In Vitro and In Vivo Characterization of Four Fibroblast Tropomyosins Produced in Bacteria: TM-2, TM-3, TM-5a, and TM-5b are Co-localized in Interphase Fibroblasts

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Abstract. Most cell types express several tropomyosin isoforms, the individual functions of which are poorly understood. In rat fibroblasts there are at least six isoforms; TM-1, TM-2, TM-3, TM-4, TM-5a, and TM-5b. TM-1 is the product of the β gene. TM-4 is produced from the TM-4 gene, and TMs 2, 3, 5a, and 5b are the products of the α gene. To begin to study the localization and function of the isoforms in fibroblasts, cDNAs for TM isoforms 2, 3, 5a, and 5b were placed into bacterial expression vectors and used to produce TM isoforms. The bacterially produced TMs were determined to be full length by sequencing the amino- and carboxy termini. These TMs were found to bind to F-actin in vitro, with properties similar to that of skeletal muscle TM. In addition, competition experiments demonstrated that TM-5b was better than TM-5a in displacing other TM isoforms from F-actin in vitro. To investigate the intracellular localization of these fibroblast isoforms, each was derivatized with a fluorescent chromophore and microinjected into rat fibroblasts. TM-2, TM-3, TM-5a, and TM-5b were each found to associate along actin filaments. There was no preferred cellular location or subset of actin filaments for these isoforms. Furthermore, co-injection of two isoforms labeled with different fluorochromes showed identical staining. At the level of the light microscope, these isoforms from the α gene do not appear to achieve different functions by binding to particular subsets of actin filaments or locations in cells. Some alternative possibilities are discussed. The results show that bacterially produced TMs can be used to study in vitro and in vivo properties of the isoforms.

THE tropomyosins (TMs)[1] are a family of actin binding proteins that are ubiquitously expressed in all tissues and cell types, although specific isoforms are characteristic of particular cell types. TMs are encoded by multiple genes and TM expression is known to be regulated by transcriptional and alternative RNA splicing mechanisms (see review by Lees-Miller and Helfman, 1991). In the rat, there are known to be 12 TM proteins expressed from three genes that likely arose through gene duplication events. The α TM gene codes for nine isoforms through the use of two promoters and alternative splicing mechanisms (Ruiz-Opazo and Nadal-Ginard, 1987; Wieczorak et al., 1988; Lees-Miller et al., 1990; Goodwin et al., 1991). These isoforms include the skeletal muscle and smooth muscle α TMs (Ruiz-Opazo and Nadal-Ginard, 1987), the three brain isoforms TMBr-1, TMBr-2, TMBr-3 (Lees-Miller et al., 1990) and four fibroblast isoforms TM-2, TM-3, TM-5a, and TM-5b (Goodwin et al., 1991). The β TM gene codes for two proteins by alternative splicing (Helfman et al., 1986) producing skeletal muscle β-TM and fibroblast TM-1, which is also found in smooth muscle where it corresponds to the smooth muscle

β-TM isoform. The TM-4 gene produces a single protein, TM-4, which is produced in several cell types (Yamawaki-Kataoka and Helfman, 1987; Lees-Miller et al., 1990).

The significance of this TM isoform diversity among muscle (skeletal, cardiac, and smooth) and nonmuscle cells is not fully understood. The repertoire of different isoforms is largely conserved from chicken to rat to man, an evolutionary span of more than 100 million years, suggesting that important functions exist among the TMs that have been maintained by selective pressure. This is exemplified in the conserved nature of the contraction mechanism of striated muscle where the expressed TM isoforms regulate, in association with the troponin complex, the highly ordered interaction of myosin heads and actin filaments in response to stimulating calcium fluxes (Ebashi et al., 1969; see also Cohen, 1975; Payne and Rudnick, 1989 for review). In contrast, although TM isoforms in smooth muscle and nonmuscle cells are largely conserved in various vertebrate species, the precise function of these proteins is still unclear. Since nonmuscle and smooth muscle cells do not contain a troponin complex, TMs must have a somewhat different role than in striated muscle. For example, one possibility is that TM may stabilize actin filaments in nonmuscle cells. This function has also been inferred from in vitro experiments which have shown that TM protects actin filaments against the

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severing action of gelsolin (Fattoum et al., 1983; Ishikawa et al., 1989) or the activities of brain depolymerizing factor (Bernstein and Bamburg, 1982). Other experiments have shown that the nonmuscle TMs prevent villin induced severing (Mooseker et al., 1982) or bundling of F-actin (Burgess et al., 1987), but they were unable to prevent bundling caused by 55-kD protein (Matsumura and Yamashiro-Matsumura, 1986). Biochemical studies have revealed differences in the properties of TMs isolated from muscle (skeletal, cardiac and smooth) and nonmuscle cells. For example, in vitro functions of striated muscle TMs include the binding of troponin T (Ebashi et al., 1969); prevention of destrin induced F-actin depolymerization (Nishida et al., 1985); inhibition of actin filament depolymerization from the pointed end (Broschat, 1990; Weigt et al., 1990); and inhibition of acto-myosin ATPase (Eaton et al., 1975). In contrast, TMs from smooth muscle and nonmuscle cells bind poorly to troponin (Pearlstone and Smillie, 1982) and have been shown to enhance the skeletal myosin or heavy meromyosin ATPase in vitro (Sobieszek and Small, 1977; Yamaguchi et al., 1984). These differences between the biochemical properties of TMs from muscle and nonmuscle cells suggest that each isoform will have a unique role in the respective cell types in which they are expressed.

Why some cell types contain only one or two TM isoforms while others express multiple isoforms is unknown. For example, rat fibroblasts contain at least six forms of TM, while skeletal muscle contains two isoforms, cardiac muscle a single isoform, and smooth muscle contains two isoforms. Microfilaments of fibroblasts represent dynamic structures that can exist in different supramolecular forms such as microfilament bundles (stress fibers), microfilament meshworks, polygonal networks, and contractile rings. The particular TM isoforms involved in these higher order actin structures are poorly defined. The multiplicity of isoforms in fibroblasts raises the possibility that specific associations of given isoforms with themselves and other cellular proteins are required for distinct functions. In nonmuscle cells TMs have been localized to actin filaments using both antibodies (Lazarides, 1975, 1982; Warren et al., 1985; Lin et al., 1988) or the microinjection of fluorescently labeled TMs from chicken smooth muscle, calf brain, and skeletal muscle (Wehland and Weber, 1980; Warren et al., 1985; Dome et al., 1988). Differences in the localization of various isoforms have been demonstrated with antibodies recognizing only the high molecular weight TMs versus those recognizing only the low molecular weight isoforms (Lin et al., 1988). The low molecular weight forms were found in both ruffles and stress fibers while the high molecular weight forms were found only in stress fibers. These differences in the distribution of different TM isoforms suggest a distinct, as of yet unidentified, role for the various isoforms.

In addition, one hypothesis that has been proposed is that changes in the relative levels of different TM isoforms in the cell may alter the organization of microfilaments. Accordingly, the alterations in microfilament structure and cell shape in transformed cells have been suggested to be the direct result of the altered patterns of TM expression observed in these cells (Matsumura et al., 1983; Hendricks and Weintraub, 1984; Cooper et al., 1985; Lin et al., 1985). Moreover, at least two reports have shown TM expression to return to normal levels in neoplastic cell lines which have spontaneously reverted to a nontransformed phenotype (Cooper et al., 1985; Fujita et al., 1990). However, at this time there is no direct evidence establishing a casual relationship between changes in TM isoform expression and alterations in cytoarchitecture.

Different biochemical properties have been reported for various nonmuscle isoforms. For example, studies of rat fibroblast TMs demonstrated that a mixture of the low molecular weight TM isoforms (termed TM-4 and TM-5) bound weakly to F-actin while the higher molecular weight isoforms (TM-1, TM-2, and TM-3) bound well (Matsumura and Yamashiro-Matsumura, 1985). Similarly, it has been shown that the low molecular weight TM isoform of equine platelets bound actin with low affinity (Côté et al., 1978) as does the low molecular weight TM isoform of brain (Broschat and Burgess, 1986). In contrast, however, the low molecular weight TMs isolated from human erythrocytes (Fowler and Bennett, 1984) and chicken intestinal epithelial cells (Broschat and Burgess, 1986) have been shown to bind with much greater affinity, similar to high molecular weight isoforms. These studies clearly indicate that different functional properties are a characteristic of specific isoforms suggesting the possibility of distinct functions in vivo. At present, few studies have addressed the function of TM isoforms in living cells. In vivo experiments which showed the alteration of intracellular granule movement following the microinjection of antibodies to TM into chick fibroblasts have suggested a function in intracellular motility (Hegmann et al., 1989).

All of the above evidence suggests fundamental roles for TM isoforms in maintaining normal cellular activity in different cell types. An understanding of the roles of this family of actin-binding proteins would further our understanding of the cell cytoskeleton and its role in cellular processes. However, it has been difficult to study the biochemical properties of the individual TM isoforms due to their very similar physical and chemical properties, which makes their isolation from cellular sources difficult. In addition, the individual isoforms have not been available for fluorescent modification to be used in microinjection studies to determine their localization in vivo. Finally, due to the conserved nature of many of the different isoforms, the in vivo localization of TM isoforms by antibodies has been hampered by the fact that most antibodies detect more than one isoform.

To begin to understand the function of the fibroblast TM diversity, we have prepared the individual isoforms using an *Escherichia coli* expression system in order to study the in vitro and in vivo properties of these proteins. In this study four fibroblast isoforms, TM-2, TM-3, TM-5a, and TM-5b, products of the α-TM gene, were expressed in a bacterial expression system. We have found that both low (TM-5a and TM-5b) and high molecular weight TMs (TM-2 and TM-3) bind to actin filaments in vitro, although TM-5b was capable of readily displacing the other isoforms from F-actin. In addition, we have microinjected fluorescently labeled TM-2, TM-3, TM-5a, and TM-5b into fibroblasts in order to see whether these isoforms behave differently in vivo. At the level of the light microscope all of the isoforms, despite their amino acid differences, were found to incorporate into the same structures of microfilaments. There was no preferred cellular location or subset of actin filaments for any of these isoforms. These results suggest that these TM isoforms do

not achieve different functions by binding to particular sub- sets of actin filaments or locations in cells. These studies also show that bacterially produced fibroblast TMs can be used to study in vitro and in vivo properties of the proteins.

Materials and Methods

TM cDNA Clones and Expression Constructs

The cDNA clones for rat fibroblast TM isoforms (TM-2, TM-3, TM-5a, and TM-5b) have been described (Goodwin et al., 1991). The coding sequences for TM-5a and TM-5b were excised from SP64 plasmids using the TM NcoI site and a HindIII site provided by the vector which was beyond the original DraI/HincII insertion site. The HindIII site was filled in using Klenow fragment and BamHI linkers added. The NcoI/BamHI fragment containing TM sequences was inserted into a NcoI/BamHI cut pET 8C expression vector (Studier et al., 1990) to form plasmids pET TM-5a and pET TM-5b. Expression plasmids pET TM-2 and pET TM-3 were made by utilizing another cDNA plasmid, SP64 TM-2, which contains the full coding sequence for TM-2. The NcoI/SacI fragment of TM-2 containing amino acids 1-234 was isolated and ligated into either pET TM-5a or pET TM-5b, with the respective NcoI/SacI fragment removed. Amino acids 235-284 were provided by the pET TM-5a or pET TM-5b plasmids, thus reconstructing TM-2 and TM-3 in the pET vector. The relationships of these expression plasmid constructs to each other and the α gene coding sequences are shown in Fig. 1 a. The predicted molecular weights for full length proteins are 32710 for TM-2, 32849 for TM-3, 28558 for TM-5a, and 28697 for TM-5b.

Purification and Characterization of TM Proteins Produced in Bacteria

The pET 8C expression system (Studier et al., 1990) uses an inducible T_7 RNA polymerase to drive transcription of the protein of interest. The cDNA clone for the protein is inserted into the pET plasmid just downstream of the T_7 promoter element. We have used the *E. coli* B strain BL21 (F-, ompt, rB-, mB-) harboring a DE3 lysogen with an IPTG inducible T_7 polymerase as host for Tm protein production. Bacteria for protein production were grown at 37°C in M9ZB media (10 g/l Bacto Tryptone, 1 g/l NH_4Cl, 3 g/l KH_2PO_4, 6 g/l Na_2HPO_4, 4 g/l glucose, 1 ml/l 1 M $MgSO_4$, 100 mM ampicillin) (DIFCO Laboratory, Detroit, MI) and induced when the OD_{600} was 0.8-0.9 by the addition of 0.1 g/l of isopropylthio-β-D-galactosidase (IPTG) (Bethesda Research Laboratories, Gaithersburg, MD). Bacteria were incubated for 2-4 h further. Before induction, the fraction of cells carrying the inducible plasmid was determined as described (Studier et al., 1990) and found to be 97-99%. The plasmids appear to have no toxic effects on the normal growth of the bacteria. Bactria were stored frozen in media with 50% glycerol at -70°C.

