Alternatively spliced exons of the β tropomyosin gene exhibit different affinities for F-actin and effects with nonmuscle caldesmon

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SUMMARY

The rat β-tropomyosin (TM) gene expresses two isoforms via alternative RNA splicing, namely skeletal muscle β-TM and fibroblast TM-1. The latter is also expressed in smooth muscle where it corresponds to smooth muscle β-TM. Skeletal muscle β-TM contains exons 7 and 10, whereas exons 6 and 11 are used in fibroblasts and smooth muscle. In order to study the properties of the alternatively spliced proteins, recombinant TMs derived from bacterial and insect cell expression systems were produced, including the normal β gene products, fibroblast TM-1 and β skeletal muscle TM, two carboxy-terminal chimeric TMs, TM-6/10 and TM-7/11, as well as a carboxyl-truncated version of each, TM-6Cla and TM-7Cla. The purified TM isoforms were used in actin filament association studies. The apparent TM association constants (Kd) were taken as the free concentration at half saturation and were found to be 6 μM for β Sk TM, 8.5 for TM-6/10, 25 μM for TM-1, and 30 μM for TM-7/11 at an F-actin concentration of 42 μM. For the truncated TMs, the values determined were higher still but the binding was not carried out to full saturation. Isoforms were also produced using the baculovirus-insect cell system which produces proteins with an acetylated amino terminus as is normally found in vivo. This modification significantly enhanced the F-actin association of TM-1 but not the β skeletal TM or the other isoforms. Fibroblast TM-2 or TM-3, both products of the α gene, enhanced the affinity of TM-1 for F-actin, demonstrating different isoforms can act cooperatively on binding to actin. This effect was not detected with the other expressed β gene products. The presence of 83 kDa nonmuscle caldesmon was found to enhance the binding of TM-1 for F-actin. This effect was dependent on the presence of both exons 6 and 11, as caldesmon had little effect on the other β gene products. Collectively these results demonstrate TMs differ in their affinity for F-actin, which can be altered by other TMs or actin-binding proteins. The β tropomyosin isoforms were fluorescently-tagged and microinjected into cultured cells to study their in vivo localization where it was found that each of the full-length TMs bound to microfilaments but, at the light microscopy level, the isoforms were not differentially localized in these fibroblasts.

Key words: β tropomyosin gene, F-actin, caldesmon, alternative splicing, tropomyosin

INTRODUCTION

Most microfilaments have tropomyosin (TM) bound along their length, in the long pitch grooves on either side of the 2-start actin filament helix. The assembly and disassembly of microfilaments is a dynamic process repeated many times in a locomoting fibroblast. In addition, once every cell cycle, the complete actin network must be disassembled in readiness for mitosis, and later reassembled. How the association of TMs with actin is regulated is unknown. In the rat, there are known to be sixteen TM proteins expressed from four genes through the use of different promoters and alternative RNA processing. In skeletal muscle, the tropomyosin exists as a heterodimer consisting of one polypeptide from the α gene (α skeletal muscle TM, 284 amino acids) and one polypeptide from the β gene (β skeletal muscle TM (β Sk TM), 284 amino acids). In smooth muscle, the heterodimer is also formed from one polypeptide from the α gene and one polypeptide from the β gene, but each is an alternatively spliced product and differs in two exons from the skeletal muscle counterparts. In cardiac tissue, the TM dimer is composed only of α gene product, two chains of the α skeletal TM. There is a wider variety of tropomyosin isoforms in nonmuscle tissues owing to the expression of the four genes, three of which are alternatively spliced. This can result in the expression of as many as eight TM isoforms in a given cell. The cloning of the cDNAs for TMs has led to a better appreciation of the complexity of tropomyosin expression in vertebrates. For example, in rat fibroblasts, the α gene produces TM-2, TM-3, TM-5α and TM-5β through the use of two promoters and alternative mRNA processing (Goodwin et al., 1991). These differ in their affinity towards F-actin in the order 5b>2=3>5a, with the 248 amino acid TM-5b capable of displacing the 284 amino acid TM-2 (Pittenger and Helfman, 1992). In this study the F-actin asso-
ciation appeared to be cooperative for a given isoform but there was not cooperativity between isoforms, with each appearing to compete for F-actin binding sites. The α gene can also produce three brain specific isoforms (Lees-Miller et al., 1990) which are differentially expressed in neuronal cells during development (Stamm et al., 1993) or induced during neurite outgrowth in PC12 cells (Weinberger et al., 1993). In most smooth muscle and nonmuscle tissues, the β gene expresses only one isoform, TM-1. This TM differs from the β gene product found in skeletal muscle (β Sk TM) by the inclusion of an alternative internal exon (exon 6 in place of exon 7) and an alternative carboxy-terminal exon (exon 11 in place of exon 10, see Fig. 1) (Helfman et al., 1986; Yamawaki-Kataoka and Helfman, 1985). The functional differences between these isoforms are unknown.

