (252) have explored a mechanism for promoting depolymerization of actin in the presence of Tm. This mechanism involves the binding of ADF to the actin filament, whereas myosin moves Tm in the opposite direction (20, 245), providing a similar mechanism to link local signaling activity to the assembly of actin filament complexes into long actin filaments (247).

The relationship between Tm and ADF/cofilin is likely to involve more facets than simply antagonism. Bryce et al. (37) observed Tm isoform-specific impact on ADF/cofilin activity and location. Tm5NM1 overexpression results in decreased ADF activity and absence of ADF from the cell periphery. In contrast, TmBr3 recruits ADF to the lamellipodial regions of the cell. Coimmunoprecipitation and colocalization studies confirm that ADF and TmBr3 coexist on the same actin filaments (37). The observation that ADF/cofilin binding to actin filaments changes the twist of the filament provides a possible explanation of how it may influence Tm binding in an isoform-specific manner (219).

The regulation of ADF/cofilin activity provides a mechanism to link local signaling activity to the assembly of isoform-specific Tm-containing actin filaments. Several pathways regulate the active state of ADF/cofilin via kinase and phosphatase activity (14). Local changes in such pathways will impact on ADF/cofilin activity, which in turn will influence Tm association with actin in an isoform-specific manner.

4. Caldesmon

Caldesmon was originally identified as a cross-linker of actin filaments regulated by calcium-calmodulin (301). Caldesmon colocalizes with Tm distribution along stress fibers (32, 357). Subsequently, caldesmon was shown to cooperate with Tm in the inhibition of myosin II activity in smooth muscle (302). Marston and co-workers (3, 92, 212, 213) have shown that caldesmon binding to an actin-Tm filament switches the filament to the “off” state with regard to myosin II engagement. Calcium-calmodulin binding to caldesmon can restore myosin binding (213). The mechanism of caldesmon function is indistinguishable from the action of the troponin complex (92, 212). Consistent with these data, caldesmon has been shown to move Tm in one direction in the groove of the actin filament, whereas myosin moves Tm in the opposite direction (110, 168). In colonic smooth muscle, acetylcholine-induced phosphorylation of both caldesmon and HSP27 collaborate to release caldesmon from Tm and promote movement of Tm to expose myosin binding sites on actin (304).

The nonmuscle isoform of caldesmon differentially collaborates with Tm isoforms in nonmuscle cells (357). Caldesmon stimulates the binding of LMW Tms to actin to the extent that their binding is indistinguishable from that of HMW Tms. This binding occurs in a calcium/calmodulin-dependent manner (357). In addition, caldesmon collaborates with both HMW and LMW Tms to block gelsolin-directed severing of actin filaments (156) and to anneal gelsolin-severed actin filaments (155, 247). Transfection of caldesmon into CHO cells which only express LMW Tms stabilized actin filaments containing Tm (336). This is consistent with a role for caldesmon in the enhanced binding of LMW Tms to actin filaments.

Caldesmon contributes two important characteristics to actin filaments. First, it has the capacity to collaborate with Tms in an isoform-specific manner to regulate actin filament structure and function. Second, its collaboration with Tm is calcium-calmodulin regulated. This provides a mechanism which links local calcium levels with the assembly of functionally distinct actin filaments at specific locations.

5. Gelsolin

The severing activity of gelsolin against actin filaments is intrinsically inhibited by Tm (83). Ishikawa et al. (155, 156) found that only HMW Tms partially protect actin filaments from severing by gelsolin. Surprisingly, LMW Tms provide no protection, even when they fully saturate the actin filament. Caldesmon collaborates with both LMW and HMW Tms to provide enhanced resistance to gelsolin (156). Caldesmon also collaborates with Tm to inhibit gelsolin-induced activation of the myosin ATPase (59). HMW, but not LMW, Tms are also capable of annealing gelsolin-severed actin filaments (155). This action of Tm is highly efficient in turning the smallest gelsolin-actin complexes into long actin filaments (247).

The view that emerges is a series of collaborative interactions that depend on local signaling, the activity of actin binding proteins, and the presence of specific types of Tm to build actin filament complexes with specific functional activity.

X. CONCLUSION: TROPOMYOSINS AS SPATIAL REGULATORS OF ACTIN FILAMENT FUNCTION

A. What We Think We Know

Tm performs one or more essential functions in organisms as diverse as yeast and humans. In all of these

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organisms, at least one of these essential functions involves the role of the actin cytoskeleton in maintaining cell viability. In addition, muscle Tm is essential for the function of the sarcomere in all striated muscles. The evolutionary diversification of actin cytoskeletal function has been paralleled by increased diversity of cytoskeletal Tms resulting from multiple genes and increasingly via alternative splicing. There is no evidence of redundancy among Tm genes (with the exception of yeast), although there may be some redundancy of isoforms encoded by the same gene. It therefore appears that specific Tms are associated with unique and essential functions of both the actin cytoskeleton and the actin filament in the sarcomere.

Tm isoforms are spatially segregated to distinct actin filament populations in a wide range of cell types. Tms may regulate both the quantity and the qualitative properties of these separate actin filament populations. The different Tms display a range of activities in terms of actin-binding affinities and interactions with actin-binding proteins. The segregation of Tms in turn suggests that these different populations of actin filaments will be functionally distinct because of the different properties of the different Tms.