After induction, the bacteria were pelleted and washed once with PBS. Bacteria were resuspended in 30 ml of 50 mM Tris, pH 7.5, 100 mM KCl, 5 mM EDTA, 1 mM DTT, 100 μg/ml PMSF, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin, and lysozyme was added to 1 mg/ml. The tubes were incubated on ice for 20 min and then frozen for 30 min at -70°C. Upon thawing, 3 ml of 10% Triton X-100 was added and the samples mixed gently for 15 min at room temperature. Samples were spun in a rotor (SW28; Beckman Instruments Inc., Palo Alto, CA) at 25,000 rpm for 2 h at 4°C. The supernatant was decanted and the pelleted debris discarded. All of the TM isoforms were expressed in BL21 (DE3) bacteria as soluble proteins. TMs were present as 10-30% of the total cellular proteins as determined by gel scanning of Coomassie blue-stained gels. The TM protein was then purified using ammonium sulfate fractionation followed by resuspension in 0.1 M KCl, 25 mM Tris-Cl, pH 7.5, and overnight dialysis versus the same buffer. TM-3 and TM-5b precipitated at 25-55% ammonium sulfate whereas TM-2 and TM-5a precipitated at 30-65% ammonium sulfate. The TMs were then pH precipitated at their isoelectric point by adding sodium acetate, pH 4.5, to 20 mM final concentration and the samples allowed to sit on ice for 20 min, followed by centrifugation (15 min, 4,500 rpm, 4°C) (H6000 rotor; Sorvall Instruments, Newton, CT). Pellets were resuspended in ~30 ml 0.1 M KCl, 25 mM Tris-Cl, pH 7.5. Solid KCl was added to 1 M KCl and after dissolving, the solution was placed in a water bath at 95°C for 10 min. Any denatured proteins were pelleted by centrifugation (Sorvall RC3B; 15 min, 4°C) and the TMs which remained in solution were recovered by pH precipitation. Alternatively, for large scale preparations, the

Table I. NH_2-terminal Sequencing

	TM-2	TM-3	TM-5a	TM-5b
Cycle				
1	M 51.7	M 2.3	A 90.0	A 134.4
2	D 47.1	D 8.7	G 67.1	G 159.2
3	A 45.7	A 15.1	S 16.9	S 27.4
4	I 24.0	I 8.1	S 15.0	S 31.6
5	K 33.1	K 4.8	S 8.1	S 21.6
6	K 41.7	K 7.6	L 39.2	L 62.6
7	K 38.2	K 7.4	E 12.9	E 29.8
8	M 40.9	M 6.7	A 37.2	A 56.3

heat treatment step followed by pH precipitation was used before AS fractionation with similar final results. Anion exchange chromatography was carried out using Q Sepharose Fast Flow (Pharmacia Fine Chemicals, Piscataway, NJ) and a gradient of 50 to 450 mM sodium chloride containing 25 mM Tris, pH 7.5, 1 mM DTT. The fractions were analyzed by electrophoresis on 12% polyacrylamide gels containing SDS-PAGE using described conditions (Laemmli, 1970) and selected fractions were pooled and pH precipitated. Before the TMs were used in subsequent experiments, they were prebound to F-actin to insure that they were 100% active. For this, TMs were incubated with F-actin for 1 h in a buffer containing 100 mM KCl, 20 mM sodium phosphate, pH 7.0, and 0.5 mM DTT. The F-actin and associated TM was pelleted by centrifugation in a Beckman Airfuge (Beckman Instruments, Palo Alto, CA) at 28 psi. The supernatants were removed and the pellets resuspended in the same buffer. After resuspension, the concentration of KCl was increased to 0.4 M and the samples warmed to 65°C for 10 min to denature the actin, which was then pelleted by centrifugation in a microfuge at 14,000 rpm and the TM was recovered from the supernatant by pH precipitation as described above, and then resuspended in 0.1 M KCL, 20 mM sodium phosphate, pH 7.0, 0.5 mM DTT, and the pH adjusted to neutral pH with 0.25 M NaOH.

NH_2-terminal Sequencing

NH_2-terminal sequencing was carried out at the Protein Core Facility at Cold Spring Harbor Laboratory (Cold Spring Harbor, NY) essentially as described (Matsudaira, 1987). Briefly, TM isoforms were electrophoresed on a 12% polyacrylamide gel and electroblotted to polyvinylidene difluoride membranes (Millipore Corp., Bedford, MA) in 10% methanol, 10 mM 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS), pH 11.0. Membranes were lightly stained with Coomassie blue, 0.1% in 50% methanol, and the bands of interest were excised for gas phase sequencing on an Applied Biosystems 475 machine (Foster City, CA) using on line PTH derivatization and subsequent high-pressure liquid chromatography on an ABI 120A machine (Applied Biosystems Inc.) No blocked termini were present and sequencing was carried out for 8 to 18 cycles. Results of the first eight cycles are shown in Table I, with the yield in pmol for each cycle.

COOH-terminal Sequencing

The amino acid sequences of the carboxy termini of two of the TM isoforms were determined by the time course of amino acids released during digestion with carboxypeptidase Y. A solution (20 micromolar) of purified TM containing 20 micromolar proline, which is not present in the TM isoforms, as internal standard was digested with 0.6 μg/ml of carboxypeptidase Y (Boehringer-Mannheim Biochemicals, Indianapolis, IN) in 0.1 M sodium acetate, pH 6.0, at 25°C. Samples of 10 μl were removed at 0, 1, 5, 15, and 30 min, 1, 2, 4, and 6 h and mixed with 10 μl concentrated acetic acid to stop the reaction and then frozen. Samples were dried in a Speed-Vac and then prepared for amino acid analysis by derivatization with PITC and analyzed on an amino acid column (Waters, Inc., Danvers, MA). The results are shown as relative molar amounts in Table II.

Filamentous Actin Binding Assay

Bacterially produced tropomyosins were tested for their ability to bind to filamentous actin (F-actin) using an in vitro sedimentation assay essentially as described (Matsumura and Yamashiro-Matsumura, 1985) in a buffer containing 100 mM KCl, 20 mM sodium phosphate, pH 7.0, and 0.5 mM DTT. Routinely, incubations were for 1 h at room temperature followed by

	Min	M	N N	L L	E	L
TM-2	1	0.86	–	–	–	–
	15	0.92	0.82	0.71	0.34	–
	30	1.0	1.56	1.2	0.39	–
	60	1.1	1.25	1.27	0.45	–
TM-5b	1	0.47	–	–	–	–
	15	0.92	0.65	0.59	0.15	–
	30	1.0	1.31	1.41	0.33	–
	60	1.05	1.61	1.91	0.43	–

sedimentation in an airfuge (Beckman Instruments) at 28 psi for 30 min (at room temperature). All of the binding assays were performed using TMs that were prebound to F-actin as described above to avoid denatured or non-binding proteins in the final preparations. F-actin concentration in the assays was routinely 1 mg/ml while the TM concentration was varied from 0.015 to 1 mg/ml. Acetone powder from rabbit skeletal muscle was prepared (Pardee and Spudich, 1982) and stored at $-20°C$ and used for actin purification by the method of Pardee and Spudich (1982). F-actin prepared by a similar procedure was also obtained as a generous gift of Dr. Leonard Stein (Department of Cardiology, State University of New York, Stonybrook, NY). Rabbit skeletal muscle TMs (α and β isoforms) were prepared as described (Smillie, 1986). Protein determinations were originally done by amino acid analysis on the purified proteins and subsequently by the method of Lowry et al. (1951) using bovine serum albumin as the standard.

Fluorescent Labeling of TM Isoforms

FITC and Lissamine rhodamine B sulfonyl chloride (LRB) were obtained in 10 mg packaging from Molecular Probes Inc. (Eugene, OR). Fluorescent labeling of purified TM isoforms was carried out as described below based on the methods used for immunoglobulins (Brandtzaeg, 1975). Briefly, FITC was dissolved in N,N-dimethylformamide to 50 mg/ml and 2 μl was added to 2 mg of TM isoform in 1 ml of 0.3 M sodium carbonate, pH 9.0, and 0.1 M potassium chloride. The sample was stirred on ice for 4 h and quenched with a molar excess of glutamine. Free dye was removed by chromatography on a 1 × 20 cm Sephadex G-25 (Pharmacia Fine Chemicals) column equilibrated with 0.1 M potassium chloride, 20 mM sodium phosphate, pH 7.0. The labeled TM protein in the void volume was collected and pH precipitated by the addition of 1 M potassium acetate pH 4.6 to 20 mM and the protein collected by centrifugation in a microcentrifuge at 4°C and 14,000 g for 5 min. The protein was then resuspended in 300–500 μl, 0.1 M KCl, 20 mM sodium phosphate, pH 7.0, and adjusted to neutral pH with 1 M NaOH and applied to a second G-25 column. The labeled TM was collected as above and resuspended in 100 mM KCl, 20 mM sodium phosphate, pH 7.0, and 0.5 mM DTT, and brought to neutral pH. For labeling of isoforms with LRB, the dye was dissolved in acetone at 10 mg/ml and 30 μl was used to label 2 mg of protein for 20 min on ice with stirring. The reaction was stopped and proteins recovered as above. Dye to protein ratios were determined using the relationship $d/p = M_r/$concentration × OD dye/extinction coefficient and the molar extinction coefficients of 76,000 and 34,000 for FITC and LRB, respectively. Reaction times were varied slightly in these reactions to yield a dye to protein ratio of 1 to 1.5 for the labeled isoforms that were used for microinjection. The samples were then brought to neutral pH and dialyzed against the actin binding buffer. The labeled proteins were then prebound to F-actin and found to bind to F-actin quite similarly to the unlabeled proteins. For most microinjection experiments, the prebound LRB labeled TM isoforms were routinely used. The labeled TMs were stored on ice and normally used within one week.

Cell Culture Methods

Cultured rat embryo fibroblasts (REF 52) were grown in DME (Gibco BRL) containing 10% Rhehatuin 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% CO_2 atmosphere at 37°C.

Microinjection of Fluorescently Tagged Isoforms into Cultured Cells

Cultured cells were grown on gridded glass coverslips (Bellco Inc.,

Vineland, NJ) in normal growth medium for 36–48 h before use. Cells were needle microinjected using published procedures (Diacumakos, 1973; Graessman et al., 1980). Micropipets were pulled on a vertical puller (model 720; David Kopf Instruments, El Cajunga, CA) from 1 mm OD thin walled glass capillaries with internal filament (WPI Inc., New Haven, CT). During injections, cells were kept in normal medium (without serum) supplemented with HEPES to 25 mM, pH 7.0, to maintain pH. Temperature was maintained at 37°C using a stage warming block designed to hold 60 mm dishes or culture chamber (constructed by M.F. Pittenger). Purified labeled TMs to be injected were first dialyzed vs. 100 mM KCl, 20 mM sodium phosphate, pH 7.0, centrifuged at 140,000 g for 20 min and the protein concentrations then determined. The cells were microinjected with a TM concentration of 2–5 mg/ml and an amount equal to 5–10% of the cell volume. This is estimated to increase the amount of a given TM isoform severalfold. Micropipets were filled from the back end by capillary action. After injection cells were incubated for 2–4 h (unless otherwise stated) in a CO_2 incubator before fixation.

