Journal of Cell Science 109, 2299-2310 (1996)
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JCS8014

N-tropomodulin: a novel isoform of tropomodulin identified as the major binding protein to brain tropomyosin

Akiya Watakabe, Ryuji Kobayashi and David M. Helfman*

Cold Spring Harbor Laboratory, PO Box 100, Cold Spring Harbor, NY 11724, USA

*Author for correspondence (e-mail: helfman@cshl.org)

SUMMARY

We have identified and characterized two proteins in rat brain that bind to the neuron-specific tropomyosin isoform, TMBr3. The two proteins were identified by blot overlay assay, in which the proteins immobilized on the membrane were probed by epitope-tagged TMBr3, followed by detection with anti-epitope antibody. We have purified these proteins using a TMBr3 affinity column. Peptide sequencing as well as immunoblotting showed that one of the two proteins is identical to tropomodulin, a tropomyosin-binding protein originally identified in erythrocytes. The cDNA for the other protein was cloned from an adult rat brain cDNA library using degenerate oligonucleotides that we designed based on the peptide sequences. Sequence analysis of the cDNA clone revealed this protein to be a novel isoform of tropomodulin which is the product of a distinct gene, and is herein referred to as N-tropomodulin. Recombinant N-tropomodulin bound to TMBr3 as well as to other low molecular mass tropomyosins (TM5a or TM5), but not to high molecular mass tropomyosins (TM2 or TMBr1). Northern blotting and RNase protection assays as well as immunoblotting showed that N-tropomodulin is expressed predominantly in brain. Furthermore, RNase protection assays revealed no alternatively spliced regions within the coding sequence. Developmentally, N-tropomodulin was detected in rat brain as early as embryonic day 14 and reaches the adult level before birth. Immunofluorescence of primary frontal cortex cell cultures showed that N-tropomodulin is specifically expressed in neurons. The neuron-specific expression of N-tropomodulin strongly suggests specialized roles of this TM-binding protein in neurons.

Key words: Tropomyosin, Tropomodulin, F-actin, Neuronal cytoskeleton

INTRODUCTION

Tropomyosins (TMs) are a family of rod-shaped proteins that bind to both grooves of filamentous actin (F-actin) forming a side filament (Pittenger et al., 1994). TMs are expressed from four genes and at least 16 distinct isoforms are generated from these genes by alternative processing (Fig. 1A). In brain, several TM isoforms are known to be expressed. TMBr3 is the major product from the α TM gene in brain (Lees-Miller et al., 1990) and is expressed specifically in neurons (Stamm et al., 1993). In developing brain, TMBr3 is detected shortly before birth and reaches maximal levels during the neonatal period (Stamm et al., 1993), which is coincident with the differentiation and maturation of neurons including massive synaptogenesis after birth. Other than TMBr3, TM4 is expressed in neurons, whose expression increases immediately after birth and then declines (Had et al., 1994). TM5 is also expressed in neurons and its intracellular localization changes from axonal to dendritic upon maturation (Weinberger et al., 1996). Considering the different and strictly regulated pattern of expression of different TM isoforms, it is likely that each TM isoform present in neurons exhibits a distinct and specific function.

The function of TM is best understood in striated muscle systems. In striated muscle, TM in association with the troponin complex (troponin T, C and I) functions as a coregulator of actomyosin contraction (for a recent review, see Farah and Reinach, 1995). TM isoforms are also thought to play diverse roles in other cell types, where the troponin complex is not present (Pittenger et al., 1994). Yeast genetics provide strong evidence for an essential role of TM in cytokinesis (Balasubramanian et al., 1992), vesicular transport (Liu and Bretscher, 1992) and morphogenesis (Drees et al., 1995). The role of TM in cytokinesis has also been suggested from studies on mammalian cells (Warren et al., 1995). In addition, mammalian studies also suggest the involvement of TM in intracellular motility (Hegmann et al., 1989) and in morphological change that is accompanied by transformation (Gimona et al., 1996; Prasad et al., 1993). Recently, genetic experiments in *Drosophila* have demonstrated the role of TM in mRNA localization in the oocyte (Erdelyi et al., 1995). While these data demonstrate some of the important functions of TMs, the tissue-specific role of TMs or isoform-specific function of TMs in most cell types is still not understood.

