Forced Expression of Chimeric Human Fibroblast Tropomyosin Mutants Affects Cytokinesis

Kerri S. Warren, Jenny L.-C. Lin, Jeff P. McDermott, and Jim J.-C. Lin

Department of Biological Sciences, The University of Iowa, Iowa City, Iowa 52242-1324

Abstract. Human fibroblasts generate at least eight tropomyosin (TM) isoforms (hTM1, hTM2, hTM3, hTM4, hTM5, hTM5a, hTM5b, and hTMsmα) from four distinct genes, and we have previously demonstrated that bacterially produced chimera hTM5/3 exhibits an unusually high affinity for actin filaments and a loss of the salt dependence typical for TM-actin binding (Novy, R.E., J. R. Sellers, L.-F. Liu, and J. J.-C. Lin, 1993. *Cell Motil. & Cytoskeleton.* 26: 248–261). To examine the functional consequences of expressing this mutant TM isoform in vivo, we have transfected CHO cells with the full-length cDNA for hTM5/3 and compared them to cells transfected with hTM3 and hTM5. Immunofluorescence microscopy reveals that stably transfected CHO cells incorporate force-expressed hTM3 and hTM5 into stress fibers with no significant effect on general cell morphology, microfilament organization or cytokinesis. In stable lines expressing hTM5/3, however, cell division is slow and sometimes incomplete. The doubling time and the incidence of multinucleate cells in the stable hTM5/3 lines roughly parallel expression levels. A closely related chimeric isoform hTM5/2, which differs only in the internal, alternatively spliced exon also produces defects in cytokinesis, suggesting that normal TM function may involve coordination between the amino and carboxy terminal regions. This coordination may be prevented in the chimeric mutants. As bacterially produced hTM5/3 and hTM5/2 can displace hTM3 and hTM5 from actin filaments in vitro, it is likely that CHO-expressed hTM5/3 and hTM5/2 can displace endogenous TMs to act dominantly in vivo. These results support a role for nonmuscle TM isoforms in the fine tuning of microfilament organization during cytokinesis.

Additionally, we find that overexpression of TM does not stabilize endogenous microfilaments, rather, the hTM-expressing cells are actually more sensitive to cytochalasin B. This suggests that regulation of microfilament integrity in vivo requires stabilizing factors other than, or in addition to, TM.

NONMUSCLE cells, unlike smooth or striated muscle cells, express multiple isoforms of tropomyosin (for review see Lees-Miller and Helfman, 1991). The generation of tropomyosin (TM)[1] isoforms involves regulated alternative splicing of an internal exon, as well as exons encoding the terminal "head and tail" regions (Lees Miller and Helfman, 1991; Pittenger et al., 1994 for reviews), domains shown to be essential for muscle TM function in vitro (Mak and Smillie, 1981; Hitchcock and Heald 1987; Cho et al., 1990; Bartegi et al., 1990; Novy et al., 1993*b*). This specific pattern of exon use in fibroblasts is conserved between human, rat, and chicken (Novy et al., 1993*c*; Pittenger et al., 1994; Bradac et al., 1989; Libri et al., 1989; Forry-Schaudies et al., 1990) and produces two size classes of TM isoforms, low and high molecular weight TMs with 248 and 284 amino acids (aa), respectively.

Representative isoforms from these two TM classes have been shown to have different localization within cultured human and chick embryo fibroblasts (Lin et al., 1988*a*), with high molecular weight TMs associated with the stable stress fibers and low molecular weight TMs found on stress fibers and in highly motile ruffle regions. In vitro characterization of nonmuscle TM isoforms from human, rat and chicken (Lin et al., 1985*a*, 1988*a*; Fowler and Bennett, 1984; Matsumura and Yamashiro-Matsumura, 1985; Pittenger and Helfman, 1992; Broschat and Burgess, 1986) have also shown isoform-specific actin-binding properties. We have reported differences for bacterially produced human fibroblast TM high and low molecular weight isoforms, as well as for chimeric combinations of high and low molecular weight isoforms (Novy et al., 1993*a*) in their ability to bind actin and enhance actin-activated HMM-ATPase activity in a manner consistent with the suggestion that low molecular weight isoforms may be more involved with regulation of

Address all correspondence to J. J.-C. Lin, Department of Biological Sciences, The University of Iowa, 138 Biology Building, Iowa City, IA 52242-1324. Tel.: (319)335-1075. Fax: (319)335-1069.

1. *Abbreviations used in this paper*: aa, amino acid; MSO, mitotic shake off; TM, tropomyosin.

motile processes and high molecular weight isoforms with microfilament protection and organization. This stabilization role for high molecular weight TM was further suggested by the in vitro findings (Ishikawa et al., 1989) that high molecular weight TM more effectively protects actin filaments from the severing activities of gelsolin than low molecular weight TM. Additionally, the down regulation of high molecular weight TM in transformed cells is coincident with the diminished, poorly organized actin cytoskeleton and rounded morphology characteristic of the transformed phenotype (Lin et al., 1984, 1985*a*; Hendricks and Weintraub, 1981; Cooper et al., 1985). A high-affinity nonmuscle TM was also found to slow the depolymerization of the actin filament pointed end in vitro (Broschat et al., 1989). There has been, however, no direct evidence for a stabilization role for TM in vivo, except that a yeast tropomyosin (TPM1) null mutant appears to have less stable actin cables (Liu and Bretscher, 1989).

Correlative data aside, the role of tropomyosin in nonmuscle cells has not been well characterized and even less is known about isoform-specific functions. We have studied bacterially produced human fibroblast TM isoforms hTM3 (high molecular weight), hTM5 (low molecular weight) and their chimeric isoforms hTM5/3 and hTM3/5, in vitro, and have found that hTM5 binds actin more strongly and amplifies actin-activated HMM-ATPase activity to a greater extent than hTM3. The chimeric mutant hTM5/3 has an affinity for F-actin that is even greater than that of hTM5 and does not demonstrate the salt dependence typical for TM-actin binding (Novy et al., 1993*a*).

To more directly study the physiological role of TM in vivo, we have separately transfected CHO cells with full-length cDNAs encoding tight-binding mutant hTM5/3 and wild-type isoforms hTM3 and hTM5. In this report we describe that, in stably transfected CHO cells, hTM3 and hTM5 colocalize with actin filaments, consistent with the distribution of endogenous tropomyosin. CHO cells stably expressing hTM5/3, however, sometimes possess disruption of F-actin bundles and a significantly increased incidence of multinuclearity that is not seen in cells expressing hTM3 or hTM5. Interestingly, expression of chimeric isoform hTM5/2, which differs from hTM5/3 only in one internal exon, also causes a high incidence of multinucleate cells. These results suggest that nonmuscle TM participates in microfilament organization and cytokinesis, and that the terminal head and tail exon-encoded regions are functionally coordinated within each tropomyosin.

Additionally, we report that overexpression of TM isoforms hTM3, hTM5, hTM5/3, and hTM5/2 in CHO cells, does not stabilize endogenous microfilaments. In fact, TM overexpression renders the cells more sensitive to cytochalasin B, with hTM3 expression having the most pronounced effect. This suggests that, although nonmuscle TM has been shown to protect actin filaments in vitro (Ishikawa et al., 1989*a*), microfilament stabilization in vivo likely requires other proteins, such as caldesmon.

Materials and Methods

Plasmid DNA Constructs

Full-length cDNAs for human fibroblast TM isoforms hTM3, hTM5, and chimeric isoform hTM5/3 (Novy et al., 1993*a*) were subcloned into the XbaI and BamHI sites (for hTM3) or the XbaI site (for hTM5/3) of pCB6hx, a slightly modified version (Warren et al., 1994) of the eucaryotic expression vector pCB6, generously provided by Dr. M. Stinski (University of Iowa, Iowa City, IA). The pCB6hx expression vector contains the neo^r gene, which allows for selection of stable clones in G418, a synthetic neomycin. Chimeric mutant hTM5/2 was created in the same manner as hTM5/3 (Novy et al., 1993*a*) with the splicing of the carboxy terminus (aa 129-284) of hTM2 onto the amino terminal fragment (aa 1-92) of hTM5, and was also subcloned into pCB6hx. The resulting recombinant plasmids are referred to as pCBhTM3, pCBhTM5, pCBhTM5/3, and pCBhTM5/2 and were prepared for the DNA transfections using Qiagen columns (Qiagen, Inc., Chatsworth, CA.).

Cell Culture and Transfection

CHO cells were maintained in DME plus 10% FCS in a humidified incubator at 37°C with 5% CO_2. DNA transfections were performed using DOTAP transfection reagent as per the manufacturer's procedure (Boehringer-Mannheim Biochemicals, Indianapolis, IN). Briefly, cells grown to 60% confluence on glass coverslips were incubated with the DOTAP-DNA mixture in DME for 6 h, and then rinsed and restored to DME plus 10% FCS. To select stable transfectants, G418 at 500 μg/ml (GIBCO BRL, Gaithersburg, MD) was added to the media 48 h after the transfection. Cells surviving 2 wk of selection were cloned, expanded and screened for expression by immunofluorescence and Western blotting. For each TM isoform studied, a G418-resistant, non-hTM-expressing line was selected to serve as a negative control, and at least three lines expressing low to high hTM amounts were selected to identify hTM expression-related effects. The stable lines used in this paper are, in order of increasing expression, hTM3C32, hTM3C15, hTM3C36 and hTM3C73 for hTM3, hTM5C2, hTM5C11, hTM5C8, and hTM5C14 for hTM5, hTM5/3C68, hTM5/3C36, hTM5/3C25 and hTM5/3C70 for hTM5/3, and hTM5/2C1, hTM5/2C5, hTM5/2C20 and hTM5/2C26 for hTM5/2.