For fixation, coverslips of injected cells were rinsed in a cytoskeletal stabilizing buffer (4 M glycerol, 10 mM EGTA, 1 mM MgCl, 25 mM Pipes, pH 6.9) and then incubated in the same buffer containing 0.2% Triton X-100 for 5 min at room temperature to allow unincorporated proteins to diffuse away. The cells were then fixed for 40 min in freshly made 4% formaldehyde (from base hydrolysis of paraformaldehyde; Polysciences Inc., Warrington, PA) in PBS, pH 7.0. The coverslips were then thoroughly rinsed with PBS and PBS containing 100 mM glycine to quench any remaining reactive formaldehyde. Where stated, the cells were then treated with primary mAbs (Tm 311; Sigma Chemical Co., St. Louis, MO) in PBS for 40 min, washed in PBS (3 × 5 min at room temperature) and fluorescent secondary goat anti-mouse antibodies (Cappel Laboratory, West Chester, PA) applied for 40 min, followed by PBS washes. Alternatively, cells were counterstained with FITC-phalloidin (Molecular Probes Inc., Eugene, Oregon) to stain F-actin in some experiments. Fluorescence microscopy was accomplished with a Zeiss Axiophot using either 40× water immersion, 63× (1.4 NA), or 100× (1.2 NA) objective lenses (Carl Zeiss Instruments, Inc., Thornwood, NY). Images were recorded on Kodak 400 or P3200 Tmax film (Eastman Kodak, Co., Rochester, NY) developed according to manufacturer's instructions.

Results

Preparation and Characterization of Bacterially Produced TMs

To prepare homogeneous preparations of rat fibroblast TM-2, TM-3, TM-5a, and TM-5b, the cDNA for each isoform was inserted into the pET 8C expression vector (Studier et al., 1990). These vectors allow full-length proteins to be made starting with the ATG codon of the cDNA and the products therefore contain only TM encoded amino acids. The pET 8C vector is shown in Fig. 1 B. The cDNA clones corresponding to the four fibroblast α gene products (see Fig. 1 A) were cloned into pET 8C using the naturally occurring NcoI site at the initiating codon and a BamHl site introduced 59-bp downstream of the stop codon in the 3'-untranslated region. These cDNAs have been previously described (Goodwin et al., 1991). The TM proteins were purified from the bacterial lysates using modifications to those methods described for isolation of tropomyosin from tissue (Bailey, 1948; Bourdillon and Baker, 1956; see Materials and Methods). The proteins were analyzed biochemically by SDS-PAGE, amino acid analysis, amino- and carboxy-terminal sequence analysis, and their ability to bind to F-actin.

The bacteria produced the soluble TM isoforms constitutively but levels were further increased by induction with IPTG. An example of the SDS-PAGE results of the crude lysates is shown in Fig. 2 A. The addition of IPTG to the growing cultures in early log phase is indicated by the plus (+) sign. The arrowheads indicate the TM isoforms which were of the expected size for full-length proteins. Representative samples of the purified TMs are shown in Fig. 2 B.
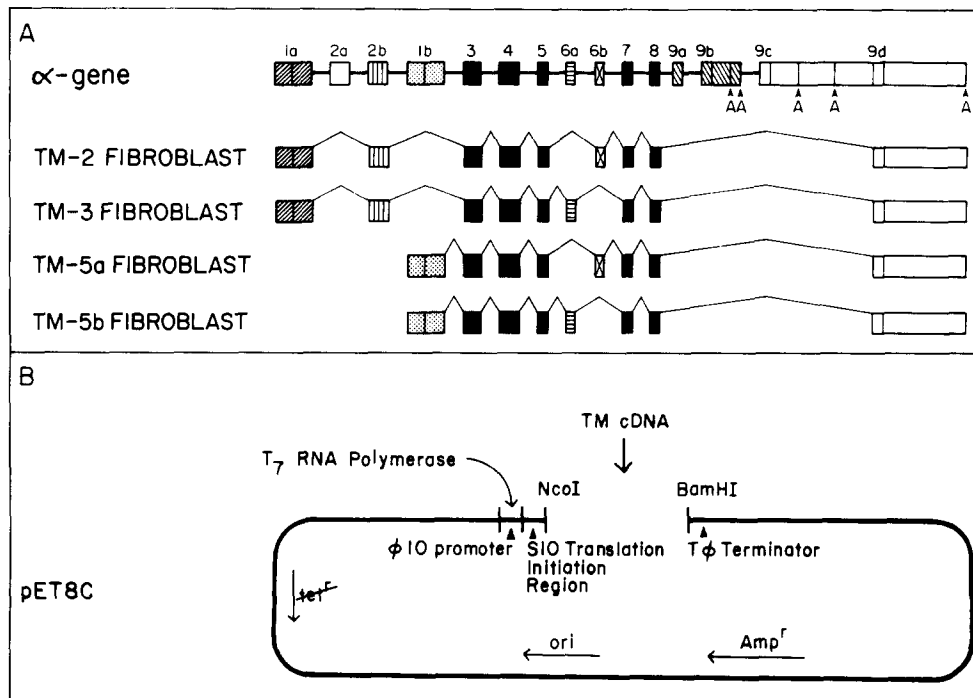
Figure 1. Diagram of the fibroblast TMs from the rat α-TM gene and the pET-8C bacterial expression plasmid. (*A*) Rat fibroblast TM isoforms TM-2, TM-3, TM-5a, and TM-5b are encoded by the α gene. The fibroblast isoforms TM-2 and TM-3 are transcribed using the promoter upstream of exon 1a, while TM-5A and TM-5B use the internal promoter upstream of exon 1b. Exon 6b is used in TM-2 and TM-5a while exon 6a is used in TM-3 and TM-5b. The solid boxes denote exons which are common to all α gene products. TMs 2, 3, 5a, and 5b all use exon 9d and the last poly A addition site (identified by the *A*). These fibroblast isoforms have identical amino acid sequences for exons 3 through 5 and 7 through 9. See Goodwin et al. (1991) for details. (*B*) Diagram of the pET 8C bacterial expression plasmid showing the pertinent regions. The gene for T₇ RNA polymerase is present in the host BL 21 bacteria as part of the lysogenic bacteriophage λ derivative DE3. Its expression is under the control of the *lacUV5* promoter which is inducible with IPTG. The T₇ polymerase is highly specific for φ10 promoter. See Studier et al. (1990) for details.

Densitometry of the Coomassie blue stained gels revealed 93–97% of the protein in each lane was present as a band of the appropriate molecular weight for full-length TMs. Faint bands could be seen near the major band and may represent modified species. The right hand lane of Fig. 2 *B* contains partially purified TMs isolated from cultured rat fibroblasts, the numbers to the right indicate the identity of the endogenous TM isoforms. Bacterial TM-2 and TM-3 appear to comigrate with their counterparts isolated from fibroblasts, while TMs 5a and 5b from bacteria exhibit a slightly faster mobility. It is unlikely that this is due to some posttranslational modification or processing occurring in the bacteria as in vitro transcription and subsequent translation in a rabbit reticulocyte extract yielded TMs with these same mobilities. Although TMs in fibroblasts are not known to be modified, this remains a possible explanation.

Amino-terminal sequencing was carried out on the full-length products to determine 8–18 amino acids of these proteins (see Table I, Materials and Methods). All bands were readily sequenced by Edman degradation and found to have an unblocked amino terminus corresponding to the correct sequence, but in the cases of TM-5a and TM-5b, the initiating methionine had been removed. Thus, for TM-2 and TM-3 the methionine was retained, while for TM-5a and TM-5b, the penultimate alanine residue was the terminus. Although these isoforms have not yet been sequenced from cellular sources, the equine platelet TM, another of the low molecular weight isoforms (247 amino acids), was also shown to have the methionine removed (Côté et al., 1978). We also used carboxypeptidase Y to determine the sequence of the carboxy terminus of the isoforms (see Table II, Materials and Methods). By the time course of the amino acids released, the sequences were determined to be correct for full-length TM isoforms. The amino acids determined for the amino- and carboxy termini for the various TMs are given in Table III.

Bacterially Produced TMs Bind F-actin In Vitro

To further characterize the biochemical properties of the bacterially produced TMs, their ability to bind and co-sediment with F-actin was tested. For this, isoforms were first prebound to F-actin and repurified as described in Materials and Methods to assure that the added TM was completely active. Decreasing concentrations of each TM isoform were incubated with a fixed amount of F-actin purified from rabbit skeletal muscle. The samples were centrifuged and tropomyosin co-sedimenting with the F-actin, as well as that remaining in the supernatant, was resolved on 12% SDS–polyacrylamide gels. The binding of the TM isoforms produced in bacteria was also compared to TMs purified from rabbit skeletal muscle (α and β skeletal isoforms, products of the α and β genes, respectively). The Coomassie blue–stained gels were quantitated by densitometry and compared with standard curves generated using known amounts of TM isoforms. Association constants were determined using the TM dimer molecular weights of 65,420, 65,698, 57,116, and 57,394 for TM-2, TM-3, TM-5a, and TM-5b, respectively. Examples of the results are shown in Fig. 3. All four of the fibroblast TMs (TM-2, TM-3, TM-5a and TM-5b) were found to bind to F-actin under these conditions. They did not sediment when F-actin was not present (data not shown but see Fig. 4, *A* and *B*, lane 5). Without the pre-binding to F-actin, TM-2, TM-3, and TM-5b were found to bind with an affinity comparable to the rabbit skeletal TMs (Fig. 3, *A*, *B*, *C*, and *E*). However, the TM-5a isoform did not bind as well as the other isoforms (Fig. 3 *D*) but eventually did show saturation binding at high con-
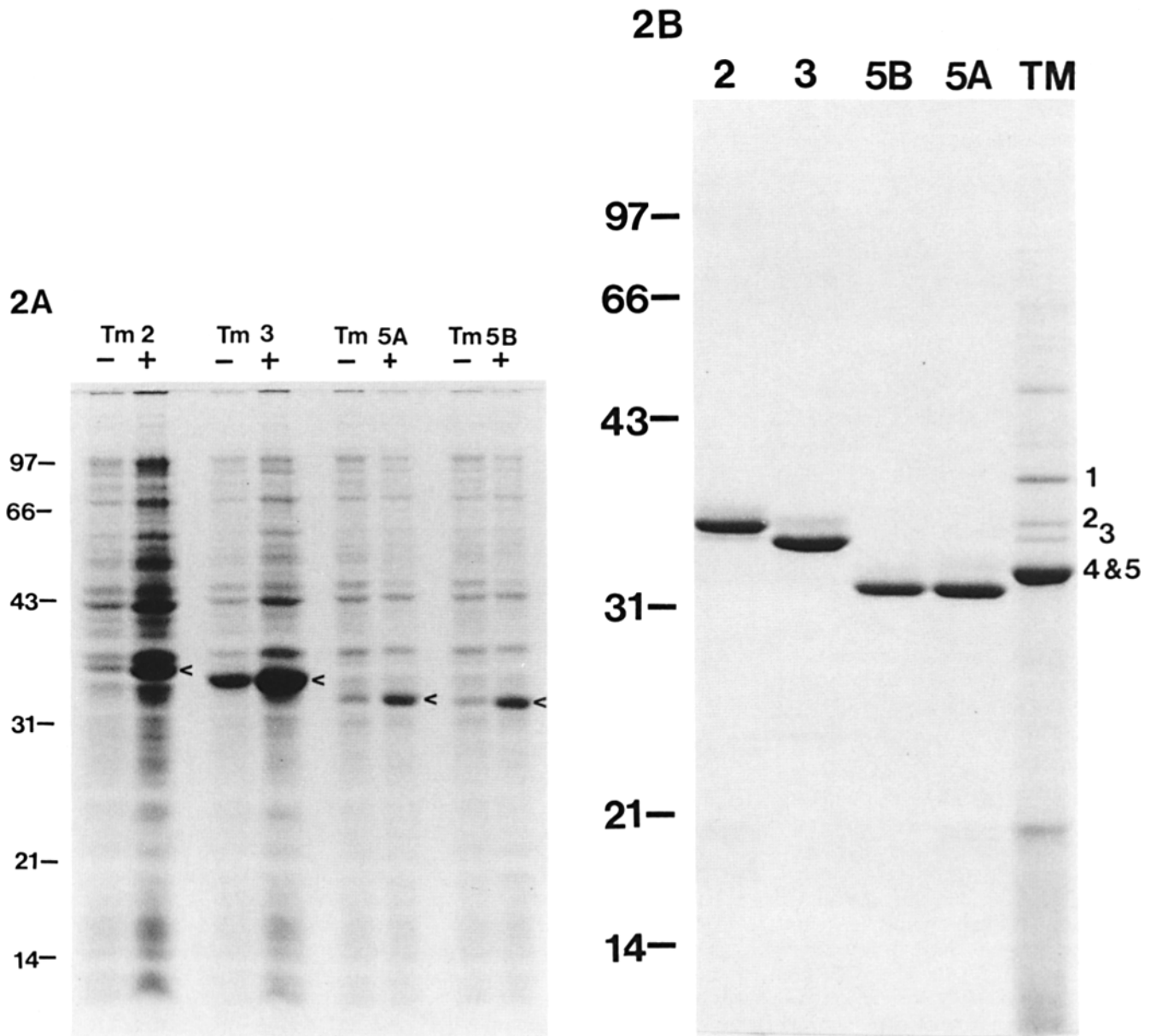
Figure 2. Induced expression of fibroblast TMs in *E. coli* and the purified isoforms. (*A*) The expression of the TM isoforms in the pET vector was constitutive, but could be induced to higher levels with IPTG. 1 ml of log growth culture was taken before induction with IPTG (− lanes) and 2 h after its addition (+ lanes). The bacteria were centrifuged and the pellet then lysed directly in 100 μl of SDS gel sample buffer. 10 μl of each sample was loaded onto a 12% gel. In *B*, 2 μg of the purified isoforms were resolved on a 12% SDS gel. For comparison, the lane marked TM contains the partially purified TMs of a fibroblast cell line, with the respective isoforms indicated to the right.

