Isoform Sorting of Tropomyosins

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Abstract

Cytoskeletal tropomyosin (Tm) isoforms show extensive intracellular sorting, resulting in spatially distinct actin-filament populations. Sorting of Tm isoforms has been observed in a number of cell types, including fibroblasts, epithelial cells, osteoclasts, neurons and muscle cells. Different Tm isoforms have differential impact on the activity of a number of actin-binding proteins and can therefore differentially regulate actin filament function. Functionally distinct sub-populations of actin filaments can therefore be defined on the basis of the Tm isoforms associated with the filaments. The mechanisms that underlie Tm sorting are not yet well understood, but it is clear that Tm sorting is a very fluid and dynamic process, with changes in sorting occurring throughout development and cell differentiation. For this reason, it is unlikely that Tm localization is determined by an intrinsic sorting signal that directs particular isoforms to a single geographical location. Rather, a molecular sink model where isoforms accumulate in actin-based structures where they have the highest affinity, is most consistent with current data. This model would predict Tm sorting to be influenced by changes to actin filament dynamics and organization and collaboration with other actin-binding proteins.

Introduction

Mammalian tropomyosins (Tms) are encoded by four genes α, β, γ and δ. These genes undergo extensive alternative splicing to give rise to a large number of isoforms. These isoforms can be divided into two broad classes consisting of high molecular weight (HMW) and low molecular weight (LMW) isoforms through the use of alternative promoters (for more details, see Chapter 2). Tm isoforms have distinct expression patterns in various tissues and throughout development (see Chapter 4) and also show distinct patterns of subcellular localisation. Differential sorting of Tm isoforms was first observed in the 1980's and since then numerous examples of intracellular sorting of nonmuscle Tms have been described in a number of cell types. It is now clear that different Tm isoforms are able to specifically sort to distinct actin structures and subcompartments of cells. With the development of new anti-Tm antibodies and tagged constructs, more and more intracellular compartments defined by Tms are being identified and the functional consequences of this sorting is becoming better understood. The mechanisms that underlie this sorting process, however, are not well understood. In this review we will outline the sorting patterns of Tm isoforms in different cell types, examine how this sorting relates to specific cell functions and review the potential mechanisms that may account for Tm sorting.

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Sorting of Tm Isoforms in Specific Cell Types

Fibroblasts

The first direct evidence of differential Tm sorting in cells was obtained using double-label immunofluorescence in fibroblasts.[1] In chicken embryo fibroblast cells, both HMW isoforms of the α gene and LMW isoforms of the γ gene localize to stress fibres, whereas only LMW isoforms also sort to ruffling membrane regions. A similar pattern is seen in human EJ (bladder carcinoma) cells, with HMW isoforms again excluded from the ruffling membrane regions.[1] Cultured mouse embryonic fibroblasts also appear to exclude HMW isoforms from the cell periphery (Fig. 1).[2]

Tm isoforms can also sort to distinct actin structures in NIH 3T3 cells. Synchronised replated cells show differential sorting of isoforms 1h after replating, with γTm isoforms sorting to a peri-nuclear region and αTm isoforms sorting to peripheral stress fibres.[3] This difference in sorting becomes less distinct as cells progress through the G1 phase of the cell cycle, with both sets of isoforms localizing to stress fibres by 5h after replating. There are still some differences, however, with enrichment of the αTm isoforms at the cell periphery compared to γTm. Further studies in NIH 3T3 cells have indicated distinct sorting patterns for the γTm isoforms Tm5NM1 and Tm5NM2. While Tm5NM1 localizes to stress fibres, the WS5/9d antibody which preferentially recognises Tm5NM2 indicates Tm5MN2 sorts to perinuclear actin structures associated with the Golgi complex.[4]

In contrast to earlier studies, microinjection of labelled recombinant αTm isoforms into rat fibroblasts showed no differences in sorting between HMW isoforms and LMW isoforms.[5] All isoforms were incorporated into microfilaments and extended to the edges of the cells. Co-injection of HMW and LMW isoforms (e.g., Tm2 and Tm5a) showed no difference in localization. Tm5b did show fainter staining in microfilaments than the other isoforms, indicating this isoform is less able to incorporate into microfilaments, most likely a result of its lower affinity for actin. The lack of sorting identified in this study may be a result of bacterially-produced proteins which lack posttranslational modifications such as acetylation, or due to an over-abundance of the exogenous protein which is therefore not mimicking the endogenous sorting pattern.[5]
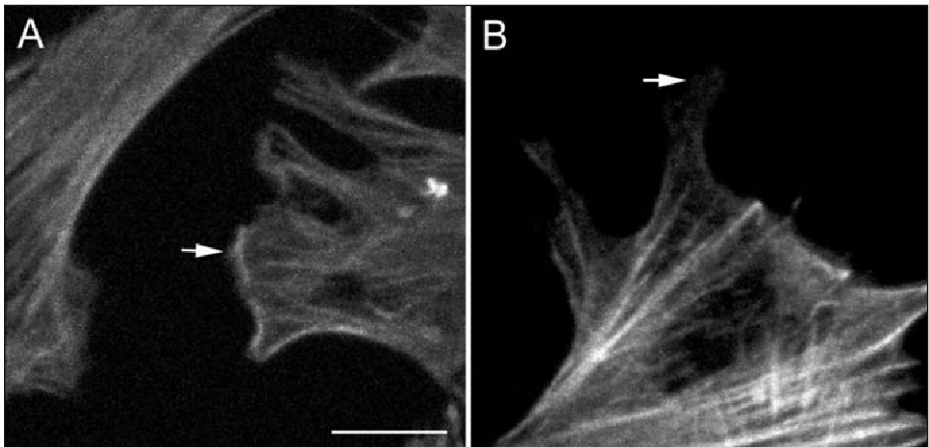

Figure 1. Tm isoforms are differentially sorted in fibroblasts. Mouse embryonic fibroblasts show differential sorting between LMW and HMW isoforms as shown by the antibodies αf9d which recognises isoforms 6, 1, 2, 3, 5a and 5b (A) and Tm311 which recognises isoforms 6, 1, 2 and 3 (B) HMW but not LMW (i.e., Tm5a/5b) isoforms appear to be excluded from the cell periphery. Scale bar, 20 μm.