Antibodies and Phalloidin

Monoclonal antibody CGβ6, generated against chicken gizzard TM and characterized previously (Lin et al., 1985*b*, 1988*a*), recognizes an epitope in the COOH-terminal exon region of hTM3 and hTM2. It does not cross react with CHO TM and therefore was used to detect hTM3, hTM5/3, and hTM5/2 in the transfected CHO cells. Anti-caldesmon antibody, C21, described previously (Lin et al., 1988*b*, 1991), was used at 250-fold dilution to recognize endogenous CHO caldesmon. Anti-TM4 antibody, LC24, was generated against bacterially produced hTM4 (COOH-terminal half), and anti-TM5 antibody, LC1, was generated from bacterially produced hTM5 (NH_2-terminal half), following described procedures (Lin et al., 1985*b*). LC24 cross-reacts strongly with hTM4, and LC1 with hTM5, as determined by ELISA and Western blotting. LC24 recognizes only CHO TM4 in immunoblots. Another anti-TM5 antibody, CG3 was characterized previously (Lin et al., 1985*b*, 1988*a*). CGβ6 and CG3 are IgM class antibodies; LC24, LC1, and C21 are IgG class. Rhodamine-phalloidin was purchased from Molecular Probes (Eugene, OR) and used at a fourfold dilution in DME plus 5% FCS. DAPI (4',6'-diamidino-2-phenylindole; Sigma Chem. Co., St. Louis, MO) was used at 0.5 μg/ml and applied as in Lourim and Lin (1992).

Immunofluorescence Microscopy

Cells grown on glass coverslips were fixed, permeabilized, and prepared for immunofluorescence microscopy much as described (Warren and Lin, 1993). Secondary antibodies included FITC-conjugated goat anti-mouse IgG (γ chain-specific; Sigma Chem. Co.), FITC-conjugated goat anti-mouse IgM (μ chain-specific; Sigma Chem. Co.) and Rhodamine-conjugated goat anti-mouse IgM (μ chain-specific; Cappel/Organon-Teknika, Durham, NC). Micrographs were taken with a Zeiss epifluorescence photomicroscope III.

Western Blot Analysis

Cells at 90% confluence were harvested and extracts processed as described previously (Warren et al., 1994). After separation by 12.5% SDS-PAGE, resolved proteins were transferred to nitrocellulose and immunoblotted as in Lin et al. (1985*b*). Amido black staining was used to demonstrate relative protein loading.

Immunoblot Quantitation of Expressed TM

Determination of the endogenous and force-expressed TM levels reported in Table I, was performed as described (Warren et al., 1994). Autoradiograph images from CGβ6, LC1, CG3, and LC24 immunoblots were collected with a Hamamatsu CCD camera, model XC77, with camera controller C2400 (Hamamatsu, Hamamatsu City, Japan) and analyzed using the Image-1/AT image processing and analysing system, version 4.13 (Universal Imaging Corporation, Westchester, PA). Intensity values of CGβ6, LC1, CG3, or LC24 autoradiograph bands from known amounts of purified, bacterially produced hTMs were used to construct standard curves, against which the CGβ6, LC1, CG3, or LC24 bands from the hTM-expressing lines were measured. Total protein concentration was measured, prior to blotting, as described by Lowry et al. (1951).

Other Cell Culture Procedures

"Mitotic shake off" (MSO) was performed as in Mariani et al. (1981). Briefly, a culture dish containing cells at 80% confluence was held in one hand and knocked sideways into the palm of the other hand. The loosened cells were drawn off the monolayer, centrifuged, and quickly replated in a 35 mm dish.

Cytochalasin B treatment involved incubation of cells grown on glass coverslips in media containing 1.0, 1.25, or 1.5 μg/ml cytochalasin B for 30 mins at 37°C. As cytochalasin B was dissolved in DMSO prior to addition to the media, control cells were exposed to DMSO alone. After treatment, cells were processed for immunoflourescence microscopy with CG3, LC24, or rhodamine-phalloidin to demonstrate the extent of microfilament disruption.

Actin Binding Assay

The actin binding-competition assay was based on the cosedimentation method of Eaton et al. (1975) and was performed as described (Novy et al., 1993*a*) using a Beckman airfuge rotor A-100/18. F-actin was purified from the acetone powder of rabbit skeletal muscle (Spudich and Watt, 1971) and bacterially produced hTMs were purified as described (Novy et al., 1993*a*). Reaction mixtures contained 9.4 μM actin and 2.0 mM hTM3 or hTM5 in F buffer (10 mM imidazole buffer, pH 7.0, 100 mM KCl, 10 mM $MgCl_2$, 0.1 mM EGTA, and 1 mM DTT) with competitors hTM5/3 or hTM5/2 in varying amounts up to 2.1 μM. The resulting bound and free fractions were analyzed and quantified as described previously (Novy et al., 1993*a*).

*Table I. Level of TM Expression in Selected Stable Lines**

Cell line	Percentage of total protein: Force-expressed hTM	Endogenous TM4
hTM3C32	0.000 ± 0.000	0.033 ± 0.001
hTM3C15	0.033 ± 0.013	0.026 ± 0.004
hTM3C36	0.050 ± 0.002	0.024 ± 0.001
hTM3C73	0.050 ± 0.002	0.022 ± 0.003
hTM5C2	0.028 ± 0.028‡	0.029 ± 0.002
hTM5C11	0.071 ± 0.005‡	0.023 ± 0.002
hTM5C8	0.075 ± 0.002‡	0.028 ± 0.008
hTM5C14	0.214 ± 0.014‡	0.026 ± 0.002
hTM5/3C68	0.000 ± 0.000	0.029 ± 0.016
hTM5/3C36	0.024 ± 0.002	0.022 ± 0.005
hTM5/3C25	0.030 ± 0.008	0.030 ± 0.010
hTM5/3C70	0.092 ± 0.004	0.033 ± 0.004
hTM5/2C1	0.000 ± 0.000	0.025 ± 0.006
hTM5/2C5	0.010 ± 0.001	0.029 ± 0.000
hTM5/2C20	0.037 ± 0.000	0.024 ± 0.001
hTM5/2C26	0.037 ± 0.001	0.026 ± 0.003

* Values shown for the hTM3, hTM5/3, and hTM5/2 lines are the average values from two independent experiments.

‡ Since the LC1 antibody crossreacts to both endogenous CHO TM5 and force-expressed hTM5, the amounts of hTM5 expression were calculated by substracting the amounts of endogenous TM5 in CHO line from the total TM5 amounts detected by LC1 antibody in each of hTM5 expressing lines.

Equivalent results were obtained whether the competitors were added at the same time or after a 30-min incubation with hTM3 or hTM5 isoform.

Results

Our previous characterization, in vitro, of bacterially produced human fibroblast hTM3, hTM5, and their chimeras hTM3/5 and hTM5/3 (Novy et al., 1993*a*) revealed that the isoforms had distinct functional properties, including the ability to bind actin, to amplify actomyosin ATPase activity and to interact with caldesmon. To study the function of TM in the nonmuscle cell and to ascertain whether the isoform-specific properties seen in vitro are physiologically relevant, we expressed hTMs in CHO cells with DNA transfection. We focused on isoform hTM5/3, because of its extreme actin binding properties, and hTM3 and hTM5, for comparison, as they are the wild-type "parent" isoforms. Full length cDNAs for hTM5/3, hTM5, and hTM3 were separately subcloned into eucaryotic expression vector pCB6hx, separately transfected into CHO cells, and assayed for expression 18–24 h after transfection, via immunofluorescence microscopy. Transient expression of hTM5/3, but not hTM3 or hTM5, resulted in severe distortions of the transfected cell shape and a disruption of F-actin bundles (data not shown). We have previously found that shape change is not routinely found with transient expression of other proteins such as actin-binding "CaD39" or non-actin-binding "CaD40" fragments of human fibroblast caldesmon (Warren et al., 1994).

Mismatched Terminal "Head and Tail" Domains Appear to be Responsible for the Disruptive Effects of hTM5/3

Fig. 1 shows a cartoon representation of the isoform-specific domains of hTM3 and hTM5 that are "mismatched," in chimeric mutant hTM5/3. Note that the sequence diversity is restricted to the exon-encoded regions of the termini as well as one internal domain. To determine whether the mismatched termini or the internal exon region are primarily responsible for the altered properties of hTM5/3, we took advantage of the fact that a chimera between hTM5 and hTM2, hTM5/2, differs from hTM5/3 solely in that internal region (Fig. 1). We therefore constructed a hTM5/2-containing expression plasmid and transfected it into CHO cells. The hTM5/2-transfected cells demonstrated the same range of aberrant morphologies as seen with transient hTM5/3 expression (data not shown). Additionally, the actin binding properties of bacterially produced hTM5/2, purified and analyzed as in Novy et al. (1993*a*), were similar to those of hTM5/3 (data not shown). Therefore, the internal exon does not seem to be the primary cause for the dominant effects of hTM5/3 on cell shape and microfilament organization.

Stable Expression of hTMs Does Not Alter Endogenous Levels of TM or Caldesmon

To more closely analyze the effects of hTM5/3, hTM5/2, hTM5 and hTM3 overexpression, we obtained clonal lines of permanently transfected cells and determined their relative expression levels by western blot analysis. As our stably transfected cells were the result of random integration of the hTM- and neo^r-containing vector, we aimed to select and characterize several independent stable lines each, of hTM3, hTM5, hTM5/3, and hTM5/2, to rule out non-TM, position-

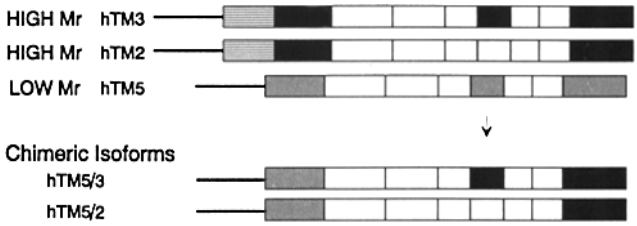

Figure 1. Schematic cartoon of the exon-encoded regions of the cDNAs for hTM3, hTM2, hTM5, and the chimeric isoforms hTM5/3 and hTM5/2. The alternatively spliced exon regions are differently shaded to show the areas of isoform diversity. Chimeric isoforms hTM5/3 and hTM5/2 differ solely in the region encoded by the internal alternatively spliced exon (*arrow*).

effect, phenotypes. Although the effects of transient transfection of hTM5/3 and hTM5/2 were often severe, we were able to obtain and expand a few clonal lines expressing different levels of hTM5/3 and hTM5/2, as well as stable lines expressing hTM3 or hTM5. A quantitative analysis of hTM expression levels from Western blots of the selected hTM lines is shown in Table I. The protein concentrations for each cell line extract were determined, known amounts of extract were subjected to immunoblotting, and the autoradiographs were analysed as described in the Materials and Methods section. Table I shows that the amount of hTM overexpression in the selected stable lines ranged from approximately one to three times that of endogenous TM4 levels, except for the high-expresing hTM5C14. It is also important to note that steady state levels of endogenous TM4, which ranged from 0.020 to 0.033% of total protein in the negative control lines, did not show hTM-expression-related changes in the lines that express hTM3, hTM5, hTM5/3, or hTM5/2.