centrations (above 25 μm dimer, data not shown). Therefore, the TM-5a sedimenting with F-actin was purified and used in a subsequent actin-binding assay. As shown in Fig. 3, *F* and *G*, the amount of TM-5a bound to actin was similar to

Table III. Sequence of NH$_2$ and COOH Terminus of Bacterially Produced TMs

Isoform	Amino terminus	Carboxy terminus
TM-2	MDAIKKKM-	-(LLEL)NNM
TM-3	MDAIKKKMQML-	Not determined
TM-5a	AGSSSLEAV-	Not determined
TM-5b	AGSSSLEAV-	-(DQT)LLELNNM

the other isoforms, suggesting that the original results with TM-5a were due to a heterogeneous protein population. Isoforms 2, 3, and 5b did not show a significant increase in F-actin affinity when such a prebinding step was included (data not shown). However, for consistancy, each of these was also prebound and repurified for use in the remaining experiments in this study. Binding assays with isoforms prebound to F-actin revealed the expected cooperativity and apparent K_as of 5 μM for TM-2, 3.5 μM for TM-3, 3.0 μM for TM-5a, 2.0 μM for TM-5b.

TM-5b Is Better than TM-5a in Displacing Other Isoforms from F-actin

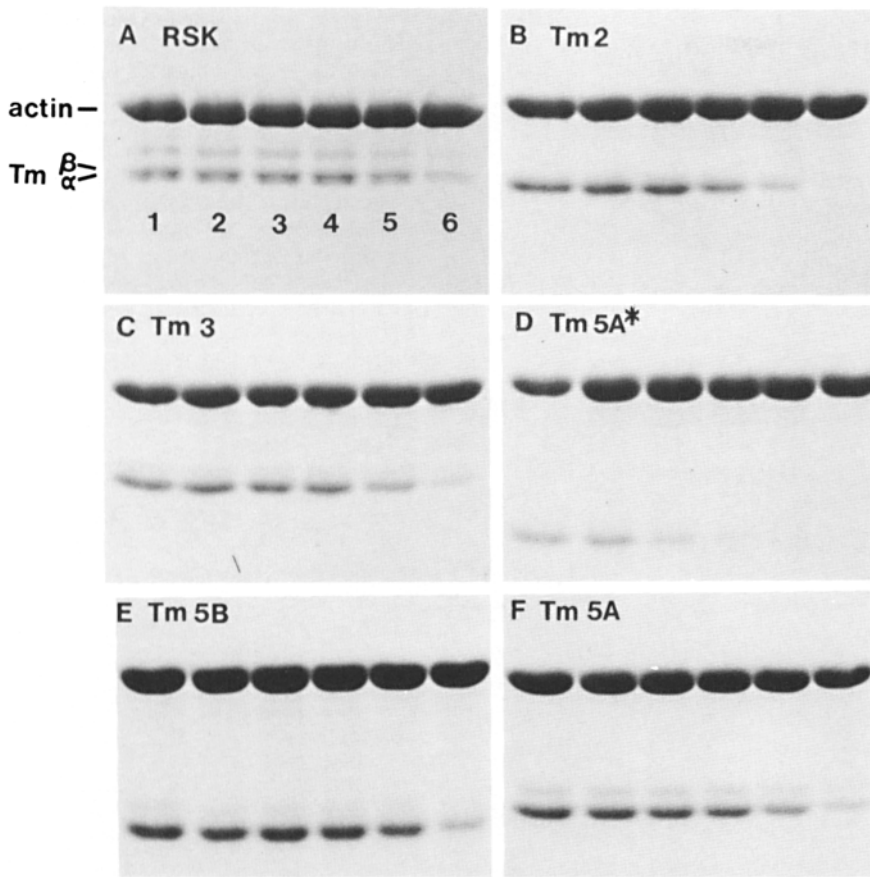The differences in the amino acid sequence between TM-5a

Figure 3. Actin-binding assay using the bacterially produced TMs; comparison with rabbit skeletal muscle TMs. Decreasing concentrations of purified isoforms of TMs 2, 3, 5a, and 5b were tested for their ability to bind to 1 mg/ml of rabbit skeletal muscle F-actin. Lanes 1 through 6 represent concentrations of 1, 0.75, 0.50, 0.25, 0.125 and 0.06 mg/ml, respectively. F-actin and bound TM were sedimented in a Beckman Airfuge and supernatants and pellets resolved on a 12% SDS–polyacrylamide gel and stained with Coomassie blue. Only the pellets are shown here. TM-2, TM-3, and TM-5b were found to bind with similar high affinity, comparable to skeletal TMs (α and β, $\diamond$ on graph). As purified, TM-5a (D and x on graph) was found to bind with a lower affinity than the other isoforms. However, when TM-5a was first bound to actin at saturating concentrations and then sedimented and repurified and used in the binding assay, it bound with an affinity similar to the other isoforms (see F and ● on graph). Subsequently, all of the TMs were prebound to F-actin and repurified before further experiments. G shows the results of binding assays using prebound isoforms to determine the apparent K_as which were 5 μM for TM-2, 3.5 μM for TM-3, 3.0 μM for TM-5a, 2.0 μM for TM-5b, and 2.5 μM for rabbit skeletal muscle TM.

and TM-5b (due to the use of exons 6a or 6b, see Fig. 1) has led to the hypothesis that TM-5b would bind actin filaments with a higher affinity than TM-5a (Goodwin et al., 1991; see Discussion below). To determine whether TM-5a and TM-5b have different affinities for F-actin in vitro, we analyzed their relative ability to displace TM-2 from actin filaments.

Isoforms TM-2, TM-5a, and TM-5b were each prebound to F-actin and repurified. F-actin was pre-incubated with saturating amounts of TM-2 for 30 min and increasing amounts of either TM-5a or TM-5b were added for an additional 60 min. The F-actin bound TMs were then pelleted by centrifugation and the proteins in the supernatants and
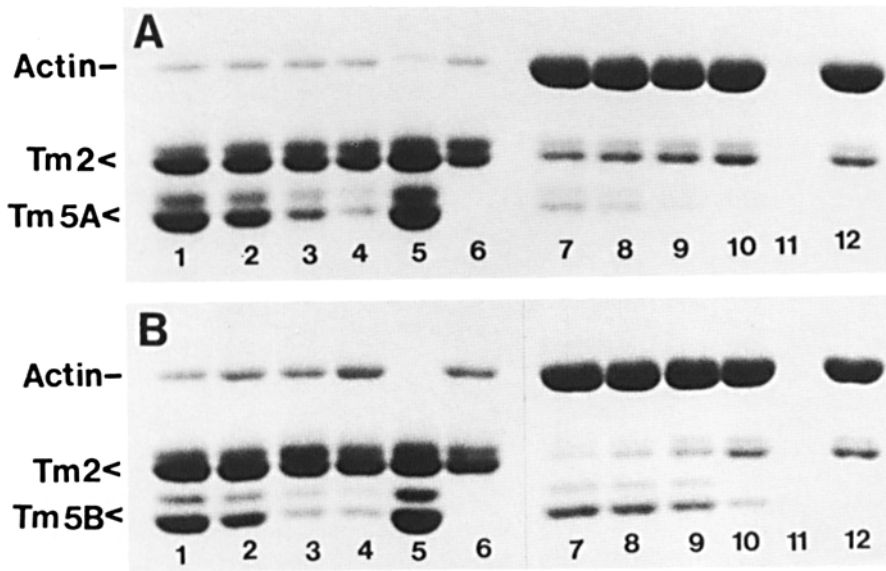
Figure 4. TM-5b is more effective than TM-5a at displacing TM-2 from F-actin. TMs were first prebound to F-actin and repurified. A constant amount of F-actin (1 mg/ml or 23 μM) was then incubated with a constant saturating amount of TM-2 (0.5 mg/ml or 15 μM) for 30 min at room temperature. Decreasing amounts of TM-5a (*A*) or TM-5b (*B*) were added for an additional hour and the F-actin with associated TMs pelleted. Supernatants and pellets were then resolved by 12% SDS–polyacrylamide gels. The supernatants are shown in lanes *1–6* and the pellets are in lanes *7–12*. Lanes *1* and *7*, 26 μM (0.75 mg/ml); lane *2* and *8*, 17 μM (0.5 mg/ml); lanes *3* and *9*, 8.7 μM (0.25 mg/ml); lanes *4* and *10*, 4.3 μM (0.125 mg/ml); lanes *5* and *11*, no actin, 15 μM TM-2, 17 μM TM-5a, or TM-5b (residual denatured actin in *A*, lane *5* is from the prebinding step); lanes *6* and *12*, no Tm-5a (*A*) or TM-5b (*B*).
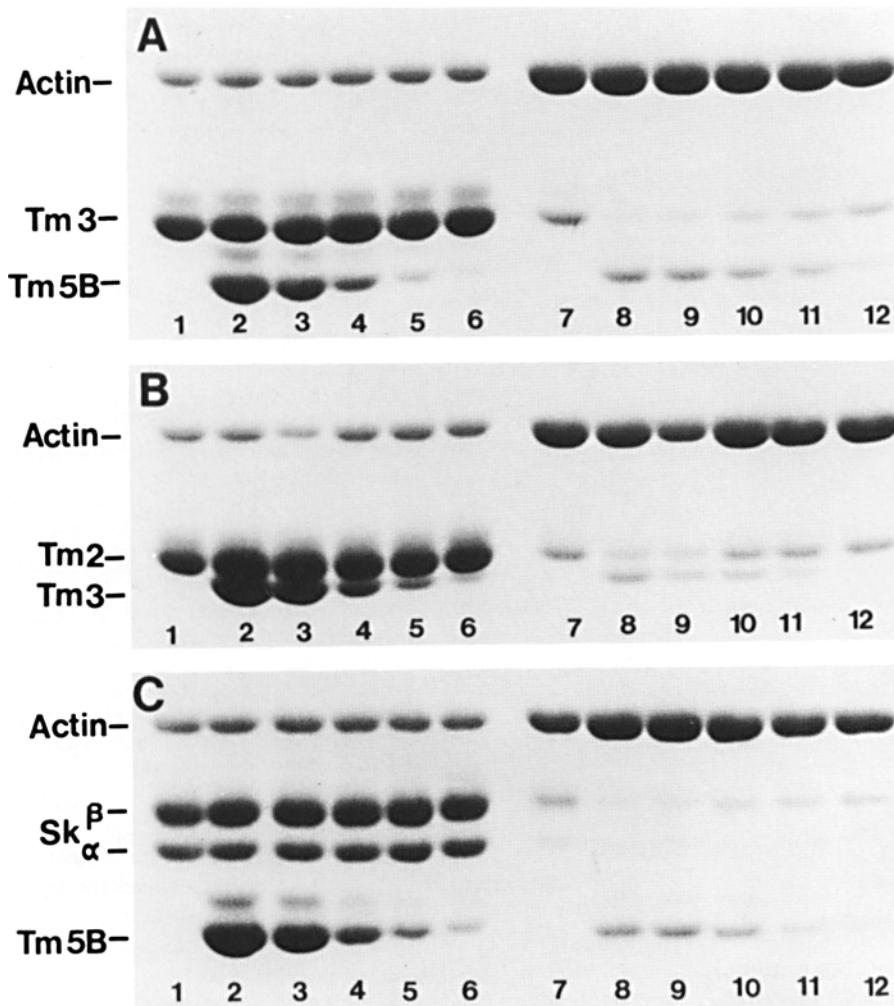
Figure 5. TM-5b efficiently displaces TM-3 or skeletal muscle TMs. Similar to Fig. 4, F-actin (1 mg/ml) was incubated with saturating amounts (0.5 mg/ml) of TM-3 (*A*), TM-2 (*B*) or skeletal muscle TMs (*C*) for 30 min at room temperature and then decreasing amounts of TM-5b (*A* and *C*) added for an additional hour. To compare TM-2 vs. TM-3 binding (*B*), 0.5 mg/ml of TM-2 was added for 30 min and decreasing amounts of TM-3 added for an additional hour. F-actin and associated TMs were pelleted and the supernatants and pellets resolved on a 12% gel. The supernatants are shown in lanes *1–6* and the pellets in lanes *7–12*. Lanes *1* and *7*, no second TM added; lanes *2* and *8*, 0.75 mg/ml second TM; lanes *3* and *9*, 0.5 mg/ml second TM; lanes *4* and *10*, 0.25 mg/ml second TM; lanes *5* and *11*, 0.125 mg/ml second TM; lanes *6* and *12*, 0.062 mg/ml second TM. TM-5b, at equal concentration, readily displaced TM-3 or the skeletal muscle TMs. However TM-3 did not readily displace TM-2.
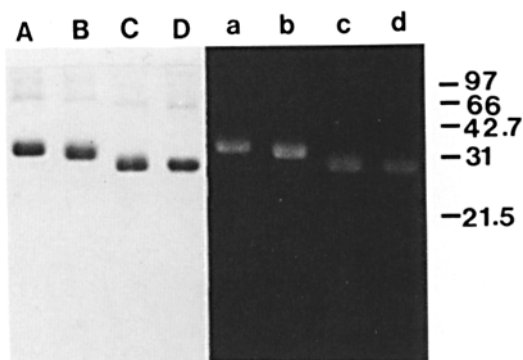
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Figure 6. Purified fibroblast TMs labeled with Lissamine rhodamine B. Fibroblast TM isoforms produced in bacteria were purified and labeled with Lissamine rhodamine B as described in Materials and Methods. The fluorescently labeled proteins were separated from unincorporated dye by column chromatography and pH precipitation. The dye to protein ratio was determined (varied from 1.1–1.3 in these samples) and 3 µg of each labeled protein was run on a 12% polyacrylamide gel. The gel was first photographed under UV illumination (lanes *a*, *b*, *c*, and *d*) and then stained with Coomassie blue and photographed on a light box (lanes *A*, *B*, *C*, and *D*). Labeled TM-2 was run in lane *A* (*a*), TM-3 in lane *B* (*b*), TM-5a in lane *C* (*c*), and TM-5b in lane *D* (*d*). Molecular weight markers are shown to the right.