Epithelial Cells

Distinct Tm sorting patterns have also been observed in epithelial cells. In T84 epithelial monolayers, the LMW isoforms Tm5a and Tm5b show enrichment at the apical membrane, while γTm isoforms and the HMW αTm isoforms are distributed throughout the cytoplasm.[6] This polarization of LMW Tm isoforms becomes more distinct with increasing differentiation. Apical/basal sorting also occurs in vivo, with human colon epithelial cells showing enrichment of HMW Tms at the basolateral surface and enrichment of Tm5NM1/2 at the apical surface.[3]

Differences in Tm localization have been observed in adhesion belts and stress fibres in LLC-PK1 epithelial cells. The LMW Tm5a/5b localize to both stress fibres and adhesion belts, while the HMW αTms localize to stress fibres only.[7] Products of the γ gene also localize to adhesion belts. When exogenous isoforms are transfected into the cells, HMW isoforms are again restricted to the stress fibres, while Tm5a/5b localize to both stress fibres and adhesion belts. Like the HMW αTm isoforms, Tm4 is excluded from adhesion belts, however this isoform binds only weakly to stress fibres.[7]

Studies of *Cryptosporidium parvum* invasion in epithelial cells have shown specific rearrangement and localization of Tm isoforms in response to invasion. In HCT-8 cells infected with *C. parvum*, γTm isoforms accumulate at the infection sites, but Tm4 does not.[8] This localization of γTm is associated with an accumulation of actin filaments at the infection sites. In CHO cells, γTm again specifically localizes to infection sites, as does Tm4 in some cases. A similar pattern of γTm accumulation is also seen in *C. parvum* infected mice in vivo. A CHO cell line stably overexpressing hTm5NM1 is more readily infected by the parasite than a line expressing a hTm5/3 mutant. This data, taken together, suggests that the functional Tm5NM1 isoform may enhance bacterial invasion.[8]

Osteoclasts

Another type of adhesion structure is seen in bone-resorbing osteoclasts. These cells show distinct sorting of Tm isoforms to the podosome attachment structure. Tm4 and the LMW isoforms Tm5a/5b are enriched in the podosomes, whereas Tm5NM1 and the HMW isoforms Tm2/3 are relatively excluded from these structures.[9] Within the podosome structure there is more specific sorting, with Tm4 being enriched at the upper surface and less so at the ventral plasma membrane, while Tm5a/5b encircle the podosomes at the base of the cell and are enriched in the upper and outer edges of the actin ring. While both Tm5NM1 and Tm2/3 are present in the cell interior, there is little colocalization between these isoforms.[9] Thus, at least four isoform specific structures are present in osteoclasts.

Neurons

Distinct and tightly regulated sorting patterns have been observed in neurons and Tm sorting in these cells has therefore been extensively studied. Intracellular localization of Tm in neurons was first studied by Burgoyne and Norman[10] who found an antibody to chicken gizzard Tm showed enriched staining in cell bodies and dendrites compared to axons. Further studies in chromaffin cells showed that a specific Tm is associated with chromaffin granule membranes.[11]

Neurons show temporally-regulated patterns of Tm localization (Fig. 2). In early cortical neurons Tm4 is localized to cell bodies and also strongly enriched in growth cones.[12] TmBr1/3 is not observed significantly in cell bodies or growth cones at this time, however appears after several days in culture[12] and is localized to the axon and presynaptic bouton in the adult neuron.[13] Tm5NM1/2 localize to the axon but not cell bodies and dendrites in early neurons in vivo, but are lost from the axon and appear in the cell body between embryonic days 15-17 in the rat.[13] This is the same time at which TmBr1/3 appear in axons, indicating that isoform "switching" has occurred. In the adult brain Tm5NM1/2 appear to have a somatodendritic localization.[13] Tm5a/5b are also temporally regulated in neurons, being present in growth cones of early neurons in culture, but not in older cultures.[14]