Representative Western blots showing hTM, endogenous TM4, as well as endogenous caldesmon levels are shown for the hTM5/3 lines in Fig. 2, for the hTM3 and hTM5/2 lines in Fig. 3, and for the hTM5 lines in Fig. 4. The CGβ6 immunoblot of Fig. 2 *A*, shows hTM5/3C36 (lane *2*), hTM5/3C25 (lane *3*), and hTM5/3C70 (lane *4*), the hTM5/3 lines chosen to represent low to high expression levels, as well as the drug-resistant non-expressor, hTM5/3C68 (lane *1*). Fig. 3 *A*, shows the hTM3 expression levels among the hTM3 stable lines, hTM3C15 (lane *2*), hTM3C36 (lane *3*), and hTM3C73 (lane *4*) and nonexpressor control line, hTM3C32 (lane *1*). A CGβ6 blot of the hTM5/2 lines is shown in Fig. 3 *E*, with the negative control line hTM5/2C1 in lane *1* and hTM5/2-expressing lines, hTM5/2C5, hTM5/2C20 and hTM5/2C26 shown in lanes *2–4*, respectively. The amido black–stained panels (Figs. 2 *D*, and 3 *D* and *H*) and the Coomassie blue–stained gel panel (Fig. 4 *A*) demonstrate relative total protein loading. Since the LC1 antibody recognizes both endogenous CHO TM5 and exogenously force-expressed hTM5, the LC1 immunoblot shall demonstrate the combined amounts of CHO TM5 and hTM5. In Fig. 4 *B*, the hTM5 stable lines, hTM5C2 (lane *2*), hTM5C11 (lane *3*), hTM5C8 (lane *4*), and hTM5C14 (lane *5*) appear to represent low to high expression levels of hTM5. The level of expression in hTM5C2 (Fig. 4 *B*, lane *2*) was not significantly different from that in the original CHO line (Fig. 4 *B*, lane *1*). Thus, the hTM5C2 may represent a drug-resistant hTM5 nonexpressor.

We previously reported that overexpression of the actin-, Ca^{2+}/calmodulin-, and TM-binding fragment of human fibroblast caldesmon, CaD39, increased the steady-state levels of endogenous TM. This was not due to an increase in TM synthesis, but to a reduction in TM turnover, presumably resulting from CaD39-enhanced TM-actin binding (Warren et al.,

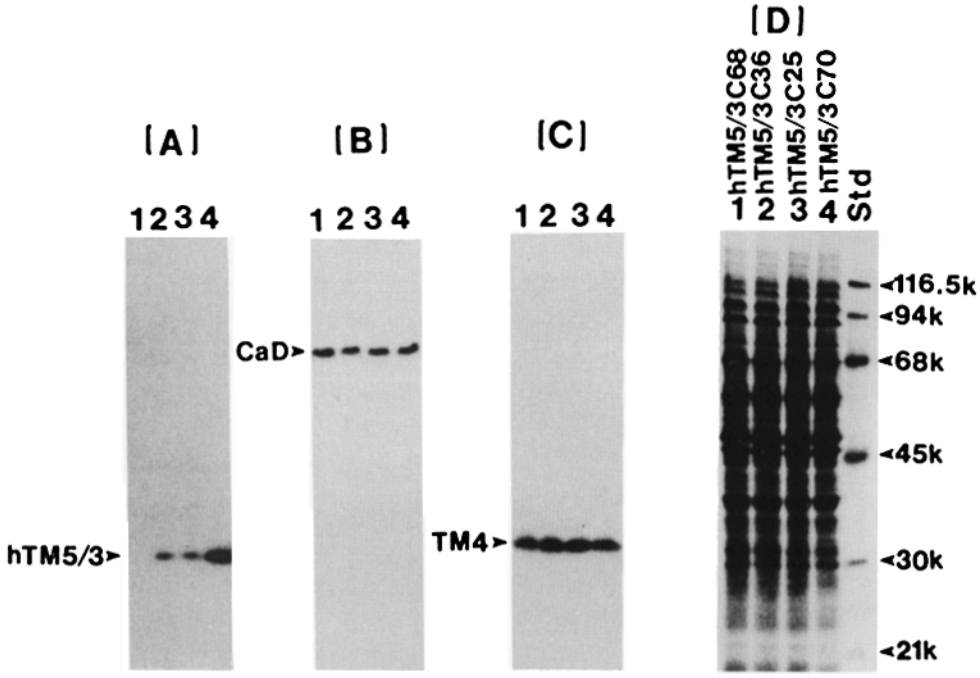

Figure 2. Western blot analysis of hTM5/3 stable lines, hTM5/3C68 (lane *1*), hTM5/3C36 (lane *2*), hTM5/3C25 (lane *3*), and hTM5/3C70 (lane *4*). The blots were run in triplicate to show the level of hTM5/3 expression with CGβ6 antibody (*A*), the endogenous caldesmon level with C21 antibody (*B*), and the level of endogenous TM4 with LC24 antibody (*C*). The amido black–stained panel (*D*) shows the total protein loadings. Control line hTM5/3C68 has no hTM5/3 expression (*A*, lane *1*) whereas hTM5/3C36, hTM5/3C25 and hTM5/3C70 have increasing hTM5/3 levels (*A*, lanes *2–4*). Note that there is no apparent hTM5/3 expression-related change in the endogenous caldesmon (*B*, lanes *1–4*) or TM4 (*C*, lanes *1–4*) of these stable lines. *TM4*, endogenous TM4; *CaD*, endogenous caldesmon.

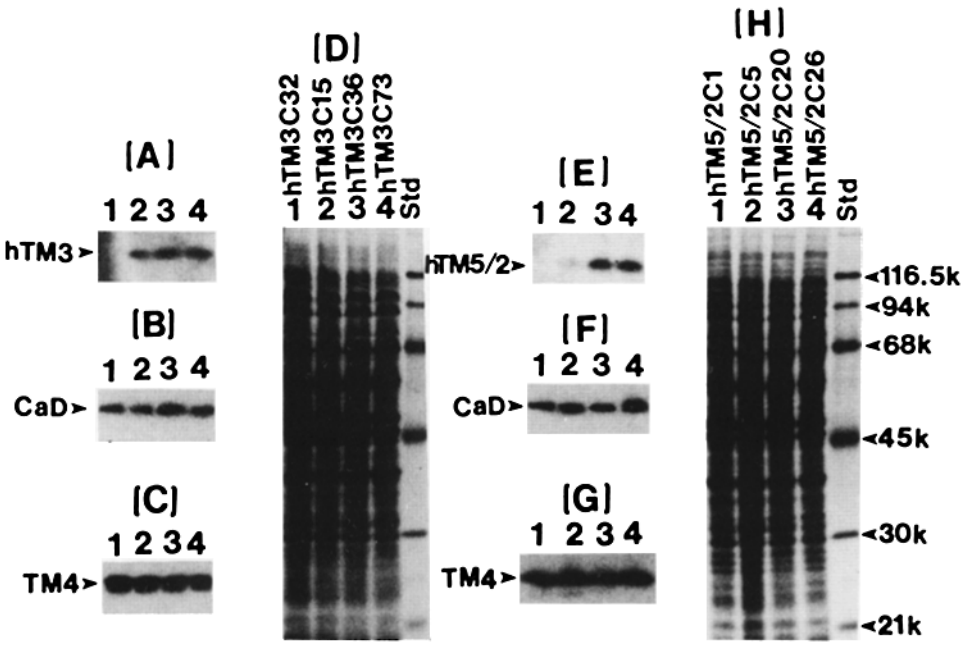

Figure 3. Western blot analysis of hTM3 (*A–D*) and hTM5/2 (*E–H*) stable lines. The experimental design is much like that of Fig. 2. *D* and *H* are amido black–stained panels to demonstrate total protein loadings for the hTM3 and hTM5/2 lines, respectively. CGβ6 antibody was used to detect the level of hTM3 (*A*) or hTM5/2 (*E*) expression. Endogenous CaD levels were probed with C21 antibody (*B* and *F*) and CHO TM4 levels were probed with LC24 antibody (*C* and *G*). There is no reproducible difference in the steady state levels of CHO CaD or CHO TM4 between the four hTM3 stable lines (lanes *1–4* of *B* and *C*) or the four hTM5/2 lines (lanes *1–4* of *F* and *G*). *TM4*, endogenous TM4; *CaD*, endogenous caldesmon.

1994). We were, therefore, curious as to whether the overexpression of TM would change caldesmon levels. The Western blots in Figs. *2–4* with anti–caldesmon antibody C21 (Figs. 2 *B*, 3 *B* and *F*, and 4 *C*) show that steady-state levels of caldesmon remain unchanged in cells expressing various amounts of hTM5/3, hTM3, hTM5, or hTM5/2. Endogenous levels of TM4 also appear undisturbed in the LC24 immunoblots of the lines expressing hTM5/3 (Fig. 2 *C*), hTM3 (Fig. 3 *C*) or hTM5/2 (Fig. 3 *G*) or hTM5 (Fig. 4 *D*). CHO TM5 levels in hTM5/2, hTM5/3, and hTM3 lines also remain constant (data not shown). It appears that force-expressed hTM5/3, hTM5/2, hTM3, and hTM5 do not appreciably alter the accumulation of endogenous CHO TM or caldesmon. The transfected, hTM-expressing, cells then possess unusual total TM to caldesmon ratios.