pellets resolved by SDS-PAGE. The results are shown in Fig. 4 with the supernatants shown in lanes *1–6* and the pellets in lanes *7–12* (see legend for details). TM-5b was more effective in binding and displacing TM-2 from actin. TM-5b, at 8.7 µM displaced >50% of the bound TM-2 in the presence of saturating amounts of TM-2, while TM-5a was much less effective. With 8.7 µM TM-5a, only 10–20% of the TM-2 was displaced. This experiment was performed four times with two preparations of each isoform and gave the same results. These results indicated TM-5b had a greater affinity than TM-5a for F-actin. TM-5b was also tested for its ability to displace TM-3 or TMs prepared from skeletal muscle (containing a mixture of α and β skeletal TMs) from F-actin.

As shown in Fig. 5, *A* and *C* (supernatants in lanes *1–6* and pellets in lanes *7–12*), TM-5b was quite efficient at displacing TM-3 or the skeletal muscle TMs. In displacing the skeletal muscle TMs, TM-5b did not preferentially displace either the α or β isoform, but displaced them with equal efficiency. We also tested the relative ability of TM-3 to displace TM-2. These two isoforms differ only in the 24 amino acid exon that also constitutes the difference between TM-5a and TM-5b (see Fig. 1). As shown in Fig. 5 *C*, these two isoforms bound to F-actin with similar affinities and TM-3 did not displace TM-2 with the efficiency that TM-5B displaced other TMs.

Fluorescent Derivatization and Microinjection of TM Isoforms

To determine whether the functional significance of the multiple fibroblast TM isoforms may be related to differences in their intracellular localization, each of the bacterially produced TMs was fluorescently labeled and introduced into cultured fibroblasts by microinjection. Each isoform was labeled in separate reactions with amine reactive fluorophores LRB and FITC. The reaction conditions were adjusted to yield a dye to protein ratio of 1 to 1.5. Examples of the LRB-labeled isoforms are shown in Fig. 6. The labeling procedure did not significantly alter the binding of the isoforms to F-actin in the binding assay (data not shown). Cultured rat embryo fibroblasts (REF 52 cells) were microinjected with the fluorescently labeled TMs and the cells were returned to the incubator for up to 4 h to allow the proteins to equilibrate with the endogenous pools and incorporate into the actin cytoskeleton. TM incorporation into the microfilaments could be seen as early as 10 min after injection and was nearly complete throughout the cytoskeleton in 30–40 min. There was not a detectable difference in TM incorporation at the cell periphery compared to the perinuclear region suggesting the dynamic incorporation occurs along the filament's length rather than at the ends (data not shown). An example of a living cell microinjected with fluorescent TM-5b and showing incorporation into the actin cytoskeleton is
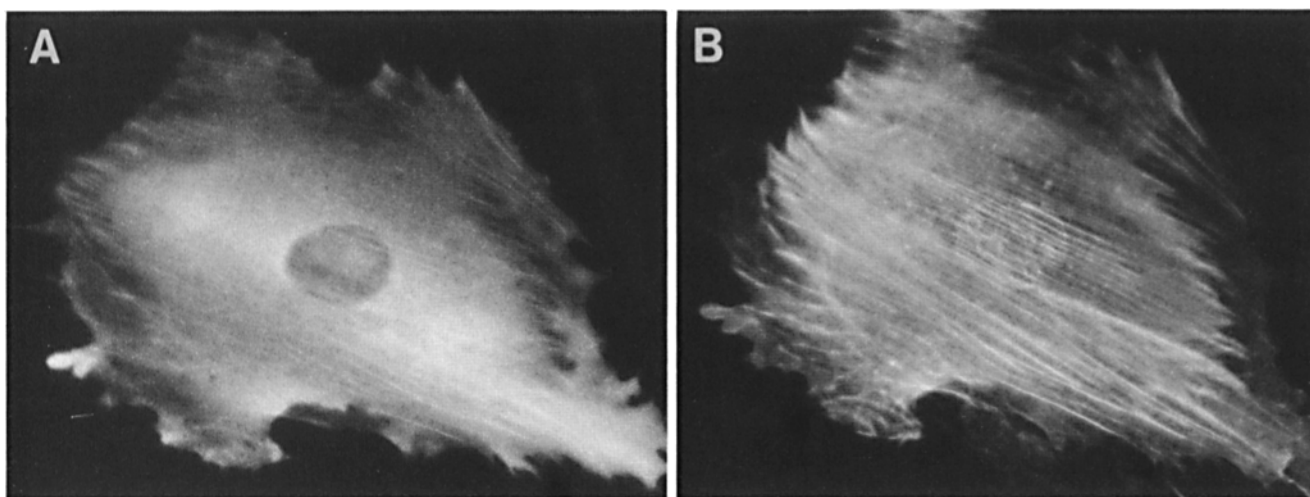

Figure 7. Distribution of microinjected TM-5b is quite similar in living and fixed cells. To show that the labeled TMs were associated with the microfilament system before fixation procedures and not as a consequence thereof, cells were microinjected and photographed with fluorescent optics while living (*A*) and following fixation (*B*). Note that the same structures are labeled and that the resolution is enhanced by extraction and fixation.
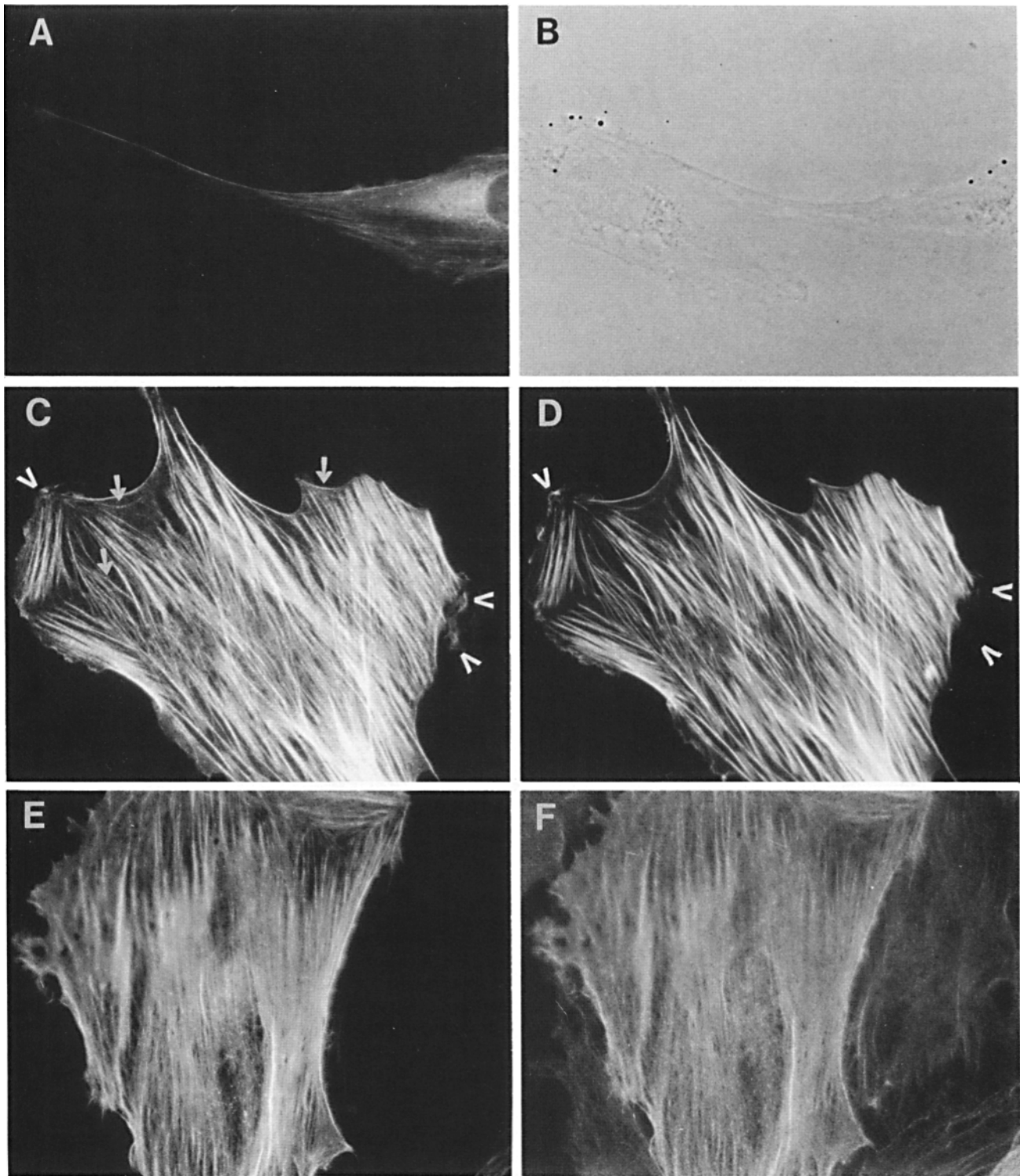
Figure 8. Microinjection of fluorescently labeled TM-2. *A*, *C*, and *E* show cells microinjected with LRB-TM-2. In *A*, TM-2 can be seen in the long cellular process as well as the cell body. An uninjected neighboring cell is also seen in the phase image in *B*. TM striations can be seen in well spread cells (*arrows* in *C*) which are not seen in FITC–phalloidin-stained microfilaments (*D*). The arrowheads point to the edges of the cell where TM-2 is present. *E* shows the injected TM-2 viewed in the rhodamine channel while *F* shows the same field with cells stained by TM antibodies and a fluorescein labeled secondary antibody.

