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Specificity of dimer formation in tropomyosins: Influence of alternatively spliced exons on homodimer and heterodimer assembly

(protein–protein interaction/protein subunit association/coiled coil/epitope tag)

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ABSTRACT **Tropomyosins consist of nearly 100% α-helix and assemble into parallel and in-register coiled-coil dimers. *In vitro* it has been established that nonmuscle as well as native muscle tropomyosins can form homodimers. However, a mixture of muscle α and β tropomyosin subunits results in the formation of the thermodynamically more stable α/β heterodimer. Although the assembly preference of the muscle tropomyosin heterodimer can be understood thermodynamically, the presence of multiple tropomyosin isoforms expressed in nonmuscle cells points toward a more complex principle for determining dimer formation. We have investigated the dimerization of rat tropomyosins in living cells by the use of epitope tagging with a 16-aa sequence of the influenza hemagglutinin. Employing transfection and immunoprecipitation techniques, we have analyzed the dimers formed by muscle and nonmuscle tropomyosins in rat fibroblasts. We demonstrate that the information for homo- versus heterodimerization is contained within the tropomyosin molecule itself and that the information for the selectivity is conferred by the alternatively spliced exons. These results have important implications for models of the regulation of cytoskeletal dynamics.**

Tropomyosins (TMs) are a family of actin filament-binding proteins that are among the major components of the thin filaments of striated and smooth muscle and the microfilaments of nonmuscle cells (1). Although TMs are expressed in all cells, different isoforms of the protein are characteristic of specific cell types. Isoform diversity of TMs in vertebrate cells is generated by a combination of four genes, multiple promoters, and alternative splicing mechanisms (2–4). We are just beginning to understand the relationship of TM isoform expression and function (5), and although a large number of TM isoforms have been identified, the specific function of these isoforms in different cell types remains to be determined.

The TM subunits consist of nearly 100% α-helix, and two chains assemble into parallel and in-register coiled-coil dimers (6, 7). TM functions as a dimer and isoform diversity allows a variety of different dimers to be formed. One of the key questions in the understanding of TM function is what mechanisms are responsible for the regulation of the assembly of homo- and heterodimers and whether cell type-specific factors are involved in this process. Rat embryo fibroblasts (REF 52 cell line) express seven nonmuscle TM isoforms simultaneously (refs. 3 and 4; W. Guo and D.M.H., unpublished work): three high molecular weight (HMW) TMs (TM1, TM2, and TM3) and four low molecular weight (LMW) TMs (TM4, TM5, TM5a, and TM5b) of 284 and 248 aa, respectively. Skeletal and smooth muscle α and β TM subunits preferentially assemble into the thermodynamically more stable α/β heterodimers (8–10). By contrast, studies of TMs from fibroblasts demonstrate that these isoforms exist as homodimers (11, 12), suggesting a more complex principle for determining the formation and stabilization of a specific and functional dimer. Because specificity is determined additionally by the relative thermodynamic stability of all possible protein–protein interactions, understanding this process requires identification of the forces that stabilize preferentially the favored complex as well as forces that destabilize the incorrect one (13). Several groups have addressed the question of homo- versus heterodimer formation, using purified muscle (10, 14, 15) or nonmuscle (11, 12) TMs. However, all of these approaches required either denaturation (10, 14) or chemical stabilization (11, 12, 16) of the TM chains. Thus, these approaches might not identify the possible involvement of cellular factors in the selective formation of TM dimers. This type of assay also seemed not to be suitable for identifying the state and composition of the TM dimers prior to the chemical and thermal treatment. In addition, isoform-specific antibodies are not available for all the isoforms expressed in a single nonmuscle cell, thereby hindering the ability to recover specific TMs in order to identify the subunits present in a given dimer.

In the present study we have used living cells as a system to generate TMs containing the correct set of posttranslational modifications essential for the correct function of TM (17–24). We investigated the dimerization of TMs in cultured cells by epitope tagging with a 16-aa sequence of influenza hemagglutinin (HA-tag) (25, 26) to allow the identification of the native composition of TM homo- and heterodimers. We used immunoprecipitation from extracts of transiently transfected REF 52 cells at low temperature, where chain exchange is minimized (14), to analyze the composition of the dimers. We demonstrate that the information for homo- versus heterodimerization is contained within the TM molecule itself and that the information for the selectivity is conferred by the alternatively spliced exons.