Stable Chimeric Mutant hTM Lines Have Cell Cycle–dependent Heterogeneous Expression Levels

All viable hTM5/3-expressing lines that we were able to isolate and expand from single clones have the peculiar trait of heterogeneous cell-to-cell expression levels as determined

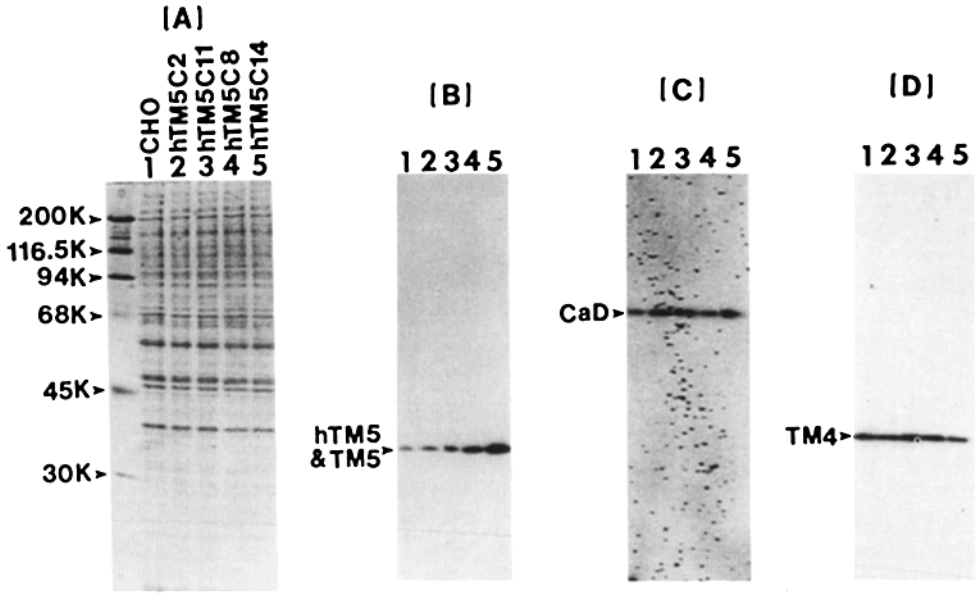

Figure 4. Western blot analysis of CHO line (lane *1*), and hTM5 stable lines, hTM5C2 (lane *2*), hTM5C11 (lane *3*), hTM5C8 (lane *4*), and hTM5C14 (lane *5*). The experimental design is much like that of Fig. 2. The blots were run in triplicate to show the level of endogenous CHO TM5 and hTM5 with LC1 antibody (*B*), the endogenous caldesmon (*CaD*) with C21 antibody (*C*), and the level of endogenous TM4 with LC24 antibody (*D*). The Coomassie blue–stained gel (*A*) shows the total protein loadings. The amount of CHO TM5 and hTM5 expressed in the hTM-5C2 (*B*, lane *2*) is not significantly different from that in CHO line (*B*, lane *1*). The hTM5C2 line may, therefore, represent a nonexpressor line. On the other hand, hTM5C11, hTM5C8, and hTM5C14 lines express increasing levels of hTM5. There is no significant difference in the steady-state levels of CHO CaD and CHO TM4 between the four hTM5 stable lines (lanes *2–5* of *C* and *D*).

by immunofluorescence microscopy. CGβ6 immunofluorescence staining in Fig. 5 *A* demonstrates that cells of hTM5/3 representative expressing line, hTM5/3C70, have different levels of expression as judged by relative CGβ6 staining intensities. Four very weakly stained cells have been outlined and marked with asterisks in Fig. 5 *A*. The heterogeneous CGβ6 intensities appear to be linked to the cell cycle because cells from all hTM5/3 lines brightly and evenly stain with CGβ6 after synchronization by mitotic shake off (Fig. 5 *B*). Homogeneous staining remains until cells lose their mitotic synchrony, at about 18 h in culture (data not shown). Heterogeneous CGβ6 staining intensities are also seen with stable hTM5/2 lines but not with hTM3 lines. The homogeneous CGβ6 staining of hTM3C73, a representative high expressing line is shown in Fig. 5 *C*.

Stable Expression of hTM5/3 and hTM5/2 Perturbs Cytokinesis in CHO Cells

Most cells of the hTM5/3 stable lines have a relatively "normal" morphology. Of the aberrant cells, some have abnormal shapes similar to the transiently transfected cells but the majority are huge with many nuclei. We found, upon comparative cell counting, that the incidence of multinuclearity increased relative to hTM5/3 expression (Table II). Cells were judged to be multinucleate if at least three nuclei were discernible. Fig. 6 shows DAPI-stained nuclei from nonexpressing hTM5/3C68 (Fig. 6 *A*) and expressing line hTM5/3C25 (Fig. 6 *B*), as examples. The nuclei in the affected cells were often bunched together, therefore, the counts included in Table II may even underestimate the percent nuclei in multinucleate cells. The multinucleate population in hTM3- or hTM5-expressing cell lines was not significantly different from that of the non-expressing control lines or for CHO cells alone. Expression of the hTM5/3-related chimera hTM5/2, however, did result in an increased incidence of multinucleate cells related roughly to the level of hTM5/2 expression.

The greatly increased presence of multinucleate cells specifically in the hTM5/3 and hTM5/2 lines is a good indication that these chimeric mutant hTMs can interfere with successful cytokinesis. We therefore sought to understand the mechanism of the cytokinesis defect by studying the hTM-expressing cells which do make it through cell division. As early as the initial cloning of the hTM5/3 and hTM5/2 lines we noticed a decrease in the growth rates that corresponded to the level of the chimeric hTM expressed. Control line hTM5/3C68 cells doubled in 15 h. The doubling times for hTM5/3C36, hTM5/3C25, and hTM5/3C70 were 17, 17, and 24 h, respectively. hTM5/2-expressing stable lines also grow more slowly than nonexpressing stable lines, but stable CHO lines expressing hTM3, hTM5, or caldesmon fragments do not have expression-related effects on their growth rates (data not shown).

To try to define the cell cycle period involved in the hTM5/3-related defect, we synchronized the cells for using MSO. This technique allows for the selection of cells which have rounded up for division without the addition of cytoskeletal-altering drugs. MSO cells from the hTM5/3 lines were collected, replated on coverslips and observed. After replating, mitotic cells completed cell division by pinching into two distinct, small, juxtaposed cells we refer to collectively as a doublet. CHO cells and nonexpressing hTM5/3C68 cells were very quick to divide and 20 min after replating, 35.4% of hTM5/3C68 cells were doublets, whereas only 17.7, 13.7, and 7.9% of TM5/3C36, hTM5/3C25, and hTM5/3C70 cells were doublets (Table III). As stable line expres-

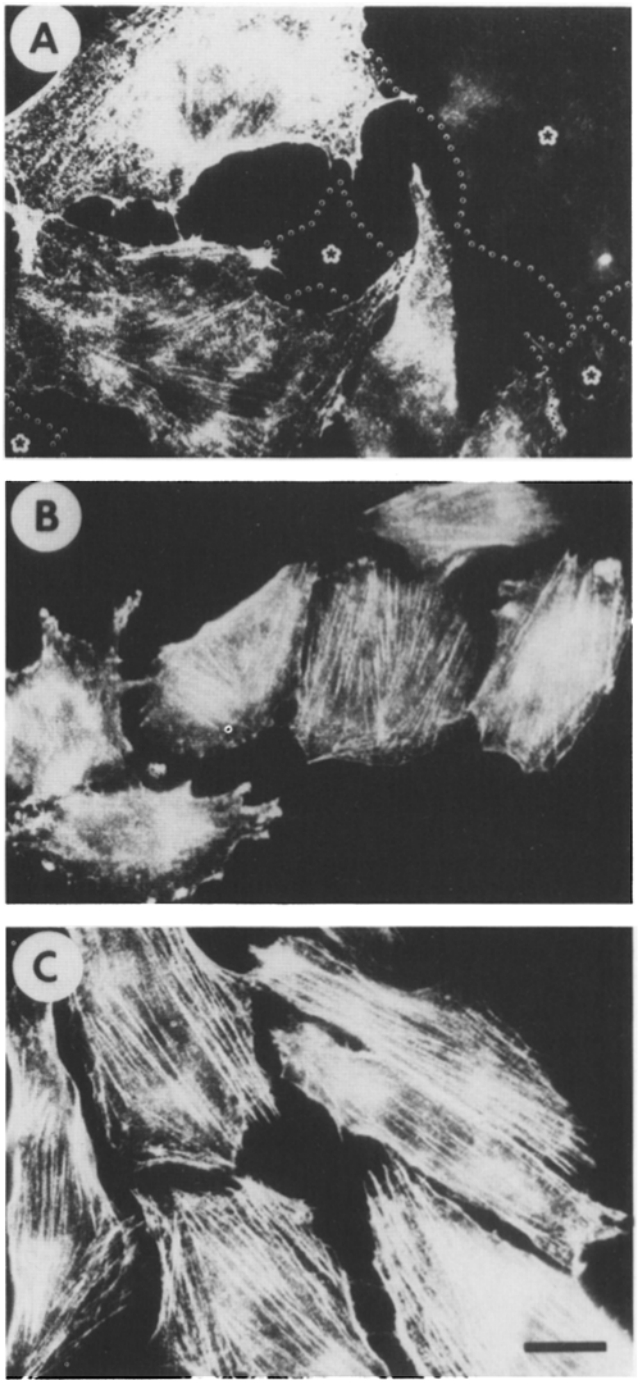

Figure 5. Immunofluorescence microscopy on cells from stable hTM5/3 (*A* and *B*) or hTM3 (*C*) lines with CGβ6. (*A*) All hTM5/3 lines, when cultured asynchronously, exhibit heterogeneous cell-to-cell CGβ6 staining (hTM5/3C70 cells shown). Four weakly staining cells are partially outlined and marked with asterisks. (*B*) CGβ6 staining levels are more homogeneous after hTM5/3-expressing cells are synchronized (hTM5/3C70 cells shown, 4 h after mitotic shake off). (*C*) hTM3 stable lines have uniform staining with CGβ6 regardless of cell cycle state. Bar, 10 μm.