While our knowledge of the TM gene family has progressed rapidly in recent years (see Lees-Miller and Helfman, 1991 for review), there is not as yet a clear understanding of the function and regulated expression of nonmuscle TM proteins. The complexity of TM isoform expression is probably not due to functional redundancy, as this complexity has been largely maintained through evolution suggesting important functions that have been conserved through selective pressures. What roles the different isoforms of TM fulfill in evolutionarily diverse organisms is unclear. TM’s function in striated muscle contraction has been well studied and tropomyosin facilitates the calcium-sensitive contraction of muscle fibers either by a steric blocking mechanism (Ebashi et al., 1969) or through allosteric interactions (Greene and Eisenberg, 1980). In smooth muscle, our understanding is less complete, but the phosphorylation of associated proteins, notably caldesmon and the myosin light chains, is involved in regulating actin and myosin association (Marston and Smith, 1985). A greater challenge still is to unravel the roles nonmuscle TMs play in different tissue types, given the variety of dynamic actin based structures present in cells. What properties the alternatively spliced exons confer on the TM isoforms is beginning to be elucidated (reviewed by Pittenger et al., 1994) but in order to better understand the characteristics of individual TMs, purified isoforms are necessary. The purification of nonmuscle TM isoforms from endogenous sources has proven difficult owing to their very similar physical and chemical properties. However, several investigators have taken advantage of the availability of cDNA clones for TMs and bacterial expression systems to generate quantities of individual isoforms for biochemical (Bartegi et al., 1990; Cho et al., 1990; Montiero et al., 1994; Novy et al., 1993a,b; Pittenger and Helfman, 1992; Urbancikova and Hitchcock-DeGregori, 1994; this paper) and cellular studies (Pittenger and Helfman, 1992; Prasad et al., 1993; this paper). The results have shown that many of the TM isoforms produced in bacteria can readily bind to F-actin in vitro (Cho et al., 1990; Heal and Hitchcock-DeGregori, 1988; Novy et al., 1993a,b; Pittenger and Helfman, 1992) but that certain TMs require an intact amino terminus or some form of amino terminal extension (Cho et al., 1990; Heal and Hitchcock-DeGregori, 1988; Montiero et al., 1994; Urbancikova and Hitchcock-DeGregori, 1994). When the isoforms that readily bind to actin filaments in vitro were introduced into cultured cells by microinjection, all were able to compete with the endogenous isoforms and incorporate into the microfilaments and in all cases, appeared to have no detrimental effects on cells (Pittenger and Helfman, 1992; Ranucci et al., 1993; this paper). As yet, the isoforms that have been introduced into cells have not shown differential localization as has been reported using an isoform-specific antibody (Lin et al., 1988).

In this paper, we report the characterization of recombinant TMs derived from the β gene using bacterial and insect cell expression systems. F-actin association of TM-1 (which contains the alternatively utilized exons 6 and 11) is shown to be affected by the presence of an acetylated amino terminus, the presence of TM-2, or the 83 kDa nonmuscle caldesmon. The β Sk TM (which contains the exons 7 and 10 in place of exons 6 and 11, respectively) showed little such effects. For those isoforms which bound well to actin in vitro, microfilament staining was easily visualized. However, no preferential localization to particular regions of the cell was seen with the labeled proteins. Our previous results also showed that the α gene products TM-2, TM-3, TM-5a and TM-5b showed indistinguishable microfilament staining when fluorescently labeled isoforms were introduced into fibroblasts by microinjection (Pittenger and Helfman, 1992). Taken together, the results suggest that most TMs do not achieve their function through differential localization within the cell. However, the organization of particular TM isoforms along actin filaments is probably not random and may be modified by the presence of other TMs or other actin binding proteins. A discussion of the organization of TMs on microfilaments and a possible model is presented.

MATERIALS AND METHODS

cDNA expression constructs

The cDNA clones for rat fibroblast TM-1 and β Sk TM have been described (Helfman et al., 1986; Yamawaki-Kataoka and Helfman, 1985). Molecular biology techniques were carried out as described (Sambrook et al., 1989). The bacterial expression plasmid pET8C (Studier et al., 1990) DNA was digested with NcoI and BamHI and the larger fragment gel purified. The TM-1 coding sequence was removed from SP64 vector by complete digestion with BamHI and partial digestion with NcoI and the correct fragment isolated by gel purification. The β Sk TM coding sequences present in clone SK22 (Helfman et al., 1986) were removed from pUC9 vector sequences by NcoI (single site) and BamHI digestion followed by gel purification. These TM coding sequences were then ligated into the NcoI/BamHI cleaved pET8C. Bacterial expression vectors to produce chimeric protein hybrids TM-7/11 and TM-6/10 were constructed by swapping the sequences 3’ of the Clal site to the SalI site between plasmids SP65 TM-1 and SP65 β Sk TM (this was done by Dr Susan Erster, for other purposes in this laboratory). Subsequently, the 3’ SalI site was converted to a BamHI site by digestion with SalI followed by nucleotide fill-in with the large fragment of Escherichia coli DNA polymerase I (Klenow fragment) and addition of BamHI linkers. The plasmids were then exhaustively digested with BamHI. The TM-7/11 construct was cut with NcoI and the proper fragment isolated. The TM-6/10 construct contained two NcoI sites so a partial digest was performed and the correct fragment isolated. These fragments were ligated overnight to the NcoI/BamHI digested pET8C. Competent E. coli was transformed with a portion of the ligation reaction, grown overnight, and colonies checked for appropriate inserts and then selected clones sequenced by the dideoxy-chain termination method (Sanger et al., 1977). Correct DNA constructs were tested for the production of TM proteins upon transformation of BL 21 (DE3) bacteria as described (Pittenger and Helfman, 1992). To produce truncated versions of TM-1 and β Sk TM lacking the alternative
of virus stock (approximately 10^5 pfu/ml) for 4 hours and 15 ml of