EXPERIMENTAL PROCEDURES

Construction of Transfection Plasmids. All TM constructs were cloned in-frame between *Xba* I and *Bam*HI sites in the pCGN expression vector (27) with or without the 16-aa HA-tag sequence. Primers were 5′-ATGGACGCCATCAAGAAGAAG-3′ for TM1, TM2, TM3, smooth muscle α-TM, and skeletal muscle α-TM; 5′-ATGGCCGGCCTCAACTCACTG-3′ for TM4; and 5′-ATGGCGGGTAGCTCGCTG-3′ for TM5a and TM5b. Each tagged TM therefore carried the HA-tag sequence at its amino terminus.

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Abbreviations: TM, tropomyosin; HMW, high molecular weight; LMW, low molecular weight; HA, hemagglutinin.

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Immunofluorescence. Rat embryo fibroblast (REF 52) cells grown to 75% confluence in Dulbecco's modified Eagle's medium with 10% fetal bovine serum were transfected with 1 μg of total DNA per construct and 25 μl of Lipofectamine (GIBCO) per 60-mm dish for 24 hr and prepared for immunofluorescence as described (28). Tagged TMs were visualized with anti-HA antibody 12CA5 and rhodamine-conjugated goat anti-mouse secondary antibody (Molecular Probes). Fluorescent images were photographed on a Zeiss Axiophot microscope using a ×63 oil-immersion lens and Kodak P3200 Tmax film. The transfection efficiency in REF 52 cells was observed to be at around 10% with the Lipofectamine method.

Immunoprecipitation. REF 52 cells after 24 hr of transfection were washed three times with ice-cold phosphate-buffered saline (pH 7.4) containing 5 mM $MgCl_2$ and 2 mM EGTA. Proteins for immunoprecipitation were extracted in 200 μl of IP buffer [20 mM imidazole/300 mM KCl/5 mM $MgCl_2$/5% (vol/vol) glycerol/1% (vol/vol) Triton X-100/1 mM phenylmethanesulfonyl fluoride/1 mM NaN_3/1 mM ATP, pH 7.0] per 60-mm dish for 60 min on ice. Cellular residue was removed by centrifugation and the extract was precleared for 60 min with protein A-Sepharose (Pharmacia) in IP buffer. The supernatant was transferred to a fresh tube washed twice for 5 min in IP buffer to reduce nonspecific binding. Five micrograms of 12CA5 antibody was added and the suspension was incubated on ice for 60 min. After addition of 30 μl of protein A-Sepharose, incubation was continued for another 60 min. The beads were washed three times in IP buffer and once in phosphate-buffered saline and then prepared for gel electrophoresis.

Electrophoresis and Western Blotting. Analytical SDS/12.5% PAGE in mini-slab gels and Western blotting onto nitrocellulose (Hybond, Amersham) were performed as described (29). Transferred proteins were visualized with a horseradish peroxidase-coupled secondary antibody and the ECL chemiluminescence detection system (Amersham).

Antibodies. Monoclonal anti-TM antibody (clone 311) that recognizes all the HMW TMs was from Sigma. Monoclonal anti-HA antibody (clone 12CA5; refs. 25 and 26) was produced as mouse ascites fluid in Cold Spring Harbor. Monoclonal anti-TM antibody (clone MP10) that recognizes the low molecular weight TMs was kindly provided by Mark Pittenger (Cold Spring Harbor Laboratory).

RESULTS

Cell Transfection. We have used the expression vector pCGN (Fig. 1*A*) for the transfection of mammalian cells with each of the nine rat TM isoforms, which are the products of the three genes depicted in Fig. 1*B*. Each TM was expressed either with or without an amino-terminal 16-aa HA-tag. Transfected rat embryo fibroblast (REF 52) cells expressed significant amounts of the tagged TM isoforms, and no premature chain-termination products were detected by Western blotting (Fig. 2). The ratio of exogenous TM subunits to the major endogenous TM subunit (TM1 in REF 52 cells) was determined to be at about 1:1. This value represents a semiquantitative estimate from Western blots of total cell extracts (with an estimated transfection efficiency of 10%) and is therefore not equivalent to the situation found in every single cell. Further, the levels of expression (as determined by immunofluorescence) additionally varied by a factor of ≈20, and cells expressing very high amounts of tagged TM contained large cisternal structures filled with immunoreactive material (data not shown).