*Table II. Percent of Nuclei in Multinucleate Cells**

Cell line	Relative expression level	Total nuclei counted	Percentage in multinucleate cells
CHO	−	1,206	0.91
hTM3C32	−	1,229	0.49
hTM3C15	++	1,204	0.83
hTM3C36	+++	1,211	0.58
hTM3C73	+++	1,306	0.54
hTM5C2	−	1,119	0.54
hTM5C11	++	1,108	0.90
hTM5C8	+++	1,091	2.47
hTM5C14	+++++	1,079	3.80
hTM5/3C68	−	1,257	1.03
hTM5/3C36	++	1,238	6.70
hTM5/3C25	++	1,256	7.25
hTM5/3C70	++++	1,340	24.78
hTM5/2C1	−	1,060	0.92
hTM5/2C5	+	1,328	5.21
hTM5/2C20	++	1,005	9.52
hTM5/2C26	++	1,446	15.77

* Criteria: cells possessing ≥3 nuclei = multinucleate.

sion levels of hTM5/3 increased the time it took to pinch in half was also increased. This phenomenon was also seen in cells expressing hTM5/2, but not for cells of the hTM3 or hTM5 lines (data not shown). These results indicate that this phase of cell division is somehow slowed by hTM5/3 or hTM5/2. The actin-based contractile ring is formed and utilized during this phase (Satterwhite and Pollard, 1992 for review), so it is possible that hTM5/3 or hTM5/2 are interfering with contractile ring formation and/or usage. In the yeast *S. pombe*, TM has already been found to be required for cytokinesis and the formation of the contractile ring (Balasubramanian et al., 1992).

We are currently investigating, in vitro, the interactions of hTM isoforms with actin binding, bundling and severing proteins as well as with actin itself, in the hope that this information will help further define the role of the chimeric mutants in the division defect and, ultimately, the role of TM in cytokinesis. We have previously characterized the in vitro actin-binding properties for bacterially produced hTM5, hTM3 and hTM5/3 (Novy et al., 1993*a*). In this study, using an actin-binding assay, we have found that bacterially produced hTM5/3 and hTM5/2 can efficiently displace hTM3 and hTM5 from actin filaments (Fig. 7). Under the saturation-binding condition with 9.4 μM actin and 2.0 μM hTM5, chimera hTM5/2 or hTM5/3 at 1.3 μM was able to compete off about half of the prebound hTM5 from actin filaments (Fig. 7 *A*). Similarly, both hTM5/2 and hTM5/3 were found to be very effective competitors for the hTM3 too (Fig. 7 *B*). Interestingly, it appeared that less amounts (0.75 μM) of hTM5/3 was required for displacing 50% of hTM3 from actin filaments, as compared to the requirement of 1.3 μM hTM5/2 (Fig. 7 *B*). This difference may be reflected by the difference in amino acid sequences between hTM5/2 and hTM5/3. The dominance of chimeric TM actin binding, in vitro, suggests that hTM5/3 and hTM5/2 may be able to exert their isoform-specific effects on CHO filament structure by displacing the endogenous TM.

hTM Overexpression Does Not Stabilize CHO Microfilaments

Nonmuscle TM has been proposed to stabilize actin-based filaments (Lin et al., 1984, 1985*a*; Hedricks and Weintraub, 1981; Leavitt et al., 1986; Cooper et al., 1985; Broschat et al., 1989; Tanaka et al., 1993) and protect them from severing agents such as gelsolin (Fattoum et al., 1983; Ishikawa et al., 1989) and villin (Bonder and Mooseker, 1983). These proposals were based on the fact that TM isoforms are down regulated in stress fiber-deficient transformed cells and also based on results from in vitro experiments. Our hTM-expressing lines provide an ideal environment in which to directly test the stabilization hypothesis, in vivo. After treatment of hTM5/3, hTM5/2, hTM5, and hTM3 stable lines with cytochalasin B, a fungal toxin which disrupts F-actin, we observed that the actin filament bundles of hTM-expressing cells were actually less resistant to treatment than those of non-hTM-expressing cells. Fig. 8 shows the extent of damage to the TM-containing microfilaments in hTM3 cells exposed to 1.0 μg/ml cytochalasin B. The CG3-stained TM-containing stress fibers are still intact in nonexpressor hTM3C32 (Fig. 8 *A*), but show gradual disruption in the low and medium level lines hTM3C15 (Fig. 8 *B*) and hTM3C36 (Fig. 8 *C*), with only brightly staining aggregates remaining in high-expressor line hTM3C73 (Fig. 8 *D*). Cells stained with phalloidin to visualize F-actin (data not shown) revealed identical patterns of filament disruption. hTM5, hTM5/3-, and hTM5/2-expressing lines were also more sensitive to cytochalasin B than non-expressing lines (data not shown). The destabilization of actin bundles in cells overexpressing TM is a new finding that implies that TM alone in vertebrate nonmuscle cells cannot fully protect actin filaments.

Discussion

The functions of TM in the nonmuscle cell have yet to be definitively assigned. Suggestions that nonmuscle TM is involved in the regulation of microfilament dynamics have been based on in vitro observations that nonmuscle TM can, when bound to F-actin, modulate the actions of other actin-binding proteins which sever (Fattoum et al., 1983; Ishikawa et al., 1989), bundle (Burgess et al., 1987), or stabilize (Ishikawa et al., 1989) actin filaments. In vivo, much less has been done to directly test nonmuscle TM function. The down regulation of TM isoforms in transformed cells correlates well with the decrease of obvious microfilament bundles (Lin et al. 1984, 1985*a*; Leavitt et al., 1986) and forced reexpression of TM in ras transformed cells has been reported to partially restore stress fibers (Prasad et al., 1993). Microinjection studies with anti-TM antibodies have implicated TM in granule movement (Hegmann et al., 1989) and TM assembly properties have been investigated with microinjection of recombinant TMs (Pittenger and Helfman, 1992). Finally, yeast genetic analyses have shown TM to be essential for actin cables in *Saccharomyces cerevisiae* (Liu and Bretscher, 1989) and cytokinesis in *Schizosaccharomyces pombe* (Balasubramanian et al., 1992). In this study, we have further approached the question of nonmuscle TM function using DNA transfection to express human fibroblast TMs and their chimeric mutants in CHO cells, and we report here

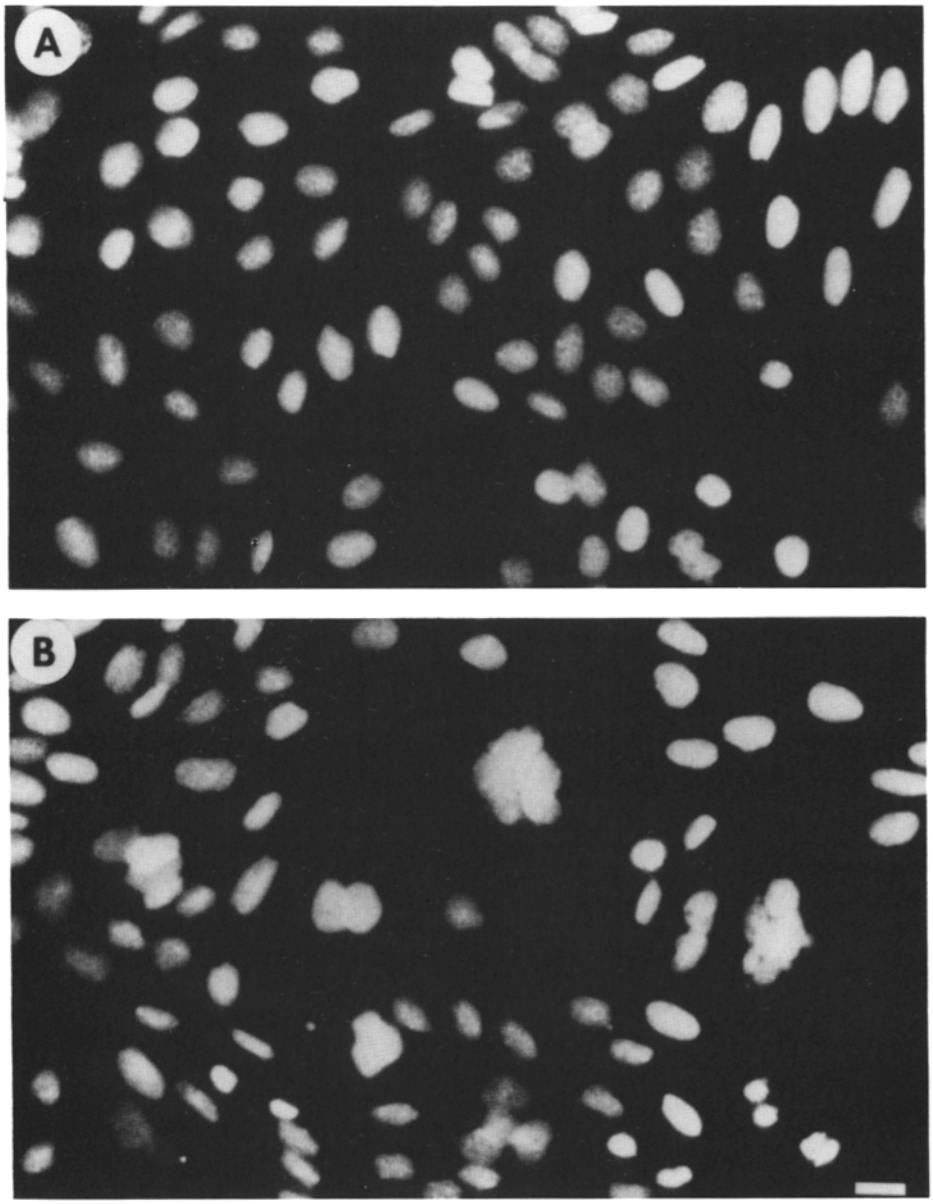

Figure 6. Cells cultured for three days on coverslips were stained with DAPI to demonstrate the increased incidence of multinucleate cells in hTM5/3-expressing lines. (*A*) Negative control line hTM5/3C68. (*B*) High-expressing line hTM5/3C70. Bar, 10 μm.

that expression of chimeric mutant hTM isoforms can perturb cytokinesis.