cm plates of SF9 cells (70% confluent) were infected with 4 ml each

expression system were purified by similar methods. Briefly, ten 150

trophoresis. The TMs expressed in bacteria were purified as previously described

bacteria BL21 (DE3) pLys-S using a full length cDNA clone in pET-

contained a myc epitope tag (EQKLISEEDL) at the amino terminus,

11d vector (Novagen, Madison, WI) and purified as described

The rat 83 kDa nonmuscle caldesmon was expressed in host

cation step involved F-actin binding and repurification as described

The full length nonmuscle caldesmon cDNA from rat was

workup on the polyhedrin promoter of the baculovirus vector pVL-

HI fragments and inserted at the Xhol/BamHI position down-

The rat 83 kDa nonmuscle caldesmon was expressed in host

was loaded onto a 5 ml anion exchange column (Mono Q resin,

repeated twice more and the final pH 4.5 pellet was resuspended in

heated to 70°C for 15 minutes and denatured proteins were pelleted

transfered to a Dounce homogenizer. Triton X-100 was added to the

Yamashiro-Matsumura, 1985; Novy et al., 1993a; Pittenger and

F-actin binding assays were performed as described (Matsumura and

were performed using 42

Core Facility at Cold Spring Harbor Laboratory. All binding studies

reported (Matsumura and Yamashiro-Matsumura, 1985; Novy et al.,

assays were quantified using scanning densitometry as previously

using the discontinuous buffer system (Laemmli, 1970). Binding

supernatants and pellets were loaded on 12% polyacrylamide gels

the expression plasmid for TM-7Cla was sequenced and the expressed

or 2-D gels, migrated virtually identically to the bacterially produced full length proteins or TMs

purified from cultured cells (data not shown). The carboxy

terminus of the TMs in bacterial expression plasmids were sequenced

using an oligo complementary to internal sequences and reading 5’

to the plasmid sequences. For several of the proteins, carboxy-terminal

sequence was carried out using the amino acid analysis of those

residues released by carboxypeptidase Y digestion over time (Pittenger and Helfman, 1992) and provided evidence of correct full

length TM proteins (data not shown). The recombinant proteins

truncated at the Ctd site were not subjected to protein sequencing but

the expression plasmid for TM-7Cla was sequenced and the expressed

proteins were of the expected molecular mass on SDS-PAGE gels.

F-actin binding assay

F-actin binding assays were performed as described (Matsumura and

Yamashiro-Matsumura, 1985; Novy et al., 1993a; Pittenger and

Helfman, 1992) or in a buffer containing 100 mM KCl, 30 mM NaCl,

20 mM sodium phosphate, pH 7.0, 0.5 mM EGTA and 1 mM DTT.

The results in the two buffers were comparable. Routinely, incubations

were performed for one hour at room temperature in a 70 µl volume,

followed by sedimentation in an Airfuge (Beckman Instruments, Palo

Alto, CA) at 28 psi for 30 minutes or a TLA-100 rotor at 65,000 rpm

in the tabletop ultracentrifuge (Beckman Instruments). After cen-

trifugation, the supernatant was removed and mixed with an equal

volume of 2X SDS gel sample buffer. The pellet was carefully resus-

pended in 140 µl of 2X SDS gel sample buffer. Samples (10 µl) of

supernatants and pellets were loaded on 12% polyacrylamide gels

using the discontinuous buffer system (Laemmli, 1970). Binding

assays were quantified using scanning densitometry as previously

reported (Matsumura and Yamashiro-Matsumura, 1985; Novy et al.,

1993a; Pittenger and Helfman, 1992) using as standards TMs whose

concentration were determined by amino acid analysis in the Protein

Core Facility at Cold Spring Harbor Laboratory. All binding studies

were performed using 42 µM actin.

Fluorescent labeling of proteins and microinjection

Fluorescent labeling of TMs produced in bacteria or SF9 cells was carried out as previously described (Pittenger and Helfman, 1992).