Incorporation of Tagged TM Isoforms into Actin Stress Fibers. Tagged TMs were incorporated into the actin-containing stress fibers of REF 52 cells within 24 hr after transfection. The immunofluorescent patterns obtained with the 12CA5 antibody (Fig. 3) are comparable with those seen

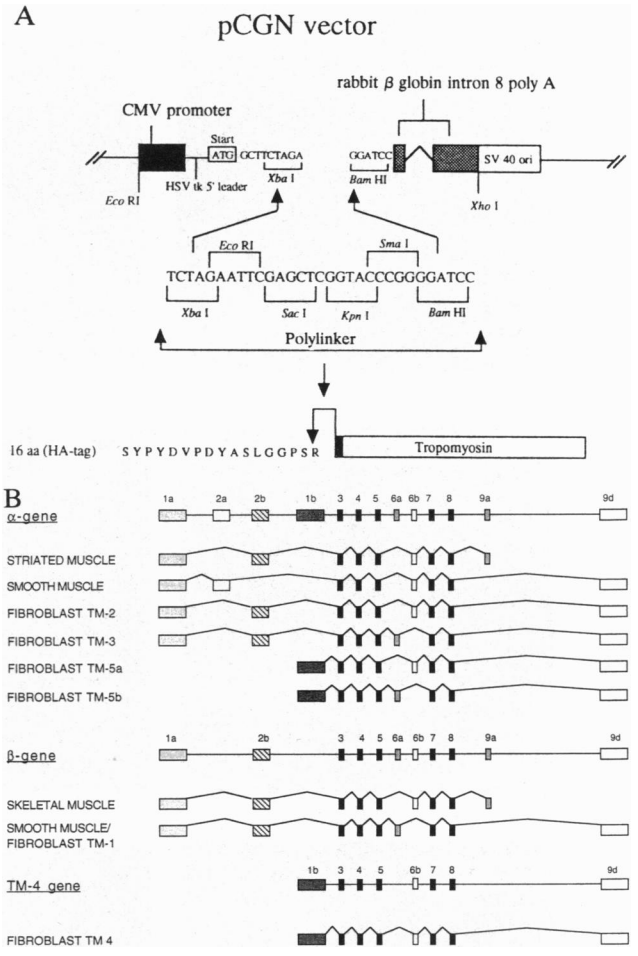

FIG. 1. (*A*) Mammalian expression vector pCGN. From left to right, labeled features include the human cytomegalovirus (CMV) promoter, the herpes simplex virus (HSV) thymidine kinase (tk) gene 5′ untranslated leader and initiation codon, the unique *Xba* I site engineered downstream of the initiation codon, the engineered *Bam*HI site, the rabbit β-globin gene segment containing splicing and poly(A)-addition signals, and the simian virus 40 (SV 40) origin of replication (ori). (*B*) Intron–exon organization of the rat TM genes and spliced TM isoforms used for transfection. Nonmuscle cells express multiple isoforms of 284 aa (TM1, TM2, and TM3) and 248 aa (TM4, TM5a, and TM5b) simultaneously, whereas muscle cells express either one (the α isoform in cardiac muscle) or two (both α and β chains in smooth or skeletal muscle) of the HMW type.

with TM-specific antibodies (30). Thus, the tag sequence at the amino terminus did not interfere with the ability of the TMs to associate with F-actin bundles. The HA-tagged TMs were also expressed in and purified from *Escherichia coli* and were found to cosediment with F-actin in an *in vitro* cosedimentation assay with rabbit skeletal muscle F-actin (data not shown). This result is consistent with previous studies demonstrating that wild-type recombinant TM was incorporated into stress fibers whether it was unacetylated (30, 31) or blocked by an 80-residue amino-terminal fusion (31) and that the patterns of

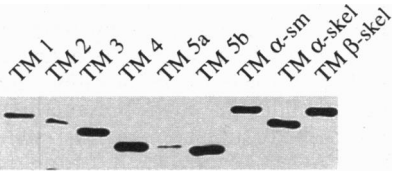

FIG. 2. Western blotting of whole cell extracts with anti-HA antibody 12CA5 demonstrating the high amounts of tagged TM isoforms present in transfected REF 52 cells.