We are interested in the role of TM isoforms in brain, especially that of the neuron-specific isoform, TMBr3. To elucidate the function of TM, it is essential to identify the proteins that interact with TM. So far, several proteins have been shown to interact with TM and to affect actin-filament organization. Tropomodulin (Tmod) was originally identified

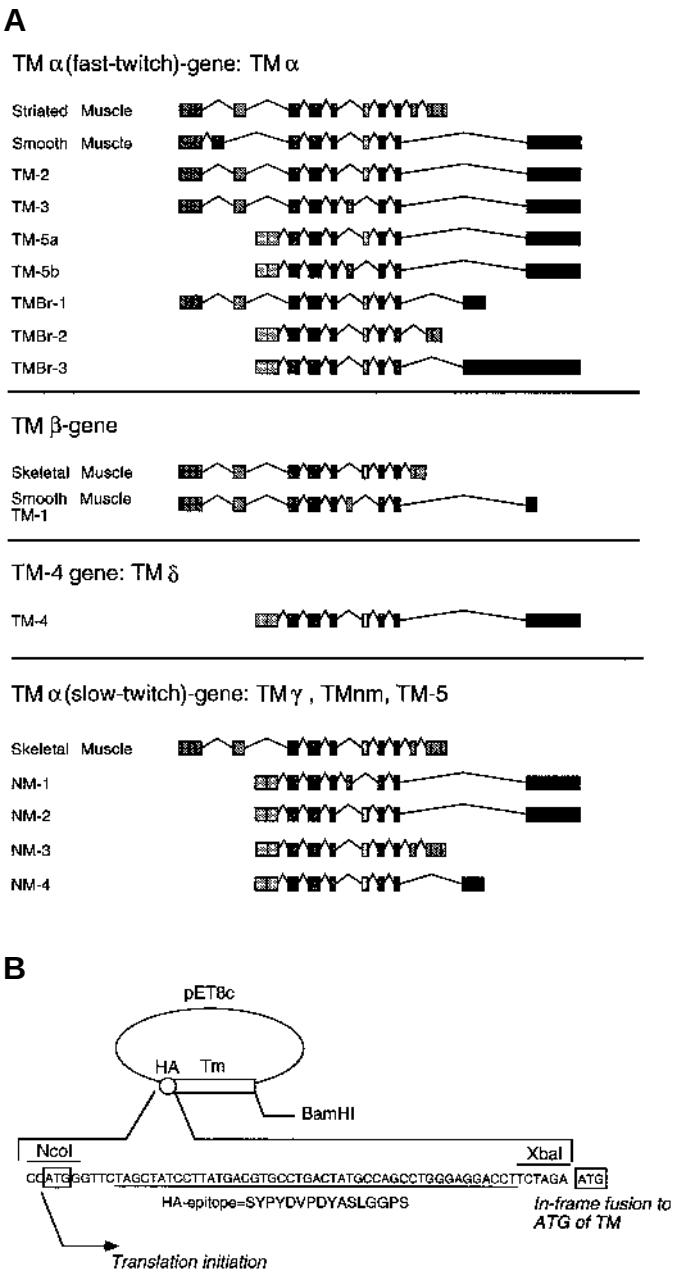

Fig. 1. (A) Schematic representation of various rat tropomyosin isoforms expressed from 4 different genes. The alternative names for each gene are also shown. To avoid confusion, in this manuscript, we refer to TMα (fast-twitch) gene simply as TMα gene, and TMα (slow-twitch) gene products as TM5 (NM-1-4). (B) Schematic representation of pET vector for HA-tagged TM expression. The vector contains a small epitope derived from the influenza virus hemagglutinin gene (HA, open circle) and the coding sequence for TM (oblong box). The nucleotide sequence around the translation initiation site is shown. The *Xba*I site originally present in pET8c vector was destroyed by filling with Klenow enzyme and the TM sequences were cloned into *Xba*I-*Bam*HI of this vector in order to fuse to the epitope-tag sequence in frame.

in erythrocytes as a binding protein to TM (Fowler, 1987) but is also expressed in a variety of other cell types including muscle (Babcock and Fowler, 1994; Fowler et al., 1993), lens (Woo and Fowler, 1994) and neurons (Ito et al., 1995; Sussman et al., 1994). Later, it was found that Tmod is a capping protein for the pointed ends of F-actin (Weber et al., 1994), and that it is required to maintain actin filament length in cardiac cells (Gregorio et al., 1995). In addition to Tmod, caldesmon (Matsumura and Yamashiro, 1993), calponin (Gimona and Small, 1996), pEL98 (18A2, p9Ka, or 42A) (Takenaga et al., 1994) and Rad (Zhu et al., 1996) all bind to TM. Of these TM-binding proteins, at least caldesmon and calponin isoforms are known to be expressed in neurons (Kira et al., 1995; Trabelsi et al., 1995). Another type of TM-interacting protein is a drebrin, a developmentally regulated neuro-protein which can inhibit the binding of TM to F-actin (Shirao, 1995).