shown in Fig. 7 *A* and labeling of microfilaments are clearly visible. The cells on this coverslip were subsequently extracted with a low concentration of non-ionic detergent in a cytoskeletal stabilizing buffer to allow the unincorporated proteins to diffuse out. The cells were then fixed with freshly

prepared formaldehyde in PBS, mounted and observed by fluorescence microscopy. Fig. 7 *B* shows the same cell as in Fig. 7 *A* after fixation and the filaments are more clearly seen. Detectable labeling of microfilaments was present as late as 48 h after microinjection, although the intensity of the
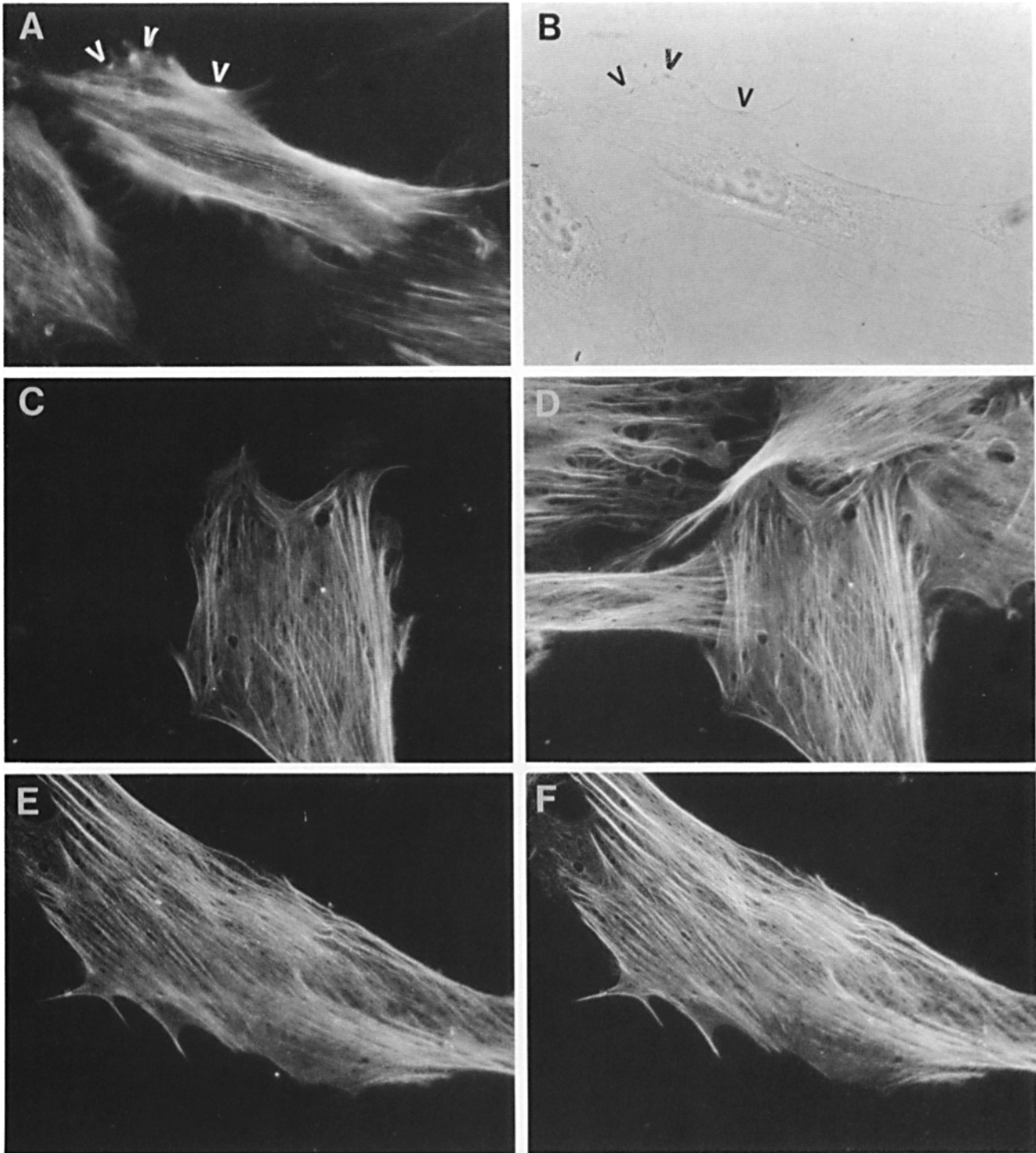
Figure 9. Microinjection of fluorescently labeled TM-3. Examples of REF 52 cells microinjected with LRB-TM-3 are shown in *A*, *C*, and *E*. *B*, *D*, and *F* show the corresponding phase (*B*) or FITC–phalloidin-stained fields (*D* and *F*). TM-3 is found out to the edge of the cell and in edge ruffles (*arrowheads*). The cells shown here do not show well developed striations in the fluorescent TM-3 labeled microfilaments although other examples have shown them. *D* shows the neighboring uninjected cells labeled with FITC-phalloidin.

fluorescence had declined greatly. In all cases, the microinjection of TM had no detectable effects on the morphology of the cells over the time period they were observed.

Bacterially Produced TMs Bind to Microfilaments In Vivo

Fluorescently labeled TM-2 was microinjected into REF 52

cells and typical results are shown in Fig. 8. Fig. 8, *A*, *C*, and *E* show the distribution of the Lissamine rhodamine B–labeled TM-2. Fig. 8 *A* is an example of TM-2 present in long cellular extensions. Fig. 8 *B* is a phase image of *A* showing the injected cell and a nearby uninjected cell. In Fig. 8 *C*, the arrowheads show TM-2 present in small ruffles at the cell periphery. There are striations present in many of the fila-
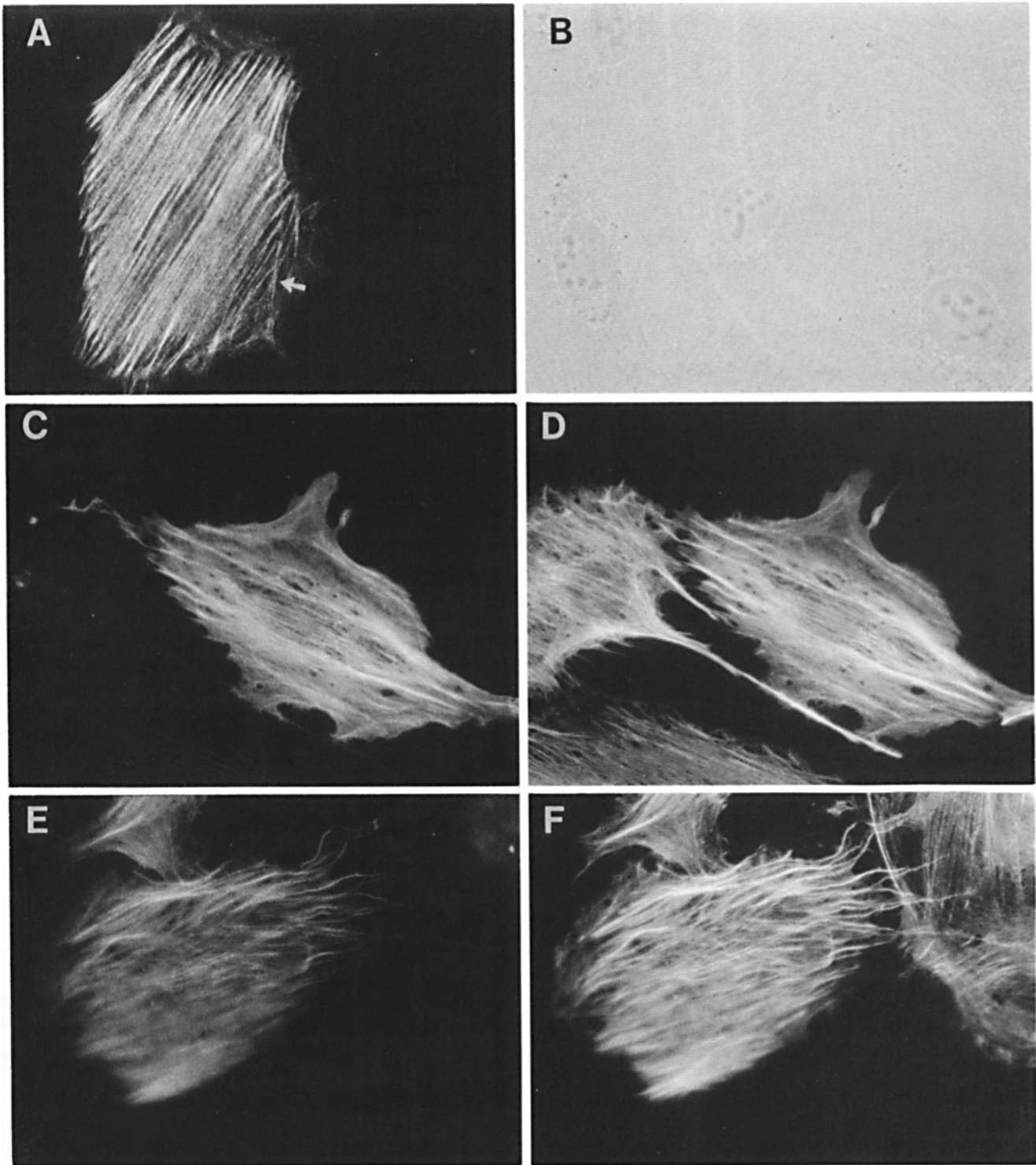
Figure 10. Labeling of microfilaments in fibroblasts microinjected with LRB-TM-5b. Visualization of microinjected LRB-TM-5b in fibro-blasts can be seen in *A*, *C*, and *E*. Striations are clearly seen in the confluent cell shown in *A*. The corresponding phase micrograph is shown in *B*. *D* and *F* show the same fields as *C* and *E*, respectively, but with filters to reveal FITC–phalloidin staining of microfilaments.

ments in this cell also (see arrows), a pattern that is com-monly seen with TM antibodies. The dark interband region has been shown to be the site of α-actinin on the filaments (Feramisco and Blose, 1980). Fig. 8 *D* is the same cell as in *C* but viewed with optics to reveal FITC–phalloidin stain-ing of F-actin. Some, but not all of the ruffles show phalloidin staining and the striations are not visible. Fig. 8 *E* shows an-

other LRB-TM-2 injected cell while panel F shows the same field with TM antibody staining in the FITC channel. Note that the injected cell appears more brightly stained with the antibody than surrounding cells but does not show any mor-phological alteration.

LRB-TM-3 microinjected cells are shown in Fig. 9. Fig. 9, *A*, *C*, and *E* show TM-3 incorporated into all regions of
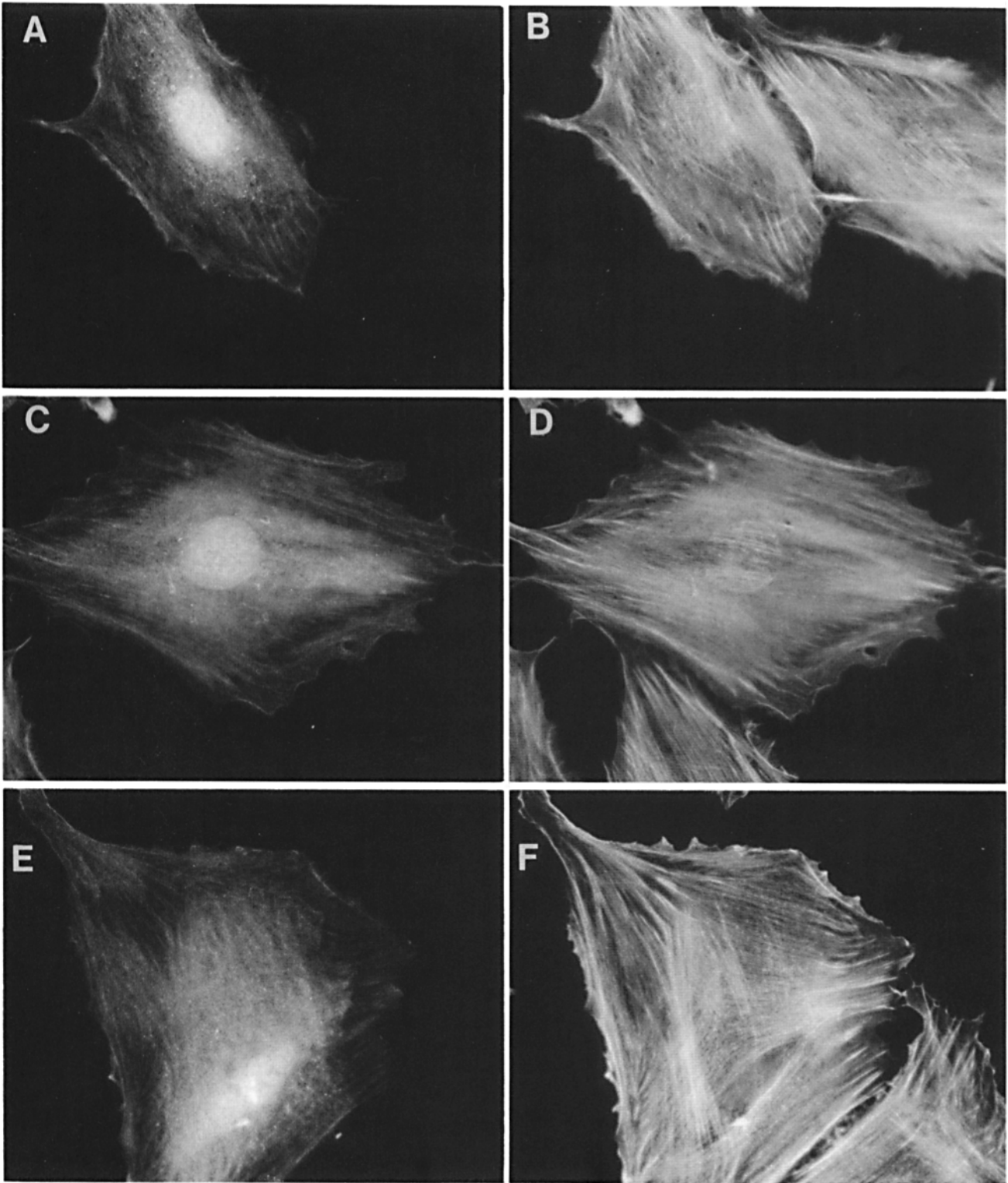
Figure 11. LRB-TM-5a labeling of microfilaments in fibroblasts. *A, C,* and *E* show REF 52 cells microinjected with LRB-TM-5a. The microfilaments are labeled throughout their length and no unlabeled microfilaments were found when compared to the phalloidin stain. The corresponding FITC–phalloidin-stained fields are shown in *B, D,* and *F.* Some staining of the nuclear region was noted with this isoform. This perinuclear staining was also seen with the other isoforms before their pre-binding to F-actin but remained with TM-5a.