In 14.5 day embryonic cortical neurons an antibody that preferentially detects Tm5NM2 shows staining in the cell body and neurites, but not the growth cone. CG3, which recognises all
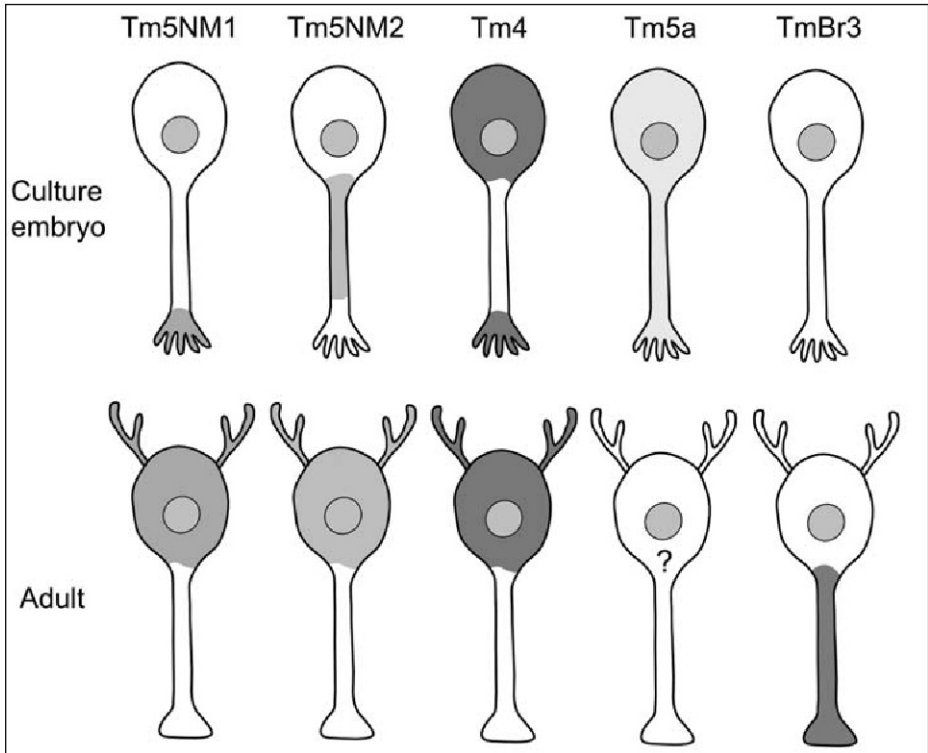
Figure 2. Tm isoform sorting in neurons is developmentally regulated. In the embryonic neuron, Tm5NM1/2, Tm4 and Tm5a show different sorting patterns. These sorting patterns are altered in mature neurons, with Tm5NM1/2 and Tm4 relocating to a somatodendritic compartment and TmBr3 replacing them in the adult axon. Figure adapted from reference 47.

isoforms from the γ gene, does stain growth cones, indicating that other γTm isoforms are present.[14] The γ/9d antibody shows Tm5NM1 is localized to growth cones in embryonic neurons.[15] Finally, in transgenic mice expressing the human Tm5NM1, this Tm shows specific localization to the growth cones and not the cell body or neurites.[15] Tm5NM2 is therefore likely to be the γ9d-containing isoform in the axons of developing neurons. The localization of γTm isoforms with different C- termini has been examined in the adult brain using antibodies specific for exons 9a, 9c and 9d. In adult neurons γ/9d appears to stain cell bodies and dendrites but not axons, whereas γ/9a and γ/9c stain cell bodies, dendrites and axons.[16]

Muscle

Muscle fibres contain three muscle-specific 9a-containing Tm isoforms which form part of the thin filament. These isoforms appear to be restricted to the thin filament, where they act as a regulatory switch to regulate contraction (see Chapter 8).[17] Despite the abundance of these muscle-specific Tms, muscle fibres also contain a number of cytoskeletal Tm isoforms. These cytoskeletal isoforms are not localized to the thin filament, but rather have specific sorting patterns within the myofibril. The γTm isoform Tm5NM1 is specifically sorted to a γ-actin based filament network adjacent to the Z-line and also to a subsarcolemmal actin-filament system at the periphery of the myofibril.[18] The Tm4 isoform is also present in a γ-actin filament system associated with the Z-line and unlike Tm5NM1, Tm4 is also present in longitudinal filaments that run perpendicular to the Z-line.[19] These longitudinal structures are associated with repair and remodelling of the myofibril. Expression of ectopic Tm3 in transgenic mouse muscle leads to accumulation of this

isoform in Z-line-adjacent filaments, but also results in a muscular-dystrophy-like phenotype, indicating the normal cytoskeletal network may be disrupted when Tms are expressed in inappropriate locations.[18]

Functional Consequences of Tm Sorting

The sorting of particular Tm isoforms to specific compartments in cells is consistent with specific functional roles for these isoforms. A number of studies have identified links between a particular Tm sorting pattern and functional consequences of this sorting.

Golgi

The localization of Tm5NM2 to a perinuclear compartment in NIH 3T3 cells indicates an association of this isoform with the Golgi complex.[4] This association has been investigated further by immunogold labelling of Tm5NM2 using both CG3 and WS5/9d antibodies. These studies confirm an interaction of γTm isoforms with short Golgi-associated microfilaments and the surface of coated vesicles derived from the Golgi.[4] Earlier studies have also shown that one or more γTm isoforms are associated with selected Golgi-derived vesicles, but Tms from the α and β genes are not.[20]

The Golgi apparatus is closely associated with an actin-based microfilament system, as well a microtubule system[21] and intermediate filaments.[22] Actin microfilaments play an important role in maintaining the shape of the Golgi structure as well as correct functioning of vesicle budding and fission.[23] The localization of specific Tms to these filaments is likely to help modulate actin function in this region, allowing finer regulation of these microfilaments.