In this paper, we employed a blot overlay technique to identify TMBr3-binding proteins in brain. Using this technique, we found that at least two proteins with a molecular mass of approximately 40 kDa can bind to TMBr3. Of these two, one proved to be tropomodulin. By purification and subsequent cDNA analysis, we found that the second protein is a novel isoform of tropomodulin. Further characterization showed that it is a neuron-specific isoform.

MATERIALS AND METHODS

Plasmid construction

pET-HA-TM5a and pET-HA-TMBr3 are derivatives of pET TM5a and pET TMBr3 (Pittenger and Helfman, 1992; Stamm et al., 1993) and contain short sequence derived from influenza hemagglutinin (HA-tag) at the N terminus (Fig. 1B). The expression plasmids for untagged N-Tmod as well as for His-tagged N- and E-Tmods were constructed by subcloning the PCR-amplified fragments containing the coding regions of Tmods into pET8c and pET11c, respectively. For the His-tag constructs, PCR fragments were subcloned together with short oligonucleotides encoding 6xHis sequence. To construct template plasmids used in RNase protection assays, either the full length of the N-Tmod coding sequence (1,056 nucleotides) or the 5′ truncated fragments (952 nt) was cloned into pBluescript II SK(+).

Expression and purification of recombinant proteins

The overexpression and the purification of recombinant TMs and HA-tagged TMs were performed as described (Pittenger and Helfman, 1992). To purify recombinant N-Tmod, the bacteria pellet was suspended in 10 mM sodium phosphate, 100 mM NaCl, pH 7.0, containing 1 mM Pefabloc (Boehringer Manheim), and sonicated. This suspension was centrifuged (Beckman 60 Ti rotor; 10,000 rpm for 20 minutes, 4°C) and the supernatant was purified by a column chromatographic method using a Hydroxyapatite column (Bio-Rad) and a MonoQ column (Pharmacia-LKB). The final peak fractions were pooled and dialyzed against 20 mM sodium phosphate, pH 7.0, 30 mM KCl, 1 mM DTT and stored at −70°C. His-tagged versions of N-Tmod and E-Tmod were purified using Ni-NTA resin (Qiagen) under native conditions according to the manufacturer's instructions.

Blot overlay assay

The blot overlay assay using antibody was performed essentially as described previously (Kriajevska et al., 1994). Proteins were separated by SDS-PAGE and transferred to a nitrocellulose membrane (Schleicher and Schuell). The membrane was incubated in blocking buffer (50 mM Tris-HCl, 150 mM NaCl, 2% BSA, pH 8.0) at room temperature for 1 hour and then incubated with recombinant HA-tagged TM (3 μg/ml) in overlay buffer (50 mM Tris-HCl, 100 mM NaCl, 0.5% BSA, 0.25% gelatin, 0.5% Nonidet P-40, 2 mM DTT, pH 8.0) at 4°C overnight. The membrane was washed 4 times for 5 minutes in buffer (50 mM Tris-HCl, 100 mM NaCl, 0.5% Nonidet P-

40, 2 mM DTT, pH 8.0) and immunostained with anti-HA antibody (clone 12CA5; Gimona et al., 1995) followed by chemiluminescence detection (DuPont).

Binding assay using His-tag tropomodulin

Samples (0.4 μg) of TMs were incubated with 0.4 μg of His-tagged Tmods in 20 μl binding buffer (50 mM Tris-HCl, pH 8.0, 100 mM NaCl, 0.5% Nonidet P-40) at room temperature for 1 hour. A 20 μl sample of Ni-NTA resin in binding buffer (1:1) was added to the solution and incubated at room temperature for 1 hour at constant mixing and 20 μl of the supernatant (the unbound proteins) were recovered and mixed with 10 μl of SDS-loading dye (Sambrook et al., 1989). The resin was washed 4 times with binding buffer and the bound proteins were eluted with 30 μl of 0.5 M imidazole, pH 7.0. Then, 20 μl of the supernatant was mixed with 10 μl of SDS-loading dye and the same volume of unbound and bound proteins were separated by SDS-PAGE and visualized by Coomassie staining.