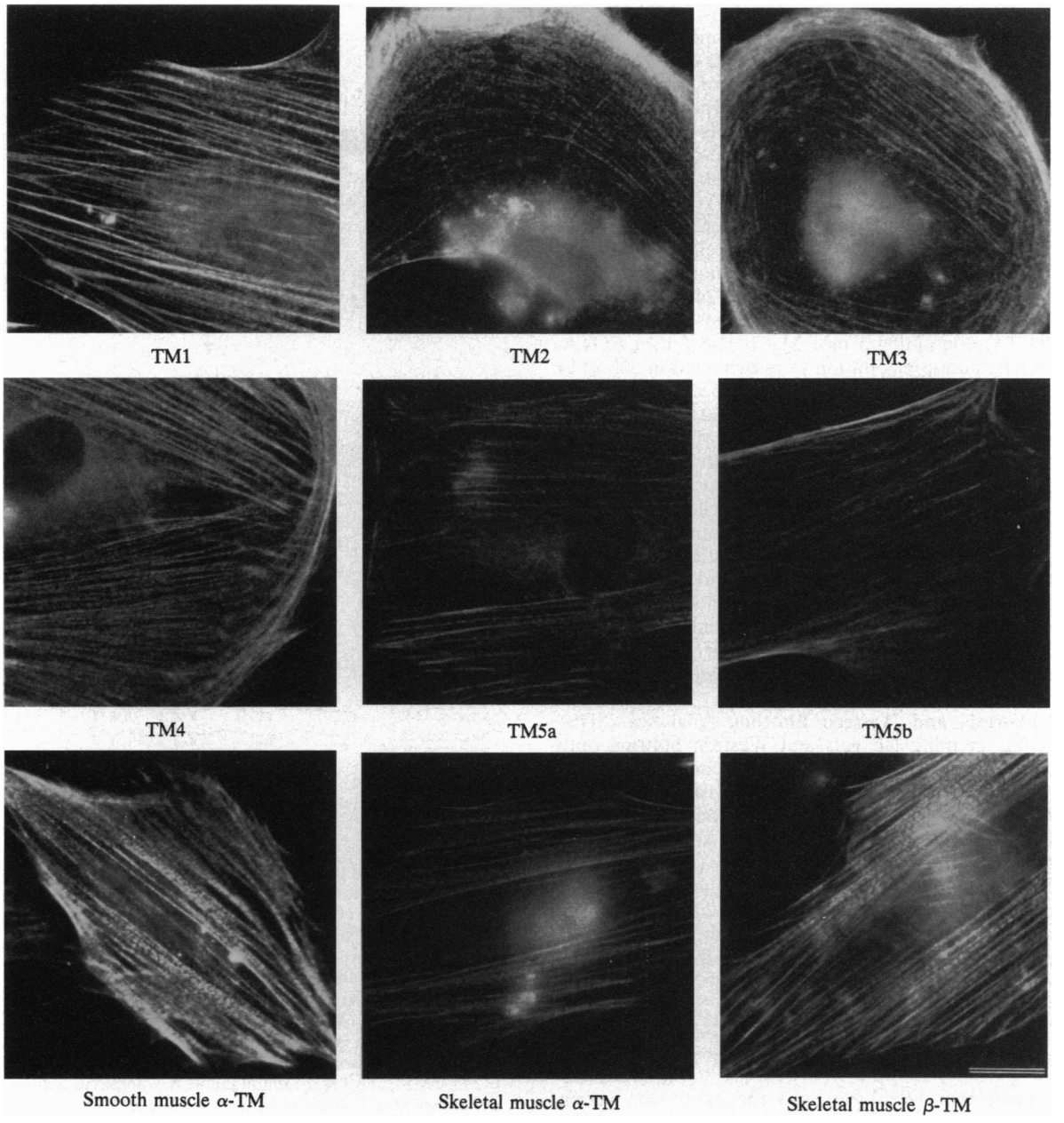

FIG. 3. Immunofluorescence showing that all HA-tagged isoforms localize and integrate into the actin-containing stress fibers of REF 52 cells. (Bar = 20 μm.)

incorporated TM were indistinguishable from that of endogenous TM (30) or isolated chicken pectoral muscle TM (31).