Why do the chimeric hTM isoforms behave so differently from the parent TM isoforms in vitro and in these transfection experiments? It has been suggested that the amino and carboxy terminal regions are required for TM-TM interactions and that both terminal regions are the major determinants of muscle TM function (Cho et al., 1990; Cho and Hitchcock De-Gregori, 1991). Our previously reported data that, in vitro, chimeric hTM5/3 displays actin binding properties distinct from those of hTM5 or hTM3, suggests that interactions between the N and C halves of an individual TM are important for TM function (Novy et al., 1993*a*). hTM5/2, the closely related chimeric isoform (see Fig. 1), has actin-binding properties similar to hTM5/3 (data not shown), further indicating that the mismatch of the terminal exon-encoded regions is responsible for the altered TM properties. Analysis of muscle TM crystal structure (Phillips et al., 1986; Sanders et al., 1988) predicts that TM is a flexible molecule with at least three conformational states. The amino half is reportedly more stable; the carboxy half more labile, especially in a segment around cys 190 (Phillips et al., 1986). We have previously proposed that a functional con-

Table III. Percent of Doublet Cells After Mitotic Shake Off

Cell line	Relative expression level	Total counted	Percent doublets after plating* 20 min	40 min	60 min
hTM5/3C68	−	1,045	35.4	39.4	44.8
hTM5/3C36	++	970	17.7	34.8	38.8
hTM5/3C25	++	1,660	13.7	26.6	28.4
hTM5/3C70	++++	1,891	7.9	22.8	32.9

* Percent doublets never reach 100% due to the significant number of cells which divide during the shake off. These small single cells are one half the size of a doublet.

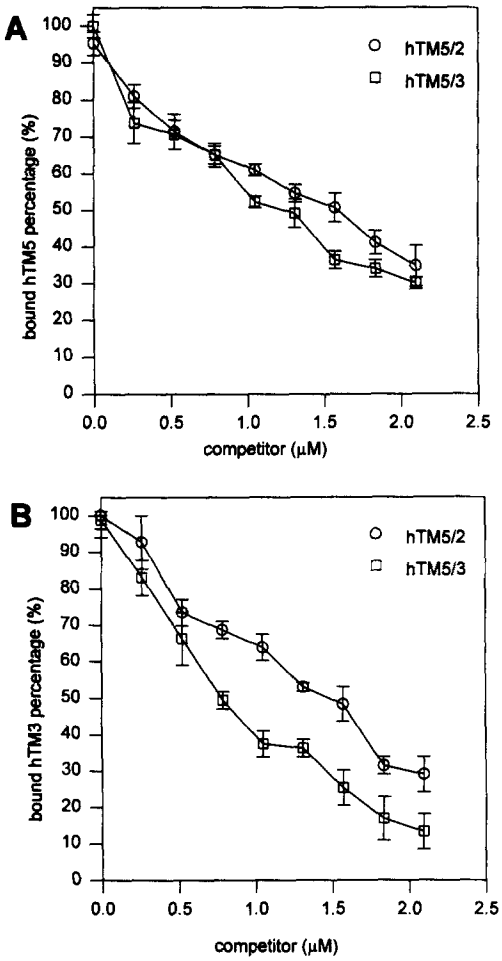

Figure 7. Competitive actin-binding assay with bacterially produced hTM3, hTM5, hTM5/2, and hTM5/3. The reaction mixtures contained 9.4 μM actin, and 2.0 μM of hTM5 (*A*) or hTM3 (*B*) in F buffer (10 mM imidazole buffer, pH 7.0, 100 mM KCl, 10 mM $MgCl_2$, 0.1 mM EGTA, and 1 mM DTT). After incubation for 30 min at room temperature, various concentrations of competitors (*hTM5/2* or *hTM5/3*) were added to the mixtures. After additional 30-min incubation, the mixtures were centrifuged at 26 psi for 20 min in a Beckman airfuge. Both supernatants and pellets were adjusted to equivalent volumes and analyzed by SDS-PAGE. The binding of hTM5 or hTM3 was determined by scanning stained gels of both supernatants and pellets. The amount of hTM5 or hTM3 bound to actin filaments in the absence of competitors was used as 100%. Data were obtained from 3 independent experiments with error bars representing standard deviations. Under this saturation condition, the prebound hTM5 (*A*) and hTM3 (*B*) were effectively competed off from actin filaments by chimera hTM5/2 or hTM5/3.

formation change exists in an analogous region in nonmuscle TM (Hegmann et al., 1988). It is possible then, that the specific combination of stable amino and labile carboxy terminal regions determines the range of conformational flexibility and also, perhaps, function. Analysis of the crystal structure of hTM5/3 may allow us to see if the inherent flexibility for hTM5/3 differs from that reported for striated or smooth muscle TM (Phillips et al., 1986; Sanders et al., 1988).

One interesting feature of the stable hTM5/3 and hTM5/2 lines is that, although they were derived from single cells, they exhibit heterogeneous staining for hTM5/3 or hTM5/2 with CGβ6 antibody (see Fig. 5 *A* for hTM5/3). The cell to cell difference is not likely a property of the CGβ6 antibody, since CGβ6 staining of the hTM3 lines is homogeneous (Fig 5 *C*). Also, the CGβ6 staining intensity of hTM5/3 or hTM5/2 lines can be evened out by synchronizing the cells (see Fig. 5 *B* for hTM5/3), suggesting that detectable hTM5/3 or hTM5/2 levels are regulated in a cyclic manner. The fact that all viable hTM5/3 and hTM5/2 we were able to maintain have this heterogeneity, and that lines expressing hTM3 (Fig 5 *C*), mouse β skeletal TM (Warren and Lin, 1993), and that caldesmon fragments CaD39 or CaD40 (Warren et al., 1994) do not, further suggested that the chimeric hTM lines that survived, may have done so because they regulated the timing of the synthesis, degradation, or distribution of the hTM5/3 or hTM5/2 with the cell cycle. Two other indications that the progression through the cell cycle might be sensitive to chimeric hTM expression come from our observations that stable line growth rate decreased with increased expression of hTM5/3 or hTM5/2, but not with increased levels of expressed hTM3 or hTM5 (not shown) and that the incidence of multinucleate cells increased roughly parallel to increased expression levels of hTM5/3 or hTM5/2 (Table II).

Cytokinesis involves a massive remodeling of the actin cytoskeleton, with both de novo filament synthesis and actin filament redistribution to the cleavage furrow (Cao and Wang 1990 *a*,*b*; Fishkind and Wang, 1993) but little is known about the mechanism of contractile ring assembly and function (for review see Satterwhite and Pollard, 1992). Important information is emerging, however, from analyses of cell cycle defective mutants in yeast. Of special interest is the identification of $cdc8^+$ as a novel tropomyosin found to be essential for the formation of the F-actin contractile ring in *S. pombe* (Balasubramanian et al., 1992). We are interested in determining whether TM is involved in contractile ring function in higher eucaryotes and are therefore closely examining the chimeric hTM cytokinesis defect. The fact that the isolated mitotic cells from chimeric hTM lines are slow to pinch in two (Table III) may be an indication that ring assembly kinetics or contraction may be altered. That cytokinesis appears to be the process most affected by stable expression of chimeric hTMs may suggest that ring formation requires an isoform-specific function that hTM5/3 or hTM5/2 do not possess, or that the crucial timing of ring assembly, function and disassembly is not achieved with hTM5/3 or hTM5/2. For example, it has been shown that tropomyosins of different association constants differentially stabilize the pointed end of actin filaments from disassembly (Broschat et al., 1989). From our in vitro studies, we know that both hTM5/2 and hTM5/3 have unusal high affinity to actin filaments (Novy et al., 1993*a*). This may suggest that chimeric tropomyosins in the contractile rings may be capable of slowing down the disassembly of actin filaments and then, lead to a defect in cytokinesis. Other cell activities involving TM function may not have such strict temporal requirements. Similar reasoning was used to explain why the *S. pombe* profilin, recently shown to be required for cytokinesis, does not appear to be essential for other processes involving actin filament formation (Balasubramanian et al., 1994).