Purification and protein sequencing of TMBr3 binding proteins

Rat brains were homogenized in 2 volumes of Buffer A (w/v) (Hepes-KOH, pH 8.0, 1 mM DTT, 1 mM Pefabloc, 1 μg/ml aprotinin). KCl and Triton X-100 were added to 40 mM and 0.9%, respectively. The homogenate was incubated on ice for 30 minutes followed by centrifugation at 55,000 rpm (Beckman 60 Ti). The pellet was homogenized in Buffer A, and 2 M KCl was slowly added to the final concentration of 600 mM. After 30 minutes of extraction, the suspension was centrifuged at 40,000 rpm (Beckman 60 Ti) and the supernatant was dialyzed against 50 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM DTT and saved for further purification. The affinity resin was prepared by coupling recombinant TMBr3 to CNBr-activated Sepharose (Pharmacia-LKB) according to the manufacturer's instructions. The TMBr3-affinity resin was mixed with the brain extract, incubated overnight at 4°C and the resin was collected into a column. The resin was washed with 5 column volumes of 50 mM Tris-HCl, pH 8.0, 1 M NaCl (fractions S1 to S5). Then bound proteins were eluted with 5 column volumes of 50 mM Tris-HCl, pH 8.0, 8 M urea (fractions U1 to U5).

The proteins in the purified fractions were precipitated by acetone, dissolved in SDS-running buffer and separated on a 10% SDS polyacrylamide gel. The protein bands were visualized by staining with Coomassie blue G (Aldrich). The TMBr3-binding proteins were excised and subjected to in-gel digestion as described previously (Sueyoshi et al., 1995). The resulting peptides were separated by HPLC using a Vydac C18 column (1.0 mm × 250 mm, 10 μm, 300 Å) and sequenced by automated protein sequencers (Applied Biosystems 470 and 477).

Cloning and characterization of E- and N-tropomodulin cDNAs

DNA and RNA manipulations including RNA isolation, RT-PCR, library screening, plasmid construction, nucleotide sequencing, northern blotting and RNase protection analyses were performed as previously described (Kawasaki, 1990; Sambrook et al., 1989). To clone N-Tmod cDNA, the degenerate primers 5′-GGATCCNGTNT-TYGARGARCCNCC-3′ and 5′-CTCTAGATTNGTRAAYTGRTA-NCC-3′ (N=G/T/A/C, Y=C/T, R=A/G) were designed based on the peptide sequence of K32 and K24 peptides (Table 1). RT-PCR using these primers and rat adult brain RNA produced an expected fragment of approximately 450 bp. This fragment was labeled and used to screen the λZap rat adult brain cDNA library (Stratagene). The cDNA clone for rat E-Tmod (original tropomodulin) was obtained from the λZap rat adult brain cDNA library (Stratagene) in the same manner except that the two degenerate primers 5′-GGAATTCATGAGTAACCA-(A/G)CA(A/G)TA-3′ and 5′-GGAATTCATGCTGAACTT(C/T)TT-(G/C)AC-3′ were used to obtain the probe fragment.

Table 1. Peptide sequences obtained from purified TMBr3 binding proteins

Band X	
K15	ALETNTHVRK
K23	GRVFI(P)K
K24	FGYQFTK
K26	NIDEDELLGK
K28	ANDPSLQEVNLNNIK
K32	PVFEEPPN(D)T
Tropomodulin	
K8	E(G)LN(S)V(I)(K)

Parentheses indicate the ambiguous amino acid position.

Antibodies and immunoblotting

A GST-fusion protein containing the C-terminal half of N-Tmod (amino acids 188-354) produced in *Escherichia coli* was purified and used as the antigen to immunize rabbits (HRP, Inc.). The antisera were affinity purified using His-tagged N-Tmod conjugated to CNBr-activated Sepharose (Pharmacia-LKB). Antibodies against human tropomodulin (E-Tmod) were a generous gift from M. K. Woo and V. M. Fowler (Scripps Inst. San Diego). Antibody against exon 9C of the TM α gene is described (Stamm et al., 1993). Antibodies against high molecular mass TM (clone TM311) and β-actin (clone AC-15) were from Sigma. Antibody against Glial fibrillary acidic protein (GFAP) was from Boehringer Manheim (clone G-A-5). Preparation of tissue extracts and immunoblotting was performed as described (Harlow and Lane, 1988; Sambrook et al., 1989).

Cell culture and immunofluorescence

Primary cultures of neurons were established from the frontal cortex of 16-day Sprague-Dawley rat embryos. After dissection, the excised tissue was washed and triturated with a Pasteur pipette in Dulbecco's modified Eagle's medium (Gibco BRL) containing 10% fetal calf serum (HyClone) and the cell suspension was plated on