Dimer Formation. The transient expression of HA-tagged or untagged exogenous TM isoforms in REF 52 cells allows the formation of a variety of dimers in addition to the dimers present in the untransfected cells (Fig. 4*A*). Besides the native homo- or heterodimers containing the endogenous TM chains (I), hybrid dimers containing either one (II) or two (III) tagged exogenous TM chains are possible. In addition, in double transfections where one tagged isoform and one untagged isoform are introduced into cells simultaneously, the formation of "native hybrids" containing two untagged chains, one endogenous and one exogenous, are possible. The differences in electrophoretic mobility due to the presence of the 16-aa tag allow the identification of both homodimers and heterodimers by SDS/12.5% PAGE. When extracts from metabolically ^{35}S-labeled transfected REF 52 cells were separated in SDS/12.5% polyacrylamide gels and autoradiographed, the expression of the tagged TM chains in these cells 24 hr after transfection was seen to be comparable to that of the endogenous TMs. Immunoprecipitations from these cell extracts further revealed that the tagged TMs were precipitated in equal amounts by the anti-HA antibody and that actin contamination was negligible (data not shown).

Western blot analysis of the immunoprecipitated tag-containing dimers demonstrated the preference of the 284-aa HMW nonmuscle isoforms TM1, TM2, and TM3 to form homodimers (Fig. 4*B*). REF 52 cells express the three HMW TM isoforms in different amounts (TM1 > TM2 >> TM3). To eliminate problems due to differential amounts of exogenous and endogenous TMs present in the extracts, cotransfections were performed with the same expression vector introducing the same TM subunit both tagged and untagged into the cells. Cotransfections of tagged TM constructs in parallel with untagged TMs did not alter the homodimer preference of the HMW nonmuscle isoforms (Fig. 4*C*).

Since fibroblast TM2 differs from smooth muscle α-TM or skeletal muscle α-TM by the use of exons 2a/2b or 9a/9d,

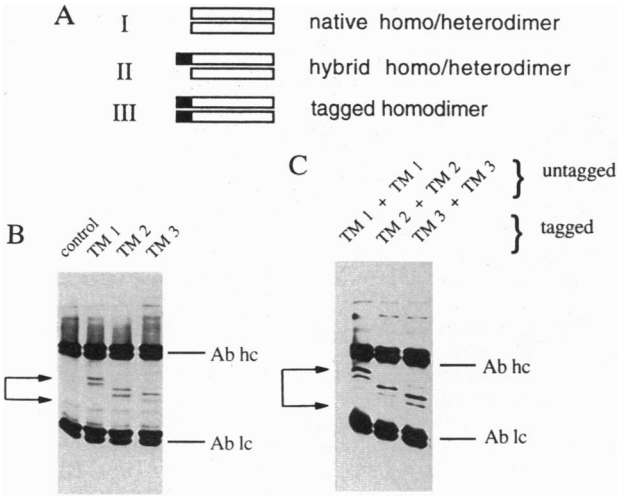

FIG. 4. (*A*) Contribution of the different possible dimers to the bands analyzed in SDS/polyacrylamide gels (see text). Open bars symbolize the TM chains; black boxes indicate the presence or absence of the HA-tag sequence. (*B*) Western blot analysis of anti-HA (12CA5) immunoprecipitates probed with a monoclonal antibody recognizing all HMW TMs (clone 311; Sigma). Single transfections of REF 52 cells with each of the nonmuscle HMW TM variants resulted in the precipitation of predominantly homodimers of the respective TM subunits. The presence of the 16-aa HA-tag increases the apparent molecular mass of the monomer chain by ≈2 kDa, allowing the identification of both tagged (upper band) and untagged (lower band) TMs according to their mobility in SDS/12.5% polyacrylamide gels. Two strongly reacting bands represent the reaction of the heavy (Ab hc) and light (Ab lc) chains of the added 12CA5 antibody with the goat anti-mouse secondary antibody. Area between arrows indicates position of TMs. When an extract of untransfected cells was used (control), no TMs were identified in the immunoprecipitate. (*C*) Double transfections using both one tagged and one untagged HMW isoform resulted in the precipitation of homodimers.