Microinjection of cultured cells was performed by published procedures (Diaucumakos, 1973; Graessmann et al., 1979) as detailed pre-

viously (Pittenger and Helfman, 1992).
Cell culture methods
Cultured rat embryo fibroblasts (REF 52) were grown in DME medium (Gibco BRL, Grand Island, NY) containing 10% rheheatin fetal calf serum (Intergen, Purchase, NY), 4,500 mg/l glucose, 50 mg/l streptomycin, 50 U/ml penicillin G. Cells were grown in a 5% CO2 atmosphere at 37°C. SF9 cells were obtained from PharMingen or the American Type Culture Collection (Rockville, MD) and were grown in TMN-FH media (Sigma Chemical Co., St Louis) supplemented with 10% fetal calf serum and 1 U/ml gentamycin, at 27°C without CO2.

RESULTS
Production of tropomyosins in bacteria and insect cells
The bacteriologically produced TMs included fibroblast TM-1 and β Sk TM, as well as two chimeric TMs, in which the C-terminal domain of each isofrom was exchanged. The resulting chimeric isoforms are herein referred to as TM-6/10 and TM-7/11 (Fig. 1). It is worth noting that these two chimeric TMs have been found to be expressed at low levels from the mouse β-TM gene (Wang and Rubenstein, 1992). In addition to the full-length TMs, two C-terminal truncated proteins were produced and are referred to as TM-6Cla and TM-7Cla (Fig. 1). The recombinant TMs were expressed constitutively in bacteria as soluble proteins but production levels could be increased approximately two-fold by the addition of IPTG (0.4 mM final concentration) to early log phase cultures. Bacteria were harvested by centrifugation and lysed by a combination of enzymatic digestion and detergent solubilization. The proteins were purified as described previously (Pittenger and Helfman, 1992) by modification of published procedures used to purify TM from tissue sources including ammonium sulfate precipitation, pH precipitation of TMs, heat denaturation of contaminating proteins, and ion exchange chromatography. Purification was assessed by SDS-PAGE (see Fig. 2) and also by western blotting, where appropriate. The yield of purified TMs expressed in bacteria varied depending on the isofrom but ranged from 10 to 40 mg per liter of bacterial culture. The full-length final products used in subsequent experiments were greater than 95% pure, the contaminants being mostly premature termination products or minor degradation products. Several of the TMs were subjected to N-terminal and C-terminal sequencing to ensure that they were full length and retained the N-terminal methionine. The bacteriologically produced TMs, TM-1 and β Sk TM, as well as the chimeras TM-6/10 and TM-7/11 were found to have the correct N-terminal sequence (see Table 1). The carboxy termini of TM-1 and TM7/11 were analysed by the release of amino acids over time with digestion by carboxypeptidase Y which gave evidence of being full length (data not shown, the amino acid sequence is 278-LLELNNL-284 and therefore somewhat redundant).

TMs were produced in SF9 cells both as suspension cultures and attached to tissue culture plates. The tropomyosins produced using the baculovirus expression system were found to have a blocked amino-terminus as expected, presumably due to acetylation which is known to occur in this expression system. The purified proteins were greater than 95% pure (see Table 1).
was encoded by exon 10 bound actin with much greater affinity than those containing exon 11. Whether this was an intrinsic property of the terminal exon or due to improper folding, etc. is not completely clear. However, eliminating the last exons by expressing the truncated TMs resulted in similarly weak binding for each of the truncated forms (see Fig. 3E and F). Several binding assays were also performed in buffers of varying ionic strength (30 to 120 mM KCl) with similar results and no change in the relative affinity of the TMs for F-actin (data not shown).

Acetylation of the amino terminus enhances TM-1 binding to filaments

When tropomyosins isolated from endogenous tissue sources have been sequenced, they have proven to have an acetylated amino terminus as is found for many proteins. Due to the head-to-tail overlap which is known to occur for certain TMs bound along the actin filament, this acetylation may be important for proper alignment and cooperative binding. In order to directly ask whether the acetylation of the amino terminus affects the actin affinity of TM-1, we expressed this isoform in an insect cell expression system. The SF9 insect cells are known to carry out protein modifications such as acetylation and limited glycosylation. When expressed in SF9 cells, TM-1 and other isoforms were unable to be sequenced from the amino terminus, presumably due to acetylation. Using this TM-1 in the F-actin binding (Fig. 5a,b) assay, a distinct increase in the binding was evident, with a decrease in the apparent association constant from 25 μM to 10 μM. When the TM α gene product TM-2 was produced in the bacterial and insect cell system and the products tested for their ability to bind F-actin, only a slight increase in association was found (Fig. 5c,d). Similarly, for the β Sk TM expressed in SF9 cells, no increase in binding was evident when compared to the bacterially expressed Sk TM. This suggests that for those TMs that readily bind F-actin, this amino terminal acetylation has limited or no effect, but that for certain isoforms it may be critical.