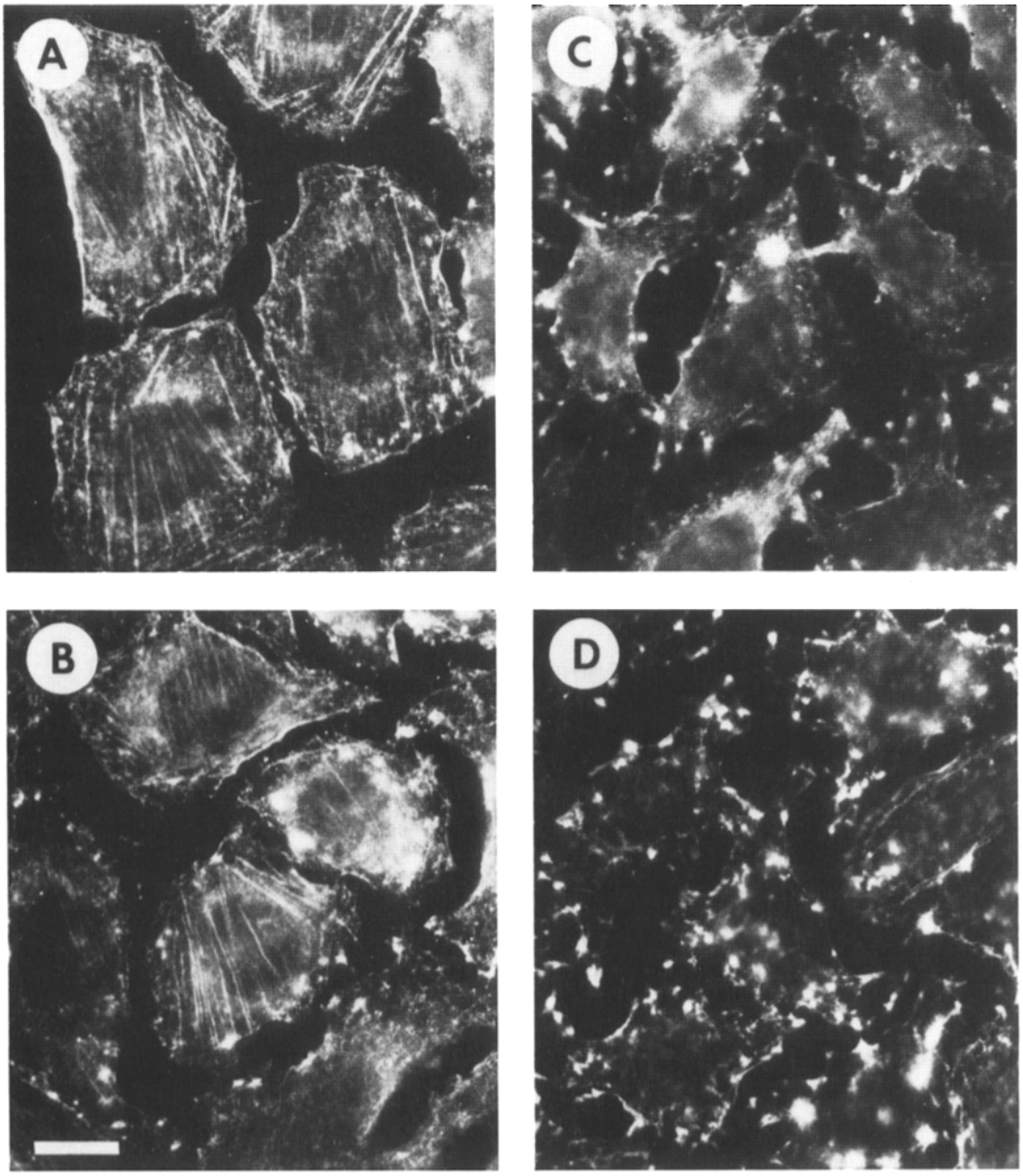

Figure 8. Cytochalasin B disruption of TM-containing microfilament bundles is more severe in stable lines expressing hTM3. CG3 staining for endogenous TM reveals the extent of microfilament rearrangement caused by a 30-min treatment of 1.0 μg/ml cytochalasin B. The nonexpressing control line, hTM3C32 (*A*), was the most resistant, with intact stress fibers still remaining in most cells. With increasing expression of hTM3 in lines hTM3C15 (*B*), hTM3C36 (*C*), and hTM3C73 (*D*), however, the disruption was increasingly more extensive. Bar, 10 μm.

In apparent contrast to proposals that nonmuscle TM serves to protect actin filament structures in the cell, we have found that stable overexpression of hTM3, hTM5, hTM5/3, or hTM5/2 does not stabilize microfilaments. In fact, with increasing levels of hTM expression, CHO cell actin structures were increasingly less resistant to cytochalasin B (Fig. 8). The disparity between the in vitro–based predictions and our study can easily be reconciled by considering TM as an essential tool used by caldesmon, and possibly other proteins, in the stabilization of actin filaments. Tropomyosin itself may not stabilize as effectively on its own. In vitro, caldesmon greatly enhances the ability of TM to bind to actin (Yamashiro-Matsumura and Matsumura, 1988; Novy et al., 1993*a*), and to protect against the severing activity of gelsolin (Ishikawa et al., 1989) and the cross-linking action of filamin (Nomura et al., 1987). In additional support of the working relationship of TM and caldesmon in vivo, we have previously reported that overexpression of an actin-, TM-, and Ca^{2+}/calmodulin-binding fragment of caldesmon (CaD39) stabilized endogenous TMs and microfilaments (Warren et al., 1994). In our hTM-transfected cells, endogenous caldesmon and TM levels do not change (Figs. 2–4),

thus the ratio of total TM to caldesmon is higher than normal. This may result in a population of filaments not fully protected, and, therefore, more susceptible to cytochalasin B. Alternatively, works from previous investigators (Lal and Korn, 1986; Hitchcock-DeGregori et al., 1988; Broschat et al., 1989) suggest little effect of tropomyosins on the barbed end of actin filaments, whereas tropomyosins are able to stabilize the pointed end from disassembly. Moreover, it has been elegantly shown that cytochalasins strongly affect the barbed end of actin filaments (for review see Cooper, 1987). Thus, it may not be too surprising that overexpression of tropomyosins in vivo, in the presence of endogenous actin-binding proteins, does not stabilize filaments from cytochalasins. However, this possibility still cannot explain the more sensitive nature of tropomyosin-overexpressing clones. Why hTM3 lines are more sensitive to actin disrupting agents than hTM5, hTM5/3, or hTM5/2 lines is not clear. Since the levels of hTM expression are not vastly different in the three sets of cell lines, with the exception of high-expressing line hTM5C14, the degree of sensitivity may involve functional isoform differences. Our previous in vitro characterization (Novy et al., 1993*a*), found that hTM3 had a lower affinity for actin and required significantly more caldesmon to achieve half maximal binding than hTM5 or hTM5/3, so it is possible that the difference in cell line sensitivity is related to the actin binding properties of the force-expressed TM.

Another line of support for a stabilizing role for TM has emerged from the study of changes that occur with cell transformation. We and others have correlated the down regulation of high molecular weight TM isoforms in transformed cells with the maintenance of the diminished microfilament organization characteristic of the transformed phenotype (Lin et al., 1984, 1985; Hendricks and Weintraub, 1981; Cooper et al., 1985; Leavitt et al., 1986). It is important to note, however, that many other actin-binding and cytoskeletal proteins are also downregulated upon transformation. These include caldesmon (Novy et al., 1991; Owada et al., 1984), gelsolin (Vanderkerckhove et al., 1990), and vinculin (Raz et al., 1986). The disorganized nature of the actin cytoskeleton in transformed cells may therefore be a result of a combined loss of factors. Even in cases where reintroduction of TM results in the partial restoration of stress fibers (Prasad et al., 1993), the role of other, perhaps TM-dependent, stabilizing factors cannot be ruled out.

In summary, our analysis of CHO cells which express wild type or chimeric isoforms of hTM lends strong support for the role of nonmuscle TM in cytokinesis. We also provide direct evidence that TM alone does not stabilize actin filaments in vivo. A search for the specific mechanism of the cytokinesis defect is underway, and we are also investigating the effects of hTM overexpression on cell motility.

We would like to thank R.E. Novy, C. Jones, and C. S. Patil for helping in the construction of expression plasmids pCBhTM3, pCBhTM5, pCBhTM5/3, and pCBhTM5/2. We are grateful to Dr. M. Stinski for his generous gift of the pCB6 expression vector.

This work was supported in part by grants HD18577 and GM40580 from the National Institutes of Health.

Received for publication 16 August 1994 and in revised form 2 February 1995.

References

Balasubramanian, M. K., D. M. Helfman, and S. M. Hemmingsen. 1992. A new tropomyosin essential for cytokinesis in the fission yeast S. pombe. *Nature (Lond.).* 360:84–87.

Balasubramanian, M. K., B. R. Hirani, J. D. Burke, and K. L. Gould. 1994. The Schizosaccharomyces pombe cdc3$^+$ gene encodes a profilin essential for cytokinesis. *J. Cell Biol.* 125:1289–1301.

Bartegi, A., C. Ferraz, A. Fattoum, J. S. Widada, F. Heitz, R. Kassab, and J.-P. Liautard. 1990. Construction, expression and unexpected regulatory properties of a tropomyosin mutant with a 31-residue deletion at the C-terminus (exon 9). *Eur. J. Biochem.* 194:845–852.

Bonder, E. M., and M. S. Mooseker. 1983. Direct electron microscopic visualization of barbed end capping and filament cutting by intestinal microvillar 95-kdalton protein (villin): a new actin assembly assay using the limulus acrosomal process. *J. Cell Biol.* 96:1097–1107.

Bradac, J. A., C. E. Gruber, S. Forry-Schaudies, and S. H. Hughes. 1989. Isolation and characterization of related cDNA clones encoding skeletal muscle β-tropomyosin and a low-molecular-weight nonmuscle tropomyosin isoform. *Mol. Cell. Biol.* 9:185–192.

Broschat, K. O., and D. R. Burgess. 1986. Low M_r tropomyosin isoforms from chicken brain and intestinal epithelium have distinct actin-binding properties. *J. Biol. Chem.* 261:13350–13359.

Broschat, K. O., A. Weber, and D. R. Burgess. 1989. Tropomyosin stabilizes the pointed end of actin filaments by slowing depolymerization. *Biochemistry.* 28:8501–8506.

Burgess, D. R., K. O. Broschat, and S. M. Hayden. 1987. Tropomyosin distinguishes between the two actin-binding sites of villin and affects actin-binding properties of other brush border proteins. *J. Cell Biol.* 104:29–40.

Cao, L.-G., and Y.-L. Wang. 1990*a*. Mechanism of the formation of contractile ring in dividing cultured animal cells. I. Recruitment of preexisting actin filaments into the cleavage furrow. *J. Cell Biol.* 110:1089–1095.

Cao, L.-G., and Y.-L. Wang. 1990*b*. Mechanism of the formation of contractile ring in dividing cultured animal cells. II. Cortical movements of microinjected actin filaments. *J. Cell Biol.* 111:1905–1911.

Cho, Y.-J., J. Liu, and S. E. Hitchcock-DeGregori. 1990. The amino terminus of muscle tropomyosin is a major determinant for function. *J. Biol. Chem.* 256:538–545.

Cho, Y.-J., and S. E. Hitchcock-DeGregori. 1991. Relationship between alternatively spiced exons and functional domains in tropomyosin. *Proc. Natl. Acad. Sci. USA.* 88:10153–10157.

Cooper, H. L., N. Feverstein, M. Noda, and R. H. Bassin. 1985. Suppression of tropomyosin synthesis, a common biochemical feature of oncogenesis by structurally diverse retroviral oncogenes. *Mol. Cell. Biol.* 5:972–983.

Cooper, J. A. 1987. Effects of cytochalasin and phalloidin on actin. *J. Cell Biol.* 105:1473–1478.

Eaton, B. L., D. R. Kominz, and E. Eisenberg. 1975. Correlation between the inhibition of the acto-heavy meromyosin ATPase and the binding of tropomyosin to F-actin: Effects of Mg^{++}, KCl, troponin I and troponin C. *Biochemistry.* 14:2718–2724.

Fattoum, A., J. H. Hartwig, and T. P. Stossel. 1983. Isolation and some structural and functional properties of macrophage tropomyosin. *Biochemistry.* 22:1187–1193.

Fishkind, D. J., and Y.-L. Wang. 1993. Orientation and three-dimensional organization of actin filaments in dividing cultured cells. *J. Cell Biol.* 123: 837–848.

Forry-Schaudies, S., N. J. Maihle, and S. H. Hughes. 1990. Generation of skeletal, smooth and low M_r non-muscle tropomyosin isoforms from the chicken tropomyosin 1 gene. *J. Mol. Biol.* 211:321–330.