The mechanisms of Tm segregation into distinct subcellular locations and the assembly of functionally distinct populations of actin filaments may be ultimately the same process. Because the type and activity of signaling pathways are spatially discrete, in any cell the activity of actin binding proteins will be similarly varied and result in the assembly of site-specific complexes. An example is shown in Figure 6 based on the findings of Bryce et al. (37). At sites where LimK1 is active, ADF/cofilin is phosphorylated and will not compete with Tm for binding to actin. If MLCK also phosphorylates myosin light chain, the assembly of Tm5NM1-containing actin filaments, which engage active myosin II, will be promoted and in turn will promote stress fiber formation. In contrast, if LimK1 is inactive, ADF/cofilin will compete with some Tms, but collaborate with TmBr3 to form actin filaments containing ADF plus TmBr3. The dynamic nature of the resulting filaments will promote formation of lamellae.

The observed segregation of actin isoforms may therefore simply reflect the preferential cooperative assembly of specific Tms at specific intracellular sites. This will also reflect formation of a unique complex with a specific functional outcome. In this way, isoform segregation and spatially segregated actin filament function may simply reflect the preferred affinities of actin, Tms, and actin binding proteins at that site. Isoform segregation may then reflect preferred assembly of specific isoforms at specific sites without the need to invoke complex transport mechanisms. This is readily reconciled with the sensitivity of isoform segregation to cytochalasin D and the intrinsic flexibility of the segregation observed in different cells.

Burgstaller and Gimona (40) have observed that the accumulation of cortactin and p190 Rho GAP at a specialized microdomain inhibits contractility at that site and correlates with the dispersal of HMW Tms. This is consistent with the model proposed in Figure 6.

B. What We Need to Know

The focus of Tm research is increasingly turning to a mechanistic understanding of how this family contributes to the diversity of actin filament function. Although there is strong evidence that Tm isoforms contribute to the formation of different actin filament-based functional complexes, there is little understanding of this process at a molecular level. Is the supply of Tm limiting the assembly of these complexes? Does Tm supply impact on actin polymerization and is the primary role of Tm to facilitate the assembly of the different functional complexes? Alternatively, do the Tms differ in the way they communicate conformational information along the length of an actin filament?

At a more specific level, the mechanism of segregation may involve directed transport or it may only be a function of local assembly of higher order complexes. If it is only the latter, why are the mRNAs targeted in neurons? Targeting mRNAs may reflect facilitation, rather than be an absolute requirement, for isoform segregation. Alternatively, if segregation is based on preferential sites of assembly of specific isoforms with local actin and actin binding proteins, can assembly take place without the appropriate Tm? If other Tms can contribute, do they differ in their capacity to incorporate into higher order structures at different sites? Underpinning these issues is really the key question. Why does the actin filament, especially in the cytoskeleton, need Tms? In addition to a direct practical role in regulating filament functional properties, we need to consider the possibility that Tm is required as a facilitator of complex formation.

Finally, while mutations have been observed in all three striated muscle Tms that lead to human disease, as yet there are no reported diseases caused by mutations in cytoskeletal Tms. Potentially this may reflect the toxicity of any mutation in a Tm which compromises cytoskeletal function or a failure to examine Tms in the appropriate human conditions. The finding that a number of muscle disease causing Tm mutations are also expressed in cytoskeletal Tms does not support the toxicity argument. The generation of Tm transgenic and knockout mice will provide the insight required to understand how Tms function in development and may identify candidate human conditions related to Tm dysfunction. The generation of muscular dystrophy in mice overexpressing Tm3 in a
cytoskeletal compartment is an example of such a finding (161). Similarly, conditional elimination of γ-actin in mouse muscle leads to a progressive myopathy (306). It therefore seems likely the cytoskeletal Tms may have the potential to contribute to a range of muscle diseases.

C. What Tms Offer Cell Physiology

At their simplest, the Tms provide a way of visualizing the organization and function of different actin filament populations. The availability of antibodies and epitope-tagged Tms provides increasingly valuable tools to visualize these populations. In addition, the fidelity of sorting of tagged Tms allows the live imaging of actin filaments containing different Tms.

The generation of mice both over- and underexpressing specific Tms will open the door to understanding the function of these specific actin filament populations. Importantly, this will allow for the analysis of these populations both in a whole tissue context and also in primary culture. This will inform our understanding of the actin cytoskeleton in cell physiology in a way that anti-actin drugs, because of their broad activity, have not been able to achieve.

In the future it may become possible to develop drugs that target the different cytoskeletal Tms. As we understand the functional role of these different actin filament populations, the value of such drugs will become substantial. At a minimum, such drugs may allow the actin cytoskeleton to be effectively targeted in chemotherapy strategies that spare the contractile apparatus of the heart and diaphragm. Indeed, it is the unsuspected specificity of the Tm composition of functionally distinct populations of actin filaments that makes Tms extraordinarily attractive targets for drug discovery. This will in turn provide particularly valuable tools for the dissection of the function of the actin cytoskeleton in cell physiology.

D. Conclusion

It is clear that dynamic regulation of the actin cytoskeleton plays a critical role in most, if not all, biological processes. The regulation of the actin cytoskeleton therefore requires precise spatial and temporal control. Together with other actin filament-regulatory molecules, the Tm family provides a unique and simple mechanism for regulating actin function. We predict that future investigation will reveal specialized Tm/actin assembly in most, if not all, biological processes that are critically dependent on actin.

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GRANTS

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