Presence of other tropomyosins can promote TM-1 association with actin filaments

Tropomyosin binding to F-actin has been shown to involve cooperative interactions, due in part to head-to-tail overlap of coiled coil dimers. In addition we have demonstrated previously using α TM isoforms expressed in bacteria, that the nonmuscle isoform TM-5b could compete and displace the TMs 2, 3 or 5a from F-actin (Pittenger and Helfman, 1992). In order to test whether the presence of other TMs would affect the binding of TM-1, mixtures of expressed isoforms were incubated with F-actin. As expected, TM-5b (expressed in bacteria) could displace TM-1 (data not shown). Surprisingly, we found that when increasing amounts of TM-1 (expressed in bacteria or insect cells) were incubated with F-actin for one hour and then equimolar amounts of TM-2 were added for an additional hour, TM-1 binding was increased at low concentrations and inhibited at higher concentrations (see Fig. 6). When TM-2 was present, TM-1 binding appeared to saturate at 15 μM (0.5 mg/ml), lower than the 45 μM (1.5 mg/ml) concentration necessary in the absence of TM-2. However, the amount of TM-1 bound at saturation was lower than in the absence of TM-2. This inhibition never precluded TM-1 binding, but amounts of TM-2 which saturate the actin filaments failed to

Fig. 2. Purified β tropomyosins following expression in bacteria. The tropomyosins were expressed in the bacterial host BL21 containing a DE 3 lysogen with the IPTG inducible T7 polymerase gene and purified as described previously (Pittenger and Helfman, 1992); 3 μg of each TM isoform was resolved on a 12% polyacrylamide gel containing SDS. Lane 1, TM-7/11; lane 2, TM-1; lane 3, β Sk TM; lane 4, TM-6/10; lane 5, TM-7Cla; lane 6, TM-6Cla. To the right are shown the positions of molecular mass markers (×10\(^{-3}\)). Arrows mark the top and bottom of the gel.
displace the bound TM-1. And the presence of TM-1 did not alter the extent of TM-2 binding. This was also true when samples were incubated for longer periods (up to 4 hours) and the result does not appear to be kinetically limited. Thus it appears that TM-1 in the presence of TM-2 binds to a limited number of sites on actin filaments and that TM-2 facilitates such TM-1 binding in a cooperative manner. From quantifying the Coomassie Blue stained gels, the molar amounts of TM bound when each TM was at saturation appeared to be 1:6, TM-1 to TM-2. TM-1 binding was similarly increased when TM-3, another 284 amino acid a gene product, was present (data not shown). It should be noted that the amount of TM-2 bound appeared to be the same in the presence or absence of TM-1, but small changes of 5-10% would be within experimental error and difficult to quantitate. However, this did not appear to be the case. Preliminary experiments involving more than two isoforms of TM became difficult to interpret without careful quantitation and are beyond the scope of the current work.

Caldesmon enhances TM-1 binding to actin filaments
Caldesmon is known to bind both actin and tropomyosin through residues in the carboxy third of the elongated molecule (Watson et al., 1990) although the exact binding sites and overlaps are not completely known. We tested whether 83 kDa
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nonmuscle caldesmon would affect the F-actin binding of the different expressed TMs. The caldesmon was expressed in bacteria which produces an unphosphorylated CaD, the form that readily binds F-actin (Yamashiro et al., 1990). TM-1 exhibited greatly enhanced binding even at subsaturating amounts of caldesmon (Fig. 7). By contrast the binding of the other TM isoforms was not dramatically affected by caldesmon (Fig. 7). The increase in binding of TM-1 lowered the half-saturating amount of TM-1 from 0.75 mg/ml to approximately 0.4 mg/ml with an estimated decrease in $K_a$ from 25 μM to about 8-10 μM. When caldesmon was first allowed to incubate with F-actin for one hour and TM-1 was subsequently added for an additional hour, the results were identical, suggesting that a TM-1:caldesmon complex is not first required for binding. When TM-1 and caldesmon were incubated in the absence of F-actin and subjected to centrifugation, no pellet was formed (data not shown). Acetylated TM-1 produced in the insect cell expression system also showed a large increase in binding in the presence of nonmuscle caldesmon (Fig. 7d), suggesting that the enhancement in binding is more than simply an alteration in the TM-1 amino terminus (see Discussion). The amount of pelleted F-actin did not change, nor did the extent of caldesmon binding when TM-1 was present. As shown in Fig. 8a, the chimeric TM-6/10 also showed an enhancement in binding in the presence of nonmuscle caldesmon, but not as dramatic as the TM-1. The expressed TM-7/11, TM-6Cla and TM-7Cla showed only a slight, if any enhancement in binding when caldesmon was present. Clearly the greatest enhancement of TM binding was seen with TM-1 which utilizes exons 6 and 11.

**Microinjection of fluorescently tagged TMs**

In order to try to identify any differences in the subcellular localization of the isoforms each of the bacterially produced β TM isoforms was fluorescently labeled by reaction with lissamine rhodamine B sulfonyl chloride and the labeled TMs used in microinjection experiments. The proteins were microinjected into REF52 cells, a rat embryo fibroblast cell line with a well-spread morphology. These cells have been used previously for the microinjection of α-TM isoforms (Pittenger and Helfman, 1992) and the expression of TMs in these cells has been well studied (Matsumura and Yamashiro-Matsumura, 1985; Matsumura et al., 1983).