the microfilament system. The arrowheads in Fig. 9 *A* point to areas of increased fluorescence which correspond to membrane ruffles in the phase image in Fig. 9 *B.* Fig. 9, *D* and *F* show the FITC–phalloidin-labeled actin filaments in the injected and neighboring cells. No morphological altera-

tions are apparent in the injected cells. The TM striations are not well developed, but still present. In general, striations were well developed in well spread, confluent cells and rarely seen in active, motile cells.

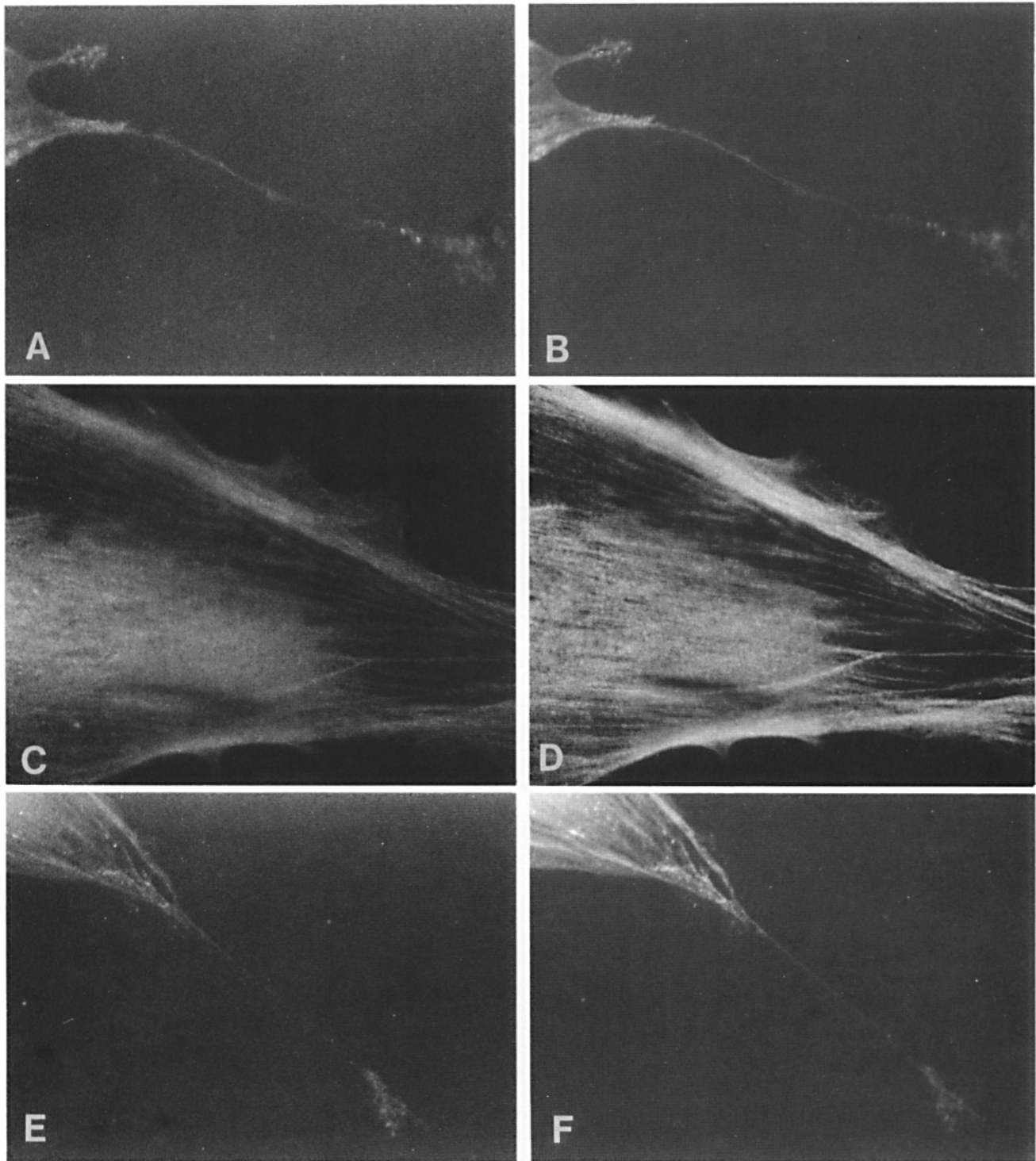Fig. 10 shows the results of microinjecting TM-5b labeled

Figure 12. Microinjection of cells with two TM isoforms labeled with contrasting fluors show coincident staining. *A* shows FITC-TM-2, while *B* shows LRB-TM-5a in the same cell. *C* and *E* are examples of FITC-TM-3 in microinjected cells and *D* and *F* show LRB-TM-5b in the respective cells. In each example, both isoforms are found throughout the cells, at the cell periphery and in long cellular extensions.

with LRB. In Fig. 10 *A*, well-developed striations are present in this injected cell of a confluent monolayer (see phase image in Fig. 10 *B*). Other examples of LRB-TM-5b microinjected cells are shown in Fig. 10, *C* and *E* while *D* and *E* show the injected cells and neighboring cells labeled with FITC–phalloidin. In these examples, it can be seen that at the level of the light microscope, all actin filaments labeled

with phalloidin are also labeled with this shorter TM isoform and the labeling of microfilaments is indistinguishable from that found with TM-2 or TM-3.

LRB labeled TM-5a was also microinjected into REF 52 cells and representative results are shown in Fig. 11. TM-5a was found to bind along microfilaments and striations could be weakly seen, but the level of incorporation into filaments

appeared to be less than with the other isoforms. Fig. 11, *A*, *C*, and *E* show cells microinjected with LRB-TM-5a while Fig. 11, *B*, *D*, and *F* show the same cells and their neighbors labeled with FITC–phalloidin. As well as the filamentous actin labeling seen with the other isoforms, some localization appeared present that weakly labeled the nuclear region. This localization was difficult to focus and did not depict known structures and may represent non-specific staining as similar nuclear region staining was also seen with the other isoforms when the labeled TMs were not prebound to F-actin.

The images of the fluorescently labeled TMs microinjected into fibroblasts always appeared as if each isoform labeled the same population of actin filaments. To investigate this further, mixtures of two TM isoforms labeled with contrasting fluorochromes were co-microinjected into cells. Examples of the results are shown in Fig. 12 at higher magnification than in previous figures. Fig. 12 *A* shows FITC-TM-2–labeled filaments while Fig. 12 *B* shows the same cell with rhodamine optics to show the co-injected LRB-TM-5a. FITC-TM-3–labeled microfilaments are shown in *C* and *E* and the corresponding labeling with LRB-TM-5b in the respective cells is shown in *D* and *F*. The staining appears coincident in both stress fibers and fine microfilaments at the cell periphery for the pairs of short and long isoforms. These experiments were repeated with the isoforms labeled with the opposite fluorochromes and with the co-injection of TM-2 and TM-5b with comparable results (data not shown).

Using microinjection, there is a range of volumes that actually enters the cells. However, the results were essentially the same whether a small or large amount of labeled-TM isoform was injected. An example of this is shown in Fig. 13, where in a field of confluent cells, the filamentous and striated labeling of actin filaments with LRB-TM-5b is clearly seen in the cell to the lower right in *B*. Two cells to the left in Fig. 13 *B* have a much higher fluorescence intensity which, upon adjusting the exposure level, can be seen to be similarly filamentous (*C*).

In summary, at the level of fluorescence microscopy, it was found that each isoform appeared to uniformly label the same actin filaments, without a preference for a particular subset of filaments or region of the cell. With few exceptions (see Fig. 8, *C* and *D*) the TM fluorescence was always coincident with phalloidin staining which is known to bind F-actin but not G-actin. There was not a preference of the lower molecular weight TMs (5a or 5b) for the ruffling edge of the cell as has been reported using antibody staining (Lin et al., 1988). Each isoform appeared to give very similar labeling of actin filaments and each was seen to label filaments in a striated fashion in confluent cells. However, TM-5a consistently produced fainter images and slight nuclear staining. This nuclear staining could not be easily focused in the microscope. Similar staining was also noted with other isoforms if the labeled isoforms were microinjected into cells without an F-actin binding step following the labeling reaction. The co-injection of labeled isoforms showed identical staining patterns in the cell and this co-localization extended to even the finest extensions of the cell periphery. Clearly, the shorter isoform TM-5b, does label actin filaments with a similar distribution as the longer isoforms TM-2 and TM-3. These longer forms are also found in fine cellular extensions where they are not part of a soluble pool that is ex-
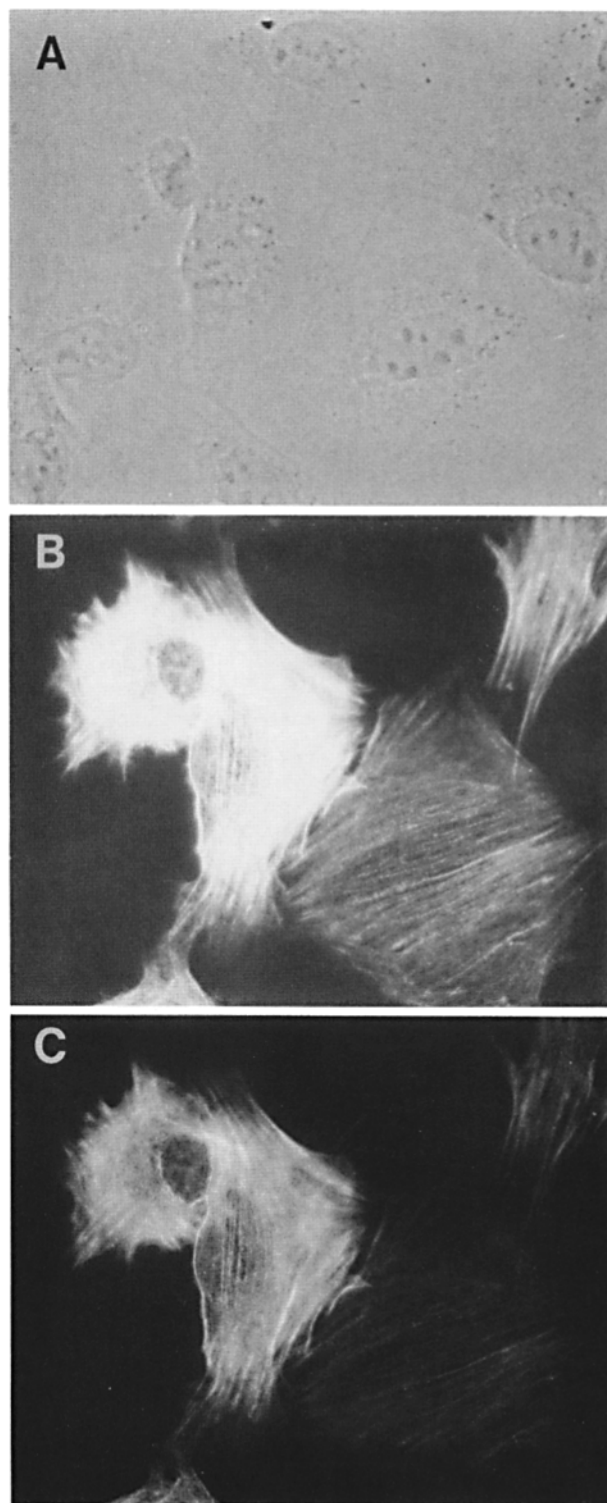

Figure 13. Pattern of microfilament staining is not altered by the amount of labeled isoform injected into the cell. A difference in the amount of LRB-5B injected into fibroblasts did not change the localization of the isoform. *A* is a phase image of confluent REF 52 cells. *B* and *C* show a short and long exposure respectively of the same field in which two cells are highly fluorescent while a third cell has a decreased level of fluorescence due to difference in the amount of injected protein. The filamentous staining is quite similar.

tractable from the cell, but are associated with the cytoskeleton. For TM-5a, the results are similar to the other isoforms except for the association with the nuclear region which may be non-specific.