Fowler, V. M., and V. Bennett. 1984. Erythrocyte membrane tropomyosin: Purification and properties. *J. Biol. Chem.* 259:5978–5989.

Hegmann, T. E., J. L.-C. Lin, and J. J.-C. Lin. 1988. Motility-dependence of the heterogenous staining of culture cells by a monoclonal anti-tropomyosin antibody. *J. Cell Biol.* 106:385–393.

Hegmann, T. E., J. L.-C. Lin, and J. J-C. Lin. 1989. Probing the role of nonmuscle tropomyosin isoforms in intracellular granule movement by microinjection of monoclonal antibodies. *J. Cell Biol.* 109:1141–1152.

Hendricks, M., and H. Weintraub. 1981. Tropomyosin is decreased in transformed cells. *Proc. Natl. Acad. Sci. USA.* 78:5633–5637.

Hitchcock-DeGregori, S. E., and R. W. Heald. 1987. Altered actin and troponin binding of amino-terminal variants of chicken striated muscle alpha-tropomyosin expressed in *Escherichia coli*. *J. Biol. Chem.* 262:9730–9735.

Hitchcock-DeGregori, S. E., P. Sampath, and T. D. Pollard. 1988. Tropomyosin inhibits the rate of actin polymerization by stabilizing actin filaments. *Biochemistry.* 27:9182–9185.

Ishikawa, R., S. Yamashiro and F. Matsumura,. 1989. Differential modulation of actin-severing activity of gelsolin by multiple isoforms of cultured rat cell tropomyosin. Potentiation of protective ability of tropomyosins by 83-kDa nonmuscle caldesmon. *J. Biol. Chem.* 264:7490–7497.

Lal, A. A. and E. D. Korn. 1986. Effect of muscle tropomyosin on the kinetics of polymerization of muscle actin. Biochemistry. 25:1154–1158.

Leavitt, J., G. Latter, L. Lutomski, D. Goldstein, and S. Burbeck. 1986. Tropomyosin isoform switching in tumorgenic human fibroblasts. *Mol. Cell. Biol.* 6:2721–2726.

Lees-Miller, J. P., and D. M. Helfman. 1991. The molecular basis for tropomyosin isoform diversity. *Bioessays.* 13:429–437.

Libri, D., M. Lemonnier, T. Meinnel, and M. Y. Fiszman. 1989. A single gene codes for the β subunits of smooth and skeletal muscle tropomyosin in the chicken. *J. Biol. Chem.* 264:2935–2944.

Lin, J. J.-C., S. Yamashiro-Matsumura, and F. Matsumura. 1984. Microfilaments in normal and transformed cells: Changes in the multiple forms of tropomyosin. *Cancer Cells.* 1:57–65.

Lin, J. J-C., D. M. Helfman, S. H. Hughes, and C.-S. Chou. 1985*a*. Tropomyosin isoforms in chicken embryo fibroblasts: Purification, characterization, and changes in rous Sarcoma virus-transformed cells. *J. Cell Biol.* 100:692–703.

Lin, J. J.-C., C.-S. Chou, and J. L.-C. Lin. 1985*b*. Monoclonal antibodies against chicken tropomyosin isoforms: production, characterization and application. *Hybridoma.* 4:223–242.

Lin, J. J.-C., T. E. Hegman and J. L.-C. Lin. 1988*a*. Differential localization of tropomyosin isoforms in cultured nonmuscle cells. *J. Cell Biol.* 107: 563–572.

Lin, J. J.-C., J. L-C. Lin, E. J. Davis-Nanthakumar, and D. Lourim. 1988*b*. Monoclonal antibodies against caldesmon, a Ca^{++}/ calmodulin- and actin-binding protein of smooth muscle and nonmuscle Cells. *Hybridoma.* 7: 273–287.

Lin, J. J.-C., E. J. Davis-Nanthakumar, J.-P. Jin, D. Lourim, R. E. Novy, and J. L.-C. Lin. 1991. Epitope mapping of monclonal antibodies against caldesmon and their effects on the binding of caldesmon to Ca^{++} caldesmon and to actin or actin-tropomyosin filaments. *Cell. Motil. & Cytoskeleton.* 20:95–108.

Liu, H., and A. Bretscher. 1989. Disruption of the single tropomyosin in yeast results in the disappearance of actin cables from the cytoskeleton. *Cell.* 57:233–242.

Lourim, D., and J. J.-C. Lin. 1992. Expression of wild-type and nuclear localization-deficient human lamin A in chick myogenic cells. *J. Cell. Sci.* 103:863–874.

Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurements with Folin phenol reagent. *J. Biol. Chem.* 193:265–275.

Mak, A. S., and L. B. Smillie. 1981. Nonpolymerizable tropomyosin: preparation, some properties and f-actin binding. *Biochem. Biophys. Res. Comm.* 101:208–214.

Mariani, B. D., D. L. Slate, and R. T. Schimke. 1981. S phase-specific synthesis of dihydrofolate reductase in Chinese hamster ovary cells. *Proc. Natl. Acad. Sci. USA.* 78:4985–4989.

Matsumura, F., and S. Yamashiro-Matsumura. 1985. Purification and characterization of multiple isoforms of tropomyosin from rat cultured cells. *J. Biol. Chem.* 260:13851–13859.

Nomura, M., K. Yoshikawa, T. Tanaka, K. Sobue, and K. Maruyama. 1987. The role of tropomyosin in the interactions of F-actin with caldesmon and actin-binding protein (or filamin). *Eur. J. Biochem.* 163:467–471.

Novy, R. E., J. J.-C. Lin, and J. L.-C. Lin. 1991. Characterization of cDNA clones encoding a human fibroblast caldesmon isoform and analysis of caldesmon expression in normal and transformed cells. *J. Biol. Chem.* 266:16817–16924.

Novy, R. E., J. R. Sellers, L.-F. Liu, and J. J.-C. Lin. 1993*a*. *In vitro* functional characterization of bacterially expressed human fibroblast tropomyosin isoforms and their chimeric mutants. *Cell Motil. & Cytoskeleton.* 26:248–261.

Novy, R. E., L.-F. Liu, C.-S. Lin, D. M. Helfman, and J. J.-C. Lin. 1993*b*. Expression of smooth muscle and nonmuscle tropomyosin in Escherichia coli and characterization of bacterially produced tropomyosins. *Biochim. Biophys. Acta.* 1162:255–265.

Novy, R. E., J. L.-C. Lin, C.-S. Lin, and J. J.-C. Lin. 1993*c*. Human fibroblast tropomyosin isoforms: Characterization of cDNA clones and analysis of tropomyosin isoform expression in human tissues and in normal and transformed cells. *Cell Motil. & Cytoskeleton.* 25:267–281.

Owada, M. K., A. Hakura, I. Kazuko, Y. Yahara, K. Sobue, and S. Kakiuchi. 1984. Occurrence of caldesmon (a caldesmon-binding protein) in cultured cells: comparison of normal and transformed cells. *Proc. Natl. Acad. Sci. USA.* 81:3133–3137.

Phillips, G. N. Jr., J. P. Fillers, and C. Cohen. 1986. Tropomyosin crystal structure and muscle regulation. *J. Mol. Biol.* 192:111–131.

Pittenger, M. F., and D. M. Helfman. 1992. *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. *J. Cell. Biol.* 118:841–858.

Pittenger, M. F., J. A. Kazzaz, and D. M. Helfman. 1994. Functional properties of nonmuscle tropomyosin isoforms. *Curr. Opin. Cell. Biol.* 6:96–104.

Prasad, G. L., R. A. Fuldner, and H. L. Cooper. 1993. Expression of transduced tropomyosin 1 cDNA suppresses neoplastic growth of cells transformed by the ras oncogene. *Proc. Natl. Acad. Sci. USA.* 90:7039–7043.

Raz, A., M. Zoller, and Ben-Ze'Ev. 1986. Cell configuration and adhesive properties of metastasizing and non-metastasizing BSp73 rat adenocarcinoma cells. *Exp. Cell Res.* 162:127–141.

Sanders, C., B. D. Sykes, and L. B. Smillie. 1988. Comparison of the structure and dynamics of chicken gizzard and rabbit cardiac tropomyosins: ^1H NMR spectroscopy and measurement of amide hydrogen exchange rates. *Biochemistry.* 27:7000–7008.

Satterwhite, L. L., and T. D. Pollard. 1992. Cytokinesis. *Curr. Opin. Cell Biol.* 4:43–52.

Spudich, J. A., and S. Watt. 1971. The regulation of rabbit skeletal muscle contraction: I. Biochemical studies of the interaction of the tropomyosin-troponin complex with actin and the proteolytic fragment of myosin. *J. Biol. Chem.* 246:4866–4871.

Tanaka, J., T. Watanabe, N. Nakamura, and K. Sobue. 1993. Morphological and biochemical analysis of contractile proteins (actin, myosin, caldesmon and tropomyosin) in normal and transformed cells. *J. Cell. Sci.* 104:595–606.

Vandekerckhove, J., G. Bauw, K. Vancompernolle, B. Honore, and J. Celis. 1990. Comparative two-dimensional gel analysis and microsequencing identifies gelsolin as one of the most prominent downregulated markers of transformed human fibroblast and epithelial cells. *J. Cell. Biol.* 111:95–102.

Warren, K. S., and J. J.-C. Lin. 1993. Forced expression and assembly of rat cardiac troponin T isoforms in cultured muscle and nonmuscle cells. *J. Muscle Res. Cell Motil.* 14:619–632.

Warren, K. S., J. L.-C. Lin, D. D. Wamboldt, and J. J.-C. Lin. 1994. Overexpression of human fibroblast caldesmon fragment containing actin-, Ca^{++}/ calmodulin-, and tropomyosin-binding domains stabilizes endogenous tropomyosin and microfilaments. *J. Cell Biol.* 125:359–368.

Yamashiro-Matsumura, S., and F. Matsumura. 1988. Characterization of 83-kD nonmuscle caldesmon from cultured rat cells: stimulation of actin binding of nonmuscle tropomyosin and periodic localization along microfilaments like tropomyosin. *J. Cell Biol.* 106:1973–1983.