The microinjection of β Sk TM and TM-6/10, isoforms which utilize exon 10, resulted in rapid and extensive incorporation into the microfilament system. As shown in Fig. 9, within 30 minutes after injection, labeling was seen throughout the cell, but was most intense near the site of injection. Labeling was also present at the cell periphery, where active reorganization of microfilaments is known to occur (Theriot and Mitchison, 1992). By 2 hours, the labeling was virtually uniform within the cell and characteristic tropomyosin stri-
itions were visible on stress fibers. Counterstaining actin filaments with fluorescein phalloidin revealed that at the light microscope level essentially all actin filaments had incorporated the microinjected TM. There appeared to be no deleterious effects on filament stability or cell viability, and labeled filaments could be found in microinjected cells as late as 48 hours. Therefore, these two isoforms, although not normally found in these cells could readily compete with endogenous TMs and incorporate into microfilaments. They did not cause morphological changes in these well spread cells, nor did there appear to be any detrimental short term effects on cells.

When bacterially expressed TM-1 and TM-7/11 were similarly labeled, microinjected and allowed to incorporate, only the faintest of filament staining was discernable (data not shown). This weak incorporation appeared to be consistent with the F-actin binding results and previous microinjection experiments (Pittenger and Helfman, 1992), suggesting that preparations of TMs that bound poorly to actin filaments in vitro were unable to label microfilaments following microinjection.

Fig. 7. Caldesmon (83 kDa) enhances the TM-1 association to F-actin. Assays were performed to analyse the effect of nonmuscle caldesmon on the binding of TM isoforms to actin filaments. For this, the TM concentration of each isoform was that at the midpoint of the binding curve which would show the greatest change, when caldesmon has an effect. Decreasing amounts of the bacterially expressed nonmuscle caldesmon were added to the constant amount of TM isoform and 1 mg/ml of F-actin. A large change in the association of TM-1 with F-actin was noted as caldesmon was added up to 0.25 mg/ml. (A) Caldesmon alone. Small arrowheads identify a caldesmon breakdown product that occurs during bacterial expression. (B) 0.125 mg/ml β Sk TM. (C) 0.25 mg/ml TM-1. Caldesmon concentrations were: lane 1, 1 mg/ml; lane 2, 0.75 mg/ml; lane 3, 0.5 mg/ml; lane 4, 0.25 mg/ml; lane 5, 0.125 mg/ml; lane 6, 0.06 mg/ml.

Fig. 8. Caldesmon has limited effect on the other β TM isoforms. The effect of caldesmon on the binding of the chimeric β TMs and the truncated forms to F-actin was tested. A limited effect was seen with TM 6/10 (A) but little or no effect was seen with TM-7/11 (B), TM-6Cla (C) or TM-7Cla (D). Concentration of TM 6/10 was 0.125 mg/ml; TM 7/11 was 0.25 mg/ml; TM-6Cla was 0.25 mg/ml; TM-7Cla was 0.25 mg/ml. As in Fig. 7, caldesmon concentrations were; lane 1, 1 mg/ml; lane 2, 0.75 mg/ml; lane 3, 0.5 mg/ml; lane 4, 0.25 mg/ml; lane 5, 0.125 mg/ml; lane 6, 0.06 mg/ml. Small arrowheads identify a caldesmon breakdown product.
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The presence of TM-2 and caldesmon within these cells apparently failed to enhance binding sufficiently. However, when TM-1 was produced in insect cells, fluorescently labeled and microinjected, extensive incorporation into microfilaments was seen (Fig. 10a). The labeling was not as intense as for β Sk TM or TM-6/10 but this may reflect the limited incorporation of TM-1 in the presence of other isoforms as seen in the F-actin binding assay (Fig. 6c).