respectively (see Fig. 1*B*), it was possible that these alternatively spliced exons were critical for the selective formation of homo- versus heterodimers. When tagged smooth muscle α-TM or skeletal muscle α-TM isoforms were cotransfected with untagged HMW nonmuscle TM isoforms, the formation of heterodimer was predominant (Fig. 5 *A* and *B*). Similarly, skeletal muscle β-TM formed heterodimers with TM1, TM2, and TM3 (Fig. 5 *A* and *B*). Thus, the muscle isoforms were capable of shifting the dimer equilibrium in the direction of the heterodimer. The selectivity of the dimer formation was further indicated by the results obtained with combinations of HMW and LMW TM isoforms. Tagged smooth muscle α-TM, skeletal muscle β-TM, or nonmuscle TM2 failed to form stable heterodimers with cotransfected LMW nonmuscle variant TM4, TM5a, or TM5b (Fig. 5*C*).

DISCUSSION

Coiled coils are found as stabilizing motifs in many different types of dimeric proteins. In the case of the basic region–leucine zipper (bZIP) family of transcription factors, Lumb and Kim (32) demonstrated that the dimerization into parallel coiled-coil dimers was controlled by the leucine zipper sequence. Similarly, O'Shea *et al.* (13) identified 8 residues in the 35-aa leucine zipper region of the oncoproteins Fos and Jun as the essential determinants for their heterodimerization into parallel, two-stranded coiled coils and demonstrated that the driving force for preferential heterodimer formation was the destabilization of the Fos homodimer. In addition, neurotrophins have been shown to be biologically active as noncovalently linked homodimers. Recently, Heymach and Shooter (33) have used a technique similar to the one applied in this work to show that three members of the neurotrophin family (nerve growth factor, brain-derived neurotrophic factor, and neurotrophin 3) were able to form heterodimers upon forced expression following transfection of cells. Our results demonstrate that amino- and carboxyl-terminal regions, as well as internal regions, contribute to the coiled-coil interactions of the TM subunits.

There is general agreement that the α-helical TM molecule unfolds and dissociates in parallel during a heating experiment, indicating regions of varying stability or many states of partially unfolded molecules. The unfolding of heterodimers is expected to be even more complex than that of homodimers, since not only domains or states of heterodimers are involved, but homodimer domains (or states) also contribute to the equilibrium state (34). In a CD study of *Rana esculenta* α/β TM, two major helix–coil transitions were observed which have been interpreted as the unfolding of independent domains (34). Ishii *et al.* (35, 36) have demonstrated that recombinant TMs carrying an amino-terminal fusion peptide are still