Lamellapodia

Lamellapodia are motile structures located at the leading edges of cells. They are characterized by a dynamic actin network comprised of short branched actin filaments which are rapidly turned over. DesMarais et al[24] reported that Tm, while present in lamellapodia, is relatively absent from the leading edge of the cell where the F-actin concentration is high, indicating a tropomyosin-free compartment in the most dynamic region of the lamellapodium. Hillberg et al[25] demonstrated endogenous HMW Tms extend out to the very edge of the lamella in MTLn3 cells, although staining is weaker within 0.1-0.2 μm of the leading edge. Likewise, HMW Tms also extend into the lamella of migrating human fibroblasts, with disorganized Tm structures in the 1-4 μm region transitioning to actin-filament associated Tm further in. These results were confirmed via expression of exogenous Tms. Both HA-tagged and GFP-tagged Tms extend to the edges of the lamellapodia. GFP-tagged Tms show a weaker signal very close to the leading edge, indicating a lower concentration of tagged Tm in this region. HA-tagged Tm4 and Tm5, both LMW, appear to be present at the leading edges of the lamella at higher concentrations than the HMW Tm1 and Tm2, confirming observations in fibroblasts that HMW isoforms are less abundant at the periphery of cells.[1,2] Localization of these tagged Tms appears to be punctate close to the leading edge, becoming more organized further into the lamella until they are integrated into actin stress fibres.[25]

Many other actin-binding and remodelling proteins are enriched in this leading edge, especially Arp2/3 and ADF/cofilin. These proteins are involved in the rapid turnover of actin filaments in this region, which leads to the highly dynamic properties of the leading edge. As Tm isoforms have been shown to inhibit the activity of these proteins, the relatively lower concentration of Tm at the leading edge may allow this rapid remodelling to occur.[25]

CFTR

The cystic fibrosis transmembrane conductance regulator (CFTR) transports chloride ions across the apical surface of epithelial cells. In T84 epithelial cells the LMW Tm5a/5b have a specific apical localization, in contrast to HMW isoforms which are more basally localized.[6] Tm5a/5b specifically localize to regions of the membrane where the CFTR receptor is inserted, indicating these isoforms may have a functional role in regulating membrane levels of this protein. Knockdown of

these isoforms using antisense oligonucleotides leads to an increase in CFTR surface expression, confirming a role for Tm5a/5b in regulating the CFTR levels in the plasma membrane.[6]

Neurons

Neurons are highly differentiated cells, which show distinct changes in Tm sorting throughout development. These changes in localization indicate that certain Tm isoforms may have specific roles at various stages of development. The Tm5NM1/2 isoforms for example are highly enriched in the developing axon, but not the mature axon, indicating a possible functional role in axonal development and outgrowth.[13] Sorting of Tm5NM1/2 occurs very early in differentiation and sorting of Tm5NM1/2 mRNA to the axonal pole may occur prior to differentiation, in cells that do not yet have processes. Tm5NM1/2 is therefore an early marker for the development of neural polarity.[26]

Both Tm5NM1/2 and Tm4 are highly enriched in the early growth cone, indicating a role for these isoforms in neurite outgrowth during development.[12,15] Tm4 is also enriched in postsynaptic terminals of neurons in the rat cerebellum, specifically in the post synaptic densities.[12] This indicates a potential role for Tm4 in synaptic function. In contrast, TmBr3 is present at presynaptic sites, indicating this isoform is likely to have a different role in synaptic function to Tm4.[12]

Mechanisms of Tm Sorting

The sorting of Tm isoforms appears to be tightly regulated, both spatially and temporally. While specific locations of Tm isoforms have been described for many cell types and changes in sorting throughout maturation, differentiation and the cell cycle have also been described, the mechanisms which control this sorting are yet to be fully understood. There are a number of potential mechanisms which may be involved and the complexity of Tm sorting indicates that there is likely to be more than one mechanism contributing to the sorting of these molecules.

mRNA Sorting

One way in which proteins can be sorted to intracellular compartments is by sorting mRNA, resulting in localized synthesis which ensures the protein is only expressed where it is required. A number of cytoskeletal proteins show localized mRNA, including actin.[27] In developing neurons in situ, Tm5NM1/2 mRNA is localized within cell bodies and enriched at the axonal pole and in the proximal region of the developing axon.[26] This correlates with an axonal localization for Tm5NM isoforms in early neurons.[13] In mature neurons the Tm5NM1/2 mRNA appears to be distributed more evenly throughout the cell body,[26] again correlating with known protein localization.[13] TmBr2 mRNA is distributed evenly throughout the cell body but excluded from the axonal pole.[26] In cultured neurons, Tm5NM1/2 but not TmBr2 mRNA again appears to have a polarized distribution , with Tm5NM1/2 mRNA present in axons and cell bodies in differentiated cells.[26] TmBr3 mRNA shows a cell body distribution in mature neurons with enrichment near the axon,[28] correlating with the known axonal distribution of the protein.[12,13]

Localization of mRNA does not perfectly correspond to protein localization, however. The protein localization is broader in neurons than the mRNA localization. Tm4 mRNA shows the same localization as Tm5NM1/2 mRNA, being enriched in the axonal pole of the cell body and the proximal axon,[28] but the Tm4 protein is widely distributed in neurons, present in cell bodies, dendrites and axons. So while these two proteins again show similar mRNA localization, their protein localization is different, indicating additional mechanisms must be involved. It may be that mRNA localization can be used to regulate sites of protein synthesis and assembly and other mechanisms are then used to direct the protein to its final location.

Exogenous Tm5NM1 lacking the 3' UTR shows identical sorting to the endogenous protein, localizing to the growth cone in neurons (Fig. 3), while another exogenous isoform not normally expressed in neurons, Tm3, localizes much more broadly to the cell bodies, neurites and growth cones.[15] In fibroblasts, exogenous Tm5NM1 with and without the 3' UTR show identical sorting to that seen for the endogenous protein.[29] These studies therefore indicate that the coding sequence alone is enough to direct sorting of Tm isoforms and the 3' UTR is not essential.
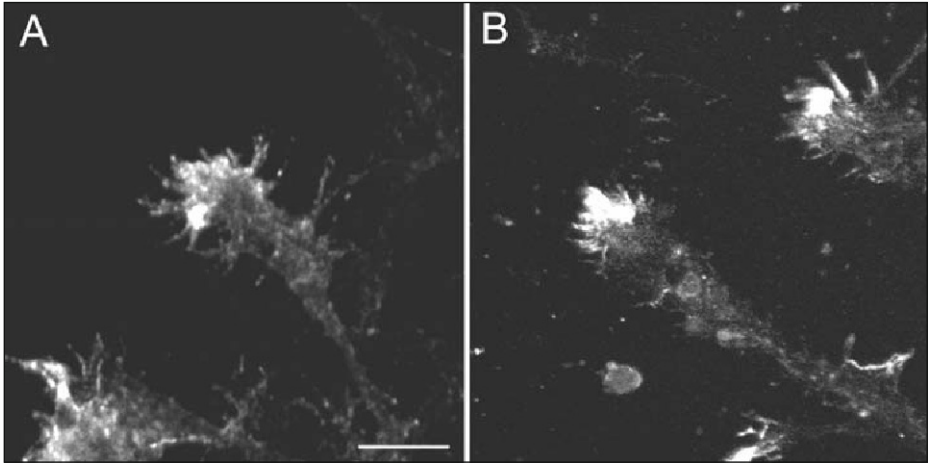
Figure 3. Exogenous Tm in transgenic mice sorts to the same compartment as endogenous Tm. Both endogenous Tm5NM1 as observed by the γ9d antibody (A) and exogenous Tm5NM1 as observed by the LC1 antibody (B) show enrichment in growth cones of mouse cortical neurons in 5 day cultures.[15] Scale bar, 10 μm.

Actin Structures

Tm sorting may be influenced by the actin isoform present in filaments, as it appears as though there are preferred associations between particular actin and Tm isoforms. Overexpression of γ-actin in myoblasts results in reduced sorting of Tm2 but not Tm5 into stress fibres,[30] suggesting competition of isoforms for inclusion into structures may play a role in sorting.

The role of actin structures in sorting of Tm isoforms has been studied using pharmacological agents that can disrupt the actin microfilament system. Treatment of neurons with cytochalasin B results in loss of specific sorting by the γTm isoforms. Washout of the drug leads to relocation of isoforms to their original locations.[14] These studies therefore indicate that an intact microfilament system is required for sorting of Tm isoforms. Intact actin structures also appear to be required for the polarized distribution of Tm5a/5b in epithelial cells. Treatment of these cells with the actin-disrupting drug cytochalasin D eliminates the polarized distribution of these isoforms.[6] Treatment with nocodazole, a microtubule-disrupting drug, does not eliminate polarized staining of Tm5a/5b, indicating that intact microtubules are not required for the polarization of Tms in epithelial cells.[6]

Microtubules form a separate cytoskeletal system from the actin microfilaments and are a major structural component of the axon. Treatment of neurons with nocodozole leads to redistribution of isoforms and a loss of Tm5a/5b and Tm5NM1/2 colocalization in axons,[14] indicating the microtubule system is involved in the maintenance of Tm sorting in neurons.

Isoforms from the γTm and αTm genes both sort to stress fibres in fibroblasts. Treatment with the drug cytochalasin D leads to loss of actin stress fibres, however stress fibres containing γTm isoforms are more resistant to cytochalasin D than those containing αTm and βTm isoforms.[3] It therefore appears that these two groups of isoforms may not bind to the same individual actin filament, but are instead associated with distinct filaments bundled together in the same stress fibre. The concept of homopolymers of Tm isoforms is also supported by the observation of isoform sorting per se. The very fact that Tm isoforms are segregated indicates individual populations of actin filaments contain primarily one isoform.

Isoform Specific Actin Affinities

There is evidence that alternative exon choice is capable of directing alternative sorting. Choice of an alternative N-terminus in the α gene (1a and 2b for HMW isoforms or 1b for LMW isoforms) can send these isoforms to different compartments in fibroblasts[2] and epithelial cells.[6] The choice of an alternative C-terminus in the γ gene can influence sorting in the brain. Isoforms with the 9d terminus are excluded from axons in adult neurons,[13] while 9a and 9c containing isoforms are present in axons, as well as cell bodies and dendrites.[16] An alternative splice choice has also been shown to direct alternative sorting in fibroblasts, with the 6a-containing Tm5NM1 sorting to stress fibres and the 6b-containing Tm5NM2 sorting to a perinuclear compartment.[4] Similarly, Tm5NM2 is targeted to the axon shaft in neurons whereas Tm5NM1 is localised to the growth cone.[15]

Exon 6 has been shown to be important for actin affinity in αTm. The 6a9d-containing isoform binds actin more strongly than the 6b9d-containing isoform and deletion of exon 6 results in loss of actin affinity, indicating that this region of the molecule is required for binding to actin.[31] This loss of actin affinity is more pronounced in isoforms containing the 9d C-terminus than in isoforms containing the 9a C-terminus, indicating that other exons can have a modulating effect and actin affinity does not depend on a single region of the protein. Note that in contrast, the choice of exon 6a or 6b in βTm does not appear to alter actin affinity, although the choice of exon 9a or 9d can.[32] This indicates that the effect on actin affinity of choosing one exon over another is not necessarily conserved between genes. It appears as though sorting of Tms in general is more conserved within genes, than between isoforms of similar exon structure from different genes. For example, a γ9c-containing isoform shows more similar sorting to γ9a-containing isoforms than to a 9c isoform from the α gene.[33] In addition, whereas Tm5b and Tm5NM1 share the same exon structure, containing the exons 1b, 6a and 9d, they show very different sorting patterns in epithelial cells.[6] All Tms exist as dimers when bound to actin and the formation of homo- or hetero-dimers can also alter actin affinity. The information for dimerization is contained within the Tm molecule and can be influenced by alternatively spliced exons (see Chapter 6).[34]

Unlike other proteins that may have a geographical targeting signal included within the alternatively-spliced region, Tm isoforms do not appear to contain an intrinsic targeting sequence that directs them to a particular compartment. Although alternative exon choice can influence sorting, as described above, this sorting is cell-type specific and also changes with development and differentiation, indicating that the process of Tm sorting is flexible and dynamic. This argues against a specific targeting signal in the manner of nuclear targeting or membrane localization signals. Rather, it appears as though multiple regions of the protein and perhaps the molecule as a whole is responsible for sorting. The differences in sorting for isoforms containing different exons may be explained by these exons influencing changes in flexibility and actin affinity, interactions with other proteins and modulation by signalling pathways.

Signalling

Organisation of the actin cytoskeleton is regulated by a number of signalling pathways. These are likely to contribute to Tm sorting by altering the composition of actin filaments in particular regions of the cell, therefore leading to changes in Tm accumulation at these sites. Members of the Rho pathway are involved in reorganisation of the cytoskeleton, with different members involved in promoting formation of different types of structures. Rho is involved in assembly of stress fibres,[35] Rac in formation of lamellipodia and membrane ruffles and Cdc42 in formation of filopodia.[36] Rac and Cdc42 alter actin polymerisation at the cell periphery through activation of the Arp2/3 complex through WAVE and WASP proteins respectively and Rho stimulates actin polymerisation to promote stress fibres through formins and can also promote myosin II actin-filament cross-linking activity through increased phosphorylation of myosin light chain.[37] Rho kinase (ROCK) is the downstream target of Rho and ROCK can also activate the downstream effector LIM kinase (LIMK). LIMK can directly regulate actin polymerisation via phosphorylation and inactivation of the actin-severing proteins ADF/cofilin and has also been implicated as a regulator

of microtubule assembly/disassembly. As well as activation by phosphorylation via ROCK, LIMK can also be activated by p21-activated kinase.[38]

Although there is no evidence of a direct link between the Rho and LIM pathways and Tm, there is some evidence that Tm can be phosphorylated. Phosphorylation is a very common post-translational modification that can not only alter protein activity, but can also alter the localization of many proteins. Tropomyosin-1 can be phosphorylated in endothelial cells in response to oxidative stress. This phosphorylation is associated with reorganisation of the actin cytoskeleton and recruitment of Tm1 into stress fibres, indicating that phosphorylation of Tm may be able to alter its localization.[39] It has been proposed that phosphorylation of Tm1 may be a major factor in actin bundling, assembly of focal contacts and generation of cellular tension.[39,40] Phosphorylation of Tm1 occurs downstream of ERK (extracellular signal-regulated kinase), although the kinase that is responsible for this phosphorylation has not yet been identified.

Lessons from Other Cytoskeletal Proteins

Many other cytoskeletal proteins show differential isoform sorting and these may provide models by which the mechanisms of Tm sorting may be understood. Sorting of cytoskeletal actin isoforms has been observed in neurons,[13] muscle[41] and other cell types,[42] although the mechanisms by which this sorting occurs have not been extensively studied. More is understood about the sorting mechanisms of myosin isoforms which, like Tms, comprise a large multigene family. These studies indicate that multiple mechanisms can contribute to the sorting of myosin isoforms.

Myosin IIA and IIB show spatial sorting within migrating endothelial cells, with myosin IIA enriched at the leading edge and IIB enriched at the trailing edge.[43] Injection of fluorescent analogues of these isoforms indicate that sorting is intrinsic to the proteins themselves and timelapse studies indicate that the different isoforms have different rates of incorporation into structures, which may explain their differential localizations. Myosin IIA is incorporated into new structures more quickly than myosin IIB and is also lost more rapidly when structures are disassembled, consistent with the presence of this isoform at the leading, but not the trailing edges of cells.[43]

Phosphorylation of myosins can alter their localization. Myosin II requires de-phosphorylation of the heavy chain in order to localize to the cleavage furrow of *Dictyostelium* during cytokinesis. This may reflect a need for the myosin to form higher order structures in the form of thick filaments before it can be properly localized to the cleavage furrow.[44] Studies in *Drosophila* S2 cells indicate the initial stage of myosin localization to the cleavage furrow requires Rho1 signalling and Rho kinase phosphorylation of the light chain of myosin II. This is thought to increase thick filament formation.[45] The same study indicates that filamentous actin is not required for the initial localization of myosin II to the cleavage furrow, but is involved in stabilising it once it gets there.

Myosin light chain isoforms show ability to compete for inclusion into structures in muscle cells, with a hierarchical order of binding specificity that mimics the developmental expression of these isoforms. This allows each new isoform expressed to have a higher binding affinity and therefore efficiently replace the previous proteins, while maintaining the stability of the structures. When multiple isoforms are expressed at once, some isoforms are preferentially sorted to the myofibrils, while those with lower affinities are distributed throughout the cytoplasm.[46]

Implications of Tm Sorting

Tm sorting is widespread in a number of cell types, including neurons, epithelial cells and fibroblasts. Although the mechanisms underlying the sorting of Tm isoforms is yet to be fully understood, it is clear that this is a very dynamic and tightly-regulated process. As more examples of Tm sorting are identified it is becoming increasingly clear that specific isoforms are associated with functionally distinct populations of actin filaments.[47] Differential sorting of isoforms is a way to regulate the amount of different Tm isoforms available at specific intracellular sites and therefore control the incorporation of Tms into specific microfilament populations. Because different Tm isoforms have diverse properties and functions with respect to actin and actin-binding proteins (see Chapters 17-21) this sorting can confer specific functional properties to the actin

microfilaments and therefore the regulation of Tm sorting can directly contribute to the regulation of actin-filament function.[48]

In the yeast *Saccharomyces cerevisiae*, loss of both Tm genes, TPM1 and TPM2, is lethal and TPM2 cannot compensate for the loss of TPM1 indicating that these two genes have distinct functions.[49] Knockout of the entire αTm or γTm genes in mice is embryonically lethal,[50-52] indicating products of each of these genes are required for cell survival. Knockout of individual isoforms within one gene has much more subtle effects however,[33] indicating that different isoforms from the same gene may be able to compensate for each other. Despite some functional redundancy within genes, it is clear that different Tm isoforms can confer different properties on the actin filament. Tm isoforms show differential abilities to bind actin[31,32] and isoform-specific regulation of the activity of actin-binding proteins myosin,[48,53] ADF/cofilin,[48] gelsolin[54] and formin.[55] These varying properties allow different Tm isoforms to differentially regulate actin filament function.

The large number of Tm isoforms may allow finer regulation of Tm function and activity, as each isoform may be regulated independently. Even isoforms with very similar properties may be regulated differently in time and space, whereas sorting of isoforms with different properties and functions may be a mechanism by which these specific functions can be restricted to the region of the cell where they are required and prevented from being expressed where they are not required. Sorting of Tm isoforms may also help direct the sorting of other cytoskeletal proteins. For example, overexpression of Tm5NM1 is able to recruit myosin II into stress fibres and displace ADF.[48]

Models of Sorting

The central question regarding the mechanism of sorting of Tm isoforms concerns the relative roles of active transport vs. passive diffusion of the molecules. Because Tm isoforms do not appear to contain an intrinsic geographical targeting signal that directs them to a particular region in all cells it is considered unlikely that an active transport system can account for the observed sorting of Tms.[47,56] Instead it appears that multiple regions of the protein and perhaps the protein as a whole are responsible for directing sorting.[56]

The simplest explanation for the accumulation of isoforms in specific structures is the 'molecular sink' model in which isoforms accumulate in structures where they have the highest affinity and are most stable.[47,56] This hypothesis is supported by drug studies, which indicate that fragmentation of actin structures can abolish Tm sorting[6,14] and also correlates with the observations that Tm sorting is flexible throughout development and alters as new structures are formed. Tm isoforms can compete for inclusion into specific actin structures, as demonstrated by the exclusion of HMW Tms from stress fibres in cells overexpressing Tm5NM1.[48]

Tm sorting can be influenced by other actin binding proteins and changes in actin organisation and dynamics. Changes in actin structure and associated proteins will influence the affinity of a particular Tm isoform in any actin-based structure and therefore alter the accumulation of specific Tms at that site. In turn, accumulation of a specific Tm isoform at a particular site in the cell will influence the properties of actin filaments by regulating the association with local actin binding proteins. Thus, collaboration between available Tms and the activity of local actin binding proteins will lead to the local assembly of functionally distinct actin filaments.