DISCUSSION

Binding of β TM gene products to F-actin

The seven or eight tropomyosin isoforms found in fibroblasts are an integral part of the microfilament system and are thought to play a role in microfilament dynamics (Ishikawa et al., 1989a,b; Nishida et al., 1985). Tropomyosins are present as coiled coil dimers, which in fibroblasts have been shown to be homodimers (Lin et al., 1985; Matsumura and Yamashiro-Matsumura, 1985; M. Gimona, A. Watakabe and D. M. Helfman, unpublished) while in smooth and striated muscle, TMs form heterodimers of one α and one β subunit. The intrinsic affinity of different TM homodimers towards F-actin has been shown to be quite different, whether the TMs are prepared from endogenous sources (Broschat and Burgess, 1986; Matsumura and Yamashiro-Matsumura, 1985) or from a heterologous expression system (Novy et al., 1993a,b; Pittenger and Helfman, 1992; Prasad et al., 1993). In order to investigate the association of β tropomyosin gene products with F-actin and microfilaments, we have expressed the cDNAs for the different isoforms in bacteria and insect cells and tested the ability of the purified isoforms to bind to actin filaments in vitro and microfilaments in vivo. Purified TM-1 expressed from bacteria bound weakly to F-actin, while the acetylated version of this protein produced by expression in insect cells was found to bind F-actin much more readily, suggesting a structural requirement that this protein fulfills. Whether this increased binding results from a better fit to binding sites on actin or is due to a head to tail interactions of overlapping adjacent TMs or perhaps another factor remains to be determined. In addition, the β Sk TM and the chimera TM-6/10, which both use the same C-terminal exon were found to have a greater F-actin affinity than TM-1 or the chimera TM-7/11. Exon 10 appears to provide for enhanced actin binding ability relative to exon 11. Hence, the ends of the linear β TM molecules have important structural information for actin association. Other investigators working with homodimers of bacterially expressed α Sk TM have shown that its binding to cultured rat fibroblasts. In order to discern whether microinjected TM isoforms would incorporate preferentially at particular regions of the cell or sites on microfilaments, fluorescently labelled TMs were microinjected into cultured rat fibroblasts (REF 52 cells). In this figure, β Sk TM was fluorescently labeled with lissamine rhodamine B and the free dye removed by chromatography. The labeled TM was bound to F-actin and subjected to centrifugation to eliminate any denatured TM. The pelleted TM was then separated from actin (see Materials and Methods) and used for microinjection. At the appropriate times following injection, the coverslips were extracted for exactly 5 minutes with 0.2% Triton X-100 in a cytoskeletal stabilizing buffer to allow unincorporated TM to diffuse away and the cells were then fixed in 4% formaldehyde for 30 minutes. (A) Two minutes after microinjection. (B) Five minutes after microinjection. (C) At 30 minutes after microinjection. (D) At 2 hours after microinjection. Arrows indicate the site of injection. The incorporation of TM into microfilaments at early time points centered around the site of injection and appeared to occur along filaments. Uniform labeling of all microfilaments was found by 2 hours post injection, and coincided with all phalloidin labeled filaments (data not shown).
F-actin requires a structural modification of the amino terminus that may be overcome by addition of as little as two amino acids (Montiero et al., 1994) or as many as eighty amino acids (Cho et al., 1990) to the amino terminal methionine of Sk α TM homodimers. It is interesting to note that the β Sk TM does not seem to require N-terminal acetylation while the α Sk TM does for the homodimers to have “high” F-actin binding. There is a high degree of conservation between the amino acid sequences of these proteins. The first 18 amino acids are identical between the α and β Sk TMs, there also being 3 conservative changes in the first 38 amino acids (see Lees-Miller and Helfman, 1991). The comparison of exons 2b from the α and β genes reveals 9 differences in 42 residues, 5 being non-conservative, usually a charged residue in β Sk TM replacing a neutral or more hydrophobic residue found in α Sk TM. In the C-terminal coding exons of the α and β genes there are 5 encoded amino acid differences, most of which are conservative in nature. It will be of interest to determine if these are sufficient to explain the differences in F-actin binding between recombinant α and β skeletal muscle isoforms.

α TM gene products enhance association of TM-1 for F-actin

In skeletal and smooth muscle, the TM exists as a coiled coil heterodimer of one α and one β TM molecule. While virtually all cell types do express proteins from several TM genes, in fibroblasts the seven or eight isoforms of TMs are likely to function as homodimers (Lin et al., 1985; Matsumura and Yamashiro-Matsumura, 1985; M. Gimona, A. Watakabe and D. M. Helfman, unpublished). These TM homodimers could bind to actin filaments and function cooperatively or competitively. For example, we have previously shown that, among the α gene products, 248 amino acid TM-5b could competitively displace the 284 amino acid TM-2 or TM-3 from actin filaments in vitro (Pittenger and Helfman, 1992). As shown in Fig. 6, the presence of TM-2 enhanced the affinity of TM-1 for F-actin but limited the extent of binding. In another series of experiments (data not shown) TM-3 had the same effect on the association of TM-1 for F-actin as TM-2 but TM-5b appeared only to compete with TM-1. When TM-1 and TM-2 were both present, TM-5b continued to compete them both, about equally, from F-actin (data not shown).

If TMs stabilize microfilaments in vivo (Ishikawa et al., 1989a; Nishida et al., 1985), cells failing to express a full complement of TM isoforms may exhibit greater microfilament instability and may never establish stable actin networks or stress fibers. This seems to be the case for many transformed cells and suppression of the synthesis of certain TM isoforms is common following oncogenic transformation by chemical carcinogens, DNA or RNA viruses (Cooper et al., 1985; Hendricks and Weintraub, 1984; Lin et al., 1985; Matsumura et al., 1983). For example, a rat embryo fibroblast cell line transformed with the DNA transforming virus adenovirus type-5 fail to express any detectable TM-1 (Matsumura et al., 1983). These cells have a pronounced spindle morphology and contain relatively few actin filaments compared to the parent cell line. Similarly NRK 1569 cells, derived from a well spread normal rat kidney cell line (NRK1570) that have been transformed by