Discussion

Biochemical Characterization of Bacterially Produced TMs

While much is known about the structure and function of TMs from striated muscle, relatively little is known about the isoforms from non-muscle cells. This is in large part due to the difficulty in isolating the individual isoforms from cells and tissues. By expressing the cDNA clones in bacteria, it is possible to obtain the individual TMs. In this paper, we demonstrate the usefulness of this approach to study the fibroblast TMs. Four fibroblast isoforms, all products of the rat α TM gene, were prepared in an *E. coli* expression system and subsequently used to study their biochemical and biological properties. These studies demonstrate that these bacterially produced TMs have biochemical properties similar to their cellular counterparts.

Amino acid sequencing of each purified TM showed no blocked amino termini and the correct sequence for the amino and carboxy termini of each isoform. It is of interest that the bacteria have removed the amino-terminal methionine from TM-5a and TM-5b but not from TM-2 and TM-3. In vertebrate cells, this modification of TMs has only been reported for the equine platelet TM (Coté et al., 1978) which is the homolog of the rat TM-4 protein (Yamawaki-Kataoka and Helfman, 1987). The function of this processing is unknown. The amino termini of TM-5a and 5b from mammalian sources have not yet been sequenced so it is unclear whether the methionine is normally removed. In *E. coli*, it has been shown that the penultimate and, sometimes, the third amino acid residue have strong bearing on NH$_2$-terminal excision by methionyl aminopeptidase and this activity depends largely on the size of the side chain at the penultimate position (Hirel et al., 1989). The TM-5a and 5b processing found here in *E. coli* is consistent with these previous findings.

Although TMs isolated from tissue sources have an acetylated amino terminus (Coté et al., 1978; Sanders and Smillie, 1978, 1985; Mak et al., 1980), the function of this acetylation in vivo is not known. It has been suggested that it may neutralize the otherwise positive charge that would exist on the amino terminus, and thereby aid the head-to-tail overlap of neighboring TMs along the actin filament (Heald and Hitchcock-DeGregori, 1988). The bacterially-derived fibroblast isoforms lack this acetylation but bind to F-actin in vitro with properties comparable to TMs isolated from skeletal muscle. In contrast, studies of skeletal muscle α-TM produced in bacteria showed that it bound poorly to actin (Hitchcock-DeGregori and Heald, 1987). These authors suggested that this was because the *E. coli* product is not acetylated. We have also produced the skeletal muscle α-TM in bacteria and observed that it does not bind as well as the fibroblast isoforms to actin filaments in vitro even when prebound to F-actin and repurified (our unpublished results). However, it is likely that the differences in the actin-binding properties between the fibroblast and striated muscle iso-

forms produced in *E. coli* are due to differences in the carboxy termini of these proteins because except for the COOH terminus, the fibroblast TM-2 is identical to the skeletal muscle α-TM. In addition, although the high and low molecular weight fibroblast isoforms reported here differ from each other at amino-terminal and internal regions, they all contain the same carboxy terminal coding region. In contrast, the skeletal muscle α-TM contains a different carboxy-terminus (exon 9b in Fig. 1). Lin and co-workers (personal communication) used bacterial expression to produce human fibroblast TM-3 and TM-5a and chicken smooth muscle α-TM for biochemical studies. Similar to our results, these authors also found that the lack of acetylation did not inhibit F-actin binding. Collectively, the foregoing results suggest that the differences in F-actin binding between the fibroblast isoforms and skeletal muscle α-TM lie in this carboxy terminal region.

Previous studies of TMs isolated from cellular and tissue sources have demonstrated that the low molecular weight TM from intestinal epithelium bound strongly to F-actin whereas the low molecular weight TMs from brain (Broschat and Burgess, 1989) or a mixture of the low molecular weight TMs from fibroblasts bound poorly (Matsumura and Yamashiro-Matsumura, 1985). We have recently shown that the low molecular weight TM from intestinal epithelium is predominantly TM-5b, whereas normal rat fibroblasts (REF-52 cells) contain predominantly TM-5a (Goodwin et al., 1991). These two isoforms differ only in the region from amino acids 153–177 due to the alternative splicing of exons 6a and 6b (see Fig. 1). There are 16 of 24 amino acid differences between these two exons. We hypothesized that, due to the amino acid differences in this region, TM-5b would have a stronger affinity for actin filaments relative to TM-5a (Goodwin et al., 1991). This region of TM-5a is identical to amino acids 189–213 of striated muscle α TM. In this segment of the striated muscle isoform, the actin binding repeat described by McLachlan and Stewart (1976) is interrupted, presumably because of the requirement for troponin T binding, needed for the regulation of muscle contraction. The amino acids in this region of TM-5b however, maintain the actin binding motif. It is worth noting that the low molecular weight isoforms from brain are comprised of TMBr-3 and TM-5a of the α-TM gene, and TM-4 from the TM-4 gene (Lees-Miller et al., 1990). These isoforms are highly conserved in the region of amino acids 153–177 (this region is encoded by exon 6b in TM-5a), and therefore could be expected to bind with lower affinity to actin as reported (Broschat and Burgess, 1989) than isoforms containing sequences similar to those found in amino acids 153–177 of TM-5b (encoded by exon 6a) (see Fig. 1). The results presented in this study showing that TM-5b is more efficient than TM-5a in displacing TM-2 from F-actin supports this hypothesis (Fig. 4). TM-5b is also capable of displacing TM-3 or skeletal muscle TMs prepared from rabbit muscle from F-actin with similar ability. We also extended the competition experiments to TM-3 and skeletal muscle TM (α and β isoforms) because we suspected that TM-3 would be most strongly bound to F-actin as it contains exon 6a, as well as being a 284 a.a. isoform capable of stronger head-to-tail overlap. We were surprised to find that TM-5b could displace TM-3 (see Fig. 5 A) and that TM-3 and TM-2 showed approximately the same binding ability. These results sug-

gest that the head-to-tail overlap or sequences contained within exon 2b of TMs 2 and 3 has a negative effect on their ability to interact with microfilaments. Experiments are in progress to distinguish these possibilities.

Bacterially Produced TMs Bind to Actin Filaments In Vivo

The work presented here shows that bacterially produced tropomyosin isoforms can be used to study the in vivo properties of these proteins. After microinjection, fluorescently labeled TM-2, TM-3, TM-5b and TM-5a were found throughout the microfilament network. However, TM-5a gave a relatively less intense fluorescence image than the other isoforms and also showed some nuclear region localization. The isoforms were all labeled at approximately the same dye-to-protein ratio, so the fainter microfilament localization is most likely due to a lower ability of TM-5a to incorporate into actin filaments relative to the other isoforms. This is consistent with the in vitro studies showing that TM-5a was displaced from actin filaments by TM-5b (Fig. 4). The nuclear localization was also found with the other fluorescently labeled TMs when they were not bound to actin and repurified prior to microinjection. However, for labeled TM-5a treated in a similar fashion this localization still persists. This nuclear localization was diffuse and therefore did not appear to represent known structures. Whether this is true localization of this isoform or represents non-specific binding of TM-5a will require additional experiments.

Previous studies to analyze the distribution of TMs in fibroblasts by microinjection were limited to TMs isolated from smooth muscle and brain. Much of this previous work was accomplished before the number of TM isoforms or an understanding of the alternative splicing patterns for TM genes was known. Our experiments extend these studies and are the first to use the fibroblast isoforms for localization in fibroblasts. The fluorescence patterns obtained with the individual microinjected fibroblast TMs are largely in agreement with previous studies using both antibodies (Lazarides, 1975, 1976; Warren et al., 1985; Lin et al., 1988) and microinjection of fluorescently labeled smooth muscle and brain TMs (Wehland and Weber, 1980; Dome et al., 1988), showing that TMs are associated with the actin-containing microfilaments of the cytoskeleton.

Although distinct functions based on differential localization of various isoforms is an intriguing model, we have not seen evidence for this in the present study. To date, differences in TM isoform localization in fibroblasts have only been demonstrated in one study using antibodies that recognize either low or high molecular weight isoforms (Lin et al., 1988). The low molecular weight forms were found in both ruffles and stress fibers while the high molecular weight forms were found only in stress fibers. In contrast, using microinjection of fluorescently labeled proteins, we did not detect differences in the localization of the isoforms in actin-containing structures. While localizations of injected tropomyosins in cellular processes and cell bodies were noticed, these were not specific for any of the α gene isoforms used and this was more clearly shown in the double label experiments (Fig. 12). Whether this reflects the limitations of using E. coli produced proteins or that other fibroblast TM isoforms will show different localizations is unknown. For example, the distribution of rat fibroblast TM-1 and TM-4,

which are the most abundant high and low molecular weight isoforms in REF-52 cells (Matsumura et al., 1983) has not yet been determined. Another concern is whether the microinjection of relatively large amounts of purified labeled TM correctly mimic the localization of the endogenous isoforms. Towards this question, it should be noted that in a field of microinjected cells there is a range of volumes that actually enters the cells. Although the total fluorescence intensity was seen to vary between injected cells, this did not result in a difference in isoform localization (see Fig. 13 and Results). Furthermore, labeled TMs were injected at a wide range of protein concentrations, and no differences in the localization of the various isoforms was detected (data not shown). It is worth noting that at the level of the light microscope the co-injection of labeled TMs appears to give coincident staining, but the resolution does not allow the determination of whether each isoform is present on the same actin filament or on an adjacent filament. Moreover, along the filaments, there are two grooves in which the tropomyosin resides. If the TM isoforms are on the same filament, what is their arrangement? Is it random or is there a specific pattern or distribution of TM isoforms along an actin filament? This question may be answered at the electron microscope level by labeling the isoforms produced in E. coli with epitope tags and using epitope antibodies or labeling the TMs with colloidal gold particles of differing diameters to locate the TM isoforms following microinjection. Finally, it is possible that TM isoforms are differentially localized, but the mechanism also involves the localization of the mRNA. For example, evidence for localization of mRNA has been shown for certain cytoskeletal proteins (Lawrence and Singer, 1986; Garner et al., 1988) as well as homeotic mRNAs (Berlath et al., 1988; see also Rebagliati et al., 1985). If such a mechanism does exist, microinjection of TM protein may not exhibit the proper subcellular distribution.

Differences in the relative amounts of each of the TM isoforms could also provide a mechanism for regulating microfilament assembly. The changes in the levels of various TM isoforms following transformation correlate with alterations in microfilament structure but no evidence providing a direct link between these events has been reported. Another possibility is that the various TM isoforms may be important for the binding of other cellular proteins to actin filaments. Accordingly, such proteins might interact with the microfilament system by associating with particular TM isoforms bound along the actin filaments. In this regard, we have seen evidence for the differential interaction of ^{125}I-labeled TM-5a and TM-5b with proteins from several tissues (Pittenger, M., and D. Helfman, unpublished results). Using similar techniques, tropomodulin, a TM-binding protein has been identified and isolated from erythrocyte membranes (Fowler, 1987). Clearly, further studies are needed in order to elucidate the significance of TM isoforms in cellular function. With the availability of the different isoforms, both biochemical and cellular properties of these proteins can be addressed in the future.

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