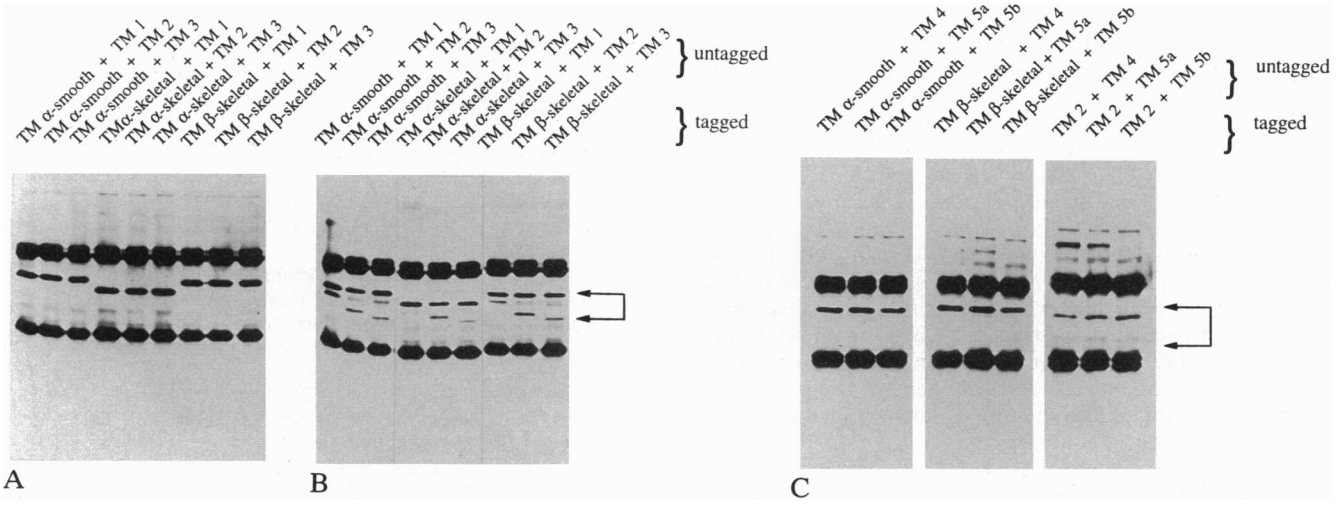

FIG. 5. (*A* and *B*) Cotransfections using HA-tagged muscle isoforms in combination with untagged HMW nonmuscle isoforms resulted in the predominant formation of stable heterodimers. Western blots of anti-HA immunoprecipitates were probed with anti-HA antibody 12CA5 (*A*) and anti-TM antibody 311 (*B*). (*C*) When either muscle TMs or a HMW nonmuscle TM (TM2) was cotransfected with LMW nonmuscle TMs, however, no stable dimers were precipitated. Western blot probed with a mixture of antibodies 12CA5 and MP10 (gift of Mark Pittenger; recognizes low molecular weight TMs). Area between arrows indicates position of TMs.

close together for local unfolding while they are separated for global unfolding. The conformation of the local unfolded region and the mechanism of the local unfolding of TM remained unsolved, however.

Whereas rat muscle cells express only one (cardiac) or two (smooth and skeletal) isoforms of TM, up to seven different TMs are present in a single rat fibroblast (refs. 4 and 30; W. Guo and D.M.H., unpublished work). We have shown that the presence of the amino-terminally tagged transfected muscle isoforms in a nonmuscle cell leads to the formation of heterodimers between endogenous and exogenous HMW nonmuscle TMs. Muscle-specific factors cannot be involved in the process of the formation (or stabilization) of heterodimers since this process is taking place in a cultured nonmuscle cell. Nonmuscle-specific factors were also not effective in maintaining the homodimeric state of the endogenous nonmuscle TMs in the presence of the transfected muscle isoforms.

Different TM isoforms differ in binding to troponin T, the formation of head-to-tail overlaps, and their affinity for F-actin (reviewed in refs. 3 and 4). A relationship between alternatively spliced exons and functional domains in TMs was found by investigating the influence of exons 2a/b and 9a/d of the smooth and striated α-TMs (5). Although the presence of exon 9a was correlated with Ca^{2+}-insensitive binding to troponin and the presence of exon 2a was correlated with changes in actin affinity, the individual exons were not recognizable as individual structural domains (5). Expression of chimeric TMs in fibroblasts recently indicated that a coordination between the amino- and carboxyl-terminal regions is required for normal TM function (37). Our data support this latter assumption and suggest that coordination between several domains in TMs is responsible for the formation of functionally relevant TM dimers. Exons 3–5, 7, and 8 are common to all TMs and appear from our studies to be insufficient to warrant stable dimerization *per se*, since stable heterodimers between LMW and HMW TMs were not observed under our assay conditions. Whether or not changes in the sequences of these conserved domains could lead to perturbation of the dimeric interactions of TM subunits is unknown. Additionally, it will be interesting to determine the influence of exon 1b (used in the 248-aa subunits of TM4, TM5, TM5a, and TM5b) on the dimerization properties of the LMW isoforms.

The functional significance of expressing a multitude of TM isoforms in nonmuscle cells and maintaining them as homodimers remains to be determined. The higher actin-binding ability of strongly head-to-tail overlapping heterodimers of smooth muscle α and β TM subunits in chicken gizzard might reflect the necessity to maintain stable association with the muscle thin filament throughout the entire length of the actin filament. For chicken gizzard TM, the heterodimer exhibits a greater ability to bind cooperatively to F-actin, due to its stronger head-to-tail overlap as compared with the α/α and β/β homodimers. It has also been suggested that the TM heterodimers represent a more flexible structure than their homodimeric counterparts (38). A higher end-to-end association of TM heterodimers in smooth muscle cells could lead to the formation of elongated TM polymers, which could reflect the requirement for a higher degree of flexibility to accommodate the structural changes in the F-actin filament during contraction and relaxation cycles (38). In contrast, a reduced ability of the homodimers to associate head-to-tail could be a necessary determinant for the more dynamic regulation of the actin cytoskeleton in nonmuscle cells.

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