An example of this collaborative model is shown in Figure 4. Consider a situation where LIM kinase (LIMK) is active and ADF/cofilin is phosphorylated and inactivated and therefore will not compete with Tms for binding to actin. If myosin light chain kinase (MLCK) is also active, myosin will be phosphorylated and activated, promoting formation of stress fibres which preferentially accumulate the Tm5NM1 isoform. In contrast, if LIMK and MLCK are inactivated, then the equilibrium shifts towards active ADF which competes with some Tms (e.g., Tm5NM1) for actin filament binding, but collaborates with TmBr3 promoting the formation of shorter filaments that incorporate TmBr3 (Fig. 4).[56] In this way, the local active actin binding proteins and a specific Tm(s) act to promote the formation of a functionally specific actin filament at a particular site in the cell. As long as the local signalling and the availability of the specific Tm remain unchanged,
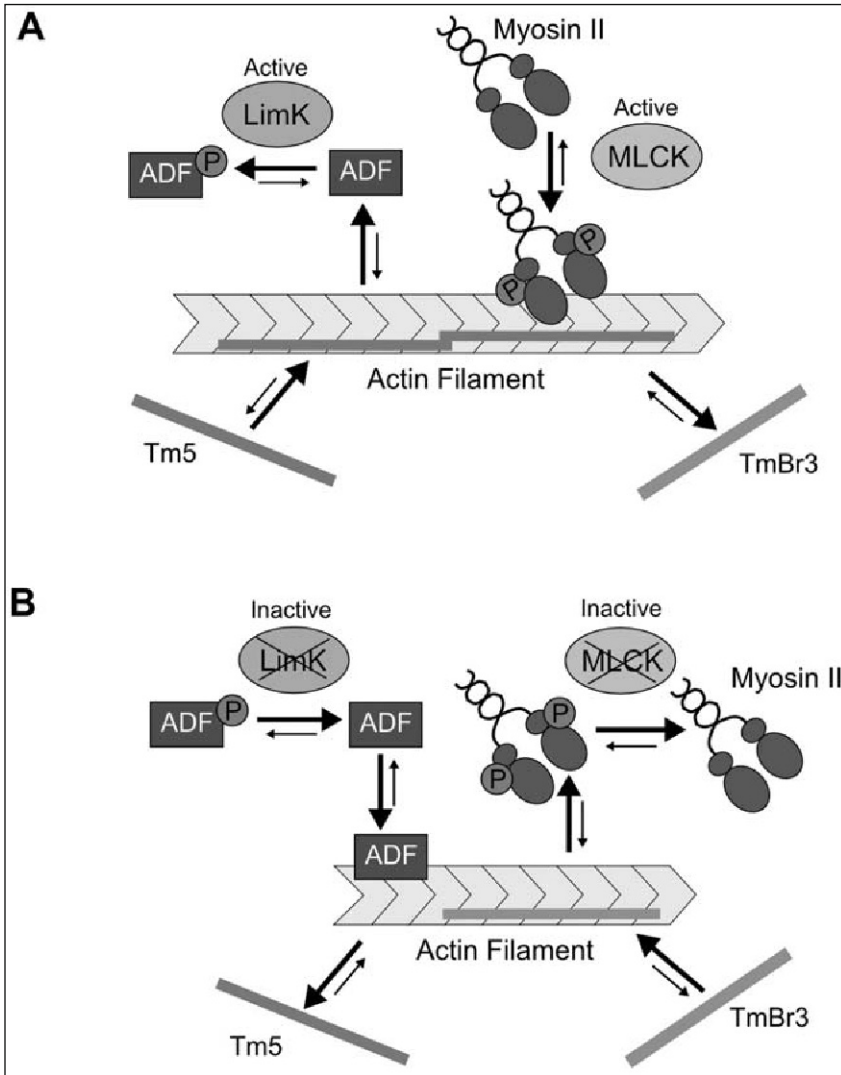
Figure 4. A collaborative model of Tm sorting. Local active actin binding proteins and specific Tms act to promote the formation of functionally specific actin filaments at a particular site in the cell. Local concentrations of all the components will drive competitive/collaborative interactions which will result in assembly of the most thermodynamically stable filaments. A) If LIM kinase and MLCK are active then ADF/cofilin will be inactivated and therefore will not compete with Tms for binding to actin and activated myosin II will promote formation of stress fibres which preferentially accumulate the Tm5NM1 isoform. B) In contrast, if LIMK and MLCK are inactivated then myosin II disengages and the equilibrium shifts towards active ADF which competes with some Tms for actin filament binding, but collaborates with TmBr3, promoting the formation of shorter filaments that favour binding of TmBr3.[56]

the type of actin filament should remain the same. The use of a single type of Tm along the length of the actin filament will also promote fidelity of function along the length of that filament.

Conversely, overexpression of Tm5NM1 will outcompete ADF/cofilin leading to elevated ADF and its subsequent phosphorylation whereas TmBr3 will promote binding of ADF/cofilin to actin

filaments.[48] Thus, local concentrations of all the components will drive competitive/collaborative interactions which will result in assembly of the most thermodynamically stable filaments. The spatial segregation of Tm isoforms may therefore be seen as the differential outcomes of these reactions at different sites in the cell.

It appears that cytoskeletal pools of Tm-containing filaments are not fixed in size.[57] Overexpression of exogenous cytoskeletal Tm leads to no decrease in expression of endogenous Tm, indicating the absence of a feedback mechanism to control cytoskeletal Tm expression. This is in contrast to sarcomeric Tms, where overexpression leads to a decrease in endogenous Tm in order to maintain the strict stoichiometry of actin and actin-binding proteins within the sarcomere.[58] Overexpressed cytoskeletal Tms accumulate in the same intracellular locations as endogenous Tm, indicating that these cytoskeletal pools are not saturated with Tm. Increased levels of Tm5NM1 lead to an increase in filamentous actin, indicating a shift in equilibrium towards increased F-actin and filament formation.[57] This suggests that the supply of individual cytoskeletal Tms is limiting for the assembly of actin filaments containing that isoform. This is most consistent with a 'molecular sink' model where Tms define both the pool size and the functional characteristics of specific actin filament populations.

Future Directions

Despite the progress that has been made in understanding how differential sorting of Tm isoforms occurs, there are a number of questions that remain unanswered. It remains to be seen if there is any kind of signal at the destination that is specific for any particular isoform, or if it is indeed simply a matter of actin affinities. It has also not yet been conclusively disproven that there is active transport involved, or what might direct this active transport if it occurred. The role of Tm in structure formation is also yet to be understood. It has been shown that some structures can be perturbed by removal of specific isoforms[6] and knockout studies have indicated that while some compensation by other isoforms can occur within genes, products from both the mammalian α and γ genes are absolutely required for life. It is not clear if an isoform is removed from a structure whether another will step in to take its place, or whether there are some types of actin-filaments that absolutely depend on specific Tm isoforms for their formation and maintenance. Study of knockout mice will be required to unambiguously answer this question.

In conclusion, Tm isoforms show extensive intracellular sorting which results in spatially distinct actin filament populations. Tm isoforms have different properties with respect to actin and actin-binding proteins and can therefore differentially regulate and confer functional differences on these actin filament populations. Although the mechanisms that control the intracellular sorting of Tm isoforms are not well understood, it appears as though many factors may contribute, including actin-filament dynamics, actin-affinity of Tm isoforms and other actin-binding proteins, all of which may favour the accumulation of particular isoforms at cellular sites. In the future, manipulation of the activity of specific actin binding proteins should reveal their role in the restriction of Tms to specific intracellular sites.

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