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Fig. 10. β gene TM isoforms show the same localization following microinjection. Expressed TM isoforms were fluorescently labeled with lissamine rhodamine B and purified as described in Fig. 9. (A) Acetylated TM-1 produced in insect cells. (B) TM-6/10 produced in bacteria. (C) β Sk TM produced in bacteria. (D) Rabbit skeletal muscle TM, a mixture of α and β isoforms. Although microfilament labeling appeared complete in 2 hours (see Fig. 9) for 6/10ch, β Sk TM or TM from rabbit skeletal muscle, those isoforms which readily bound F-actin, best images for TM-1 were obtained 12-18 hours after microinjection. Hence, these photographs were all taken 12-14 hours after injection. TM-1 produced in bacteria as well as the two truncated isoforms TM-6Cla and TM-7Cla failed to incorporate into the microfilaments of injected cells to levels which allowed visualization.
the v-ras containing Kirsten RNA virus grow as quite rounded cells, loosely attached to the substrate. These cells do not produce detectable TM-2 or TM-3 proteins (Matsumura et al., 1983). Recently, it has been reported that the 3′ untranslated region of α tropomyosin mRNA may suppress transformation by an as yet unidentified mechanism (Rastinejad et al., 1993).

**Caldesmon promotes TM-1 binding to F-actin**

Nonmuscle 83 kDa caldesmon (CaD) is known to have a periodic localization along microfilaments (Yamashiro-Matsumura and Matsumura, 1988) and binds tropomyosin through residues in its C-terminal half (Watson et al., 1990). Lin and co-workers have recently shown that forced expression of the carboxy-terminal half of nonmuscle CaD can stabilize TM association and enhance microfilament stability in CHO cells (Warren et al., 1994). To investigate the effect of caldesmon on the association of β TM isoforms with actin filaments, the full length bacterially expressed 83 kDa CaD protein was included in the TM:F-actin association assays. As we have shown in Fig. 7, caldesmon has a strong positive effect on the binding of TM-1 to F-actin. This isoform utilizes the alternatively spliced exons 6 (internal) and 11 (carboxy-terminal). The binding of the isoform β Sk TM and chimaera TM-6/10 was affected in a minimal but positive manner when caldesmon was present, while little or no effect was seen with the expressed chimera TM-7/11. The expressed, truncated proteins TM-6Cla and TM-7Cla showed virtually no increase in actin binding when caldesmon was present. Hence, among these TM proteins, there is a requirement for the exons 6 and 11 to achieve the maximal enhancement of TM binding by 83 kDa caldesmon.

The enhancement of TM-1 binding to F-actin in the presence of 83 kDa CaD agrees well with the reports of 140 kDa smooth muscle caldesmon enhancing the binding of smooth muscle TM or recombinant β smooth muscle TM (identical to TM-1) to actin filaments (Novy et al., 1993a). The 83 kDa caldesmon has not been as extensively studied as the smooth muscle counterpart, however, similar properties include the binding of TM and calmodulin, as well as filamentous actin and the inhibition of actomyosin ATPase. The interaction of 83 kDa caldesmon with F-actin has been shown to be regulated by Ca²⁺/calmodulin or phosphorylation by the mitotically active cdc2 kinase (Yamashiro et al., 1990). The association of 83 kDa caldesmon with the cytoskeleton is known to be inhibited by elevated Ca²⁺/calmodulin levels, and increases in levels of intracellular Ca²⁺ would be expected to allow activation of actomyosin ATPase activity and increases in cellular motile activities. Similarly, the release of CaD (see Fig. 11) followed by a weakening of the TM-1:microfilament association, may allow severing proteins such as gelsolin, to cleave the unprotected actin filaments efficiently. As an example, in a motile fibroblast that is undergoing changes in morphology, local microfilament reorganization involving TMs and CaD could be responsive to Ca²⁺ levels prompted from intracellular stores or membrane fluxes by receptor-based mechanisms. The sensitivity of CaD to cdc2 kinase phosphorylation may promote the pronounced alterations of the cytoskeleton that must occur prior to mitosis. As the cell then progresses through the cell cycle, the dramatic changes that occur throughout the cytoskeleton in readiness for mitosis could be controlled by phosphorylation events on CaD and other components of the cytoskeleton.

**Fluorescently tagged isoforms microinjected into cells do not show differential subcellular localization**

The incorporation of microinjected β Sk TM and TM-6/10 produced in bacteria was rapid and extensive throughout the fibroblast cytoskeleton as was the TM purified from rabbit muscle (Figs 9 and 10). The bacterially produced TM-1 failed to incorporate, but the acetylated TM-1 from the insect cell expression system was found in the characteristic striations along stress fibers. When the TM localization was compared with fluorescein-phalloidin staining, all actin filaments appeared to have the introduced TMs bound along their length. No difference could be discerned between the localization of these microinjected TMs. It would be intriguing to know whether the periodic CaD localization previously reported (Bretscher and Lynch, 1985; Yamashiro et al., 1990) correlates with the periodic staining of TM-1.

While microinjection of labeled TM protein into fibroblasts has not yet shown a difference in isoform localization, antibodies that can distinguish between certain isoforms have been used to show such differences in 293 cells (Lin et al., 1988) or the more highly differentiated porcine intestinal epithelial
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REFRERENCES


Alternatively spliced exons of the β tropomyosin gene


(Received 9 January 1995 - Accepted 22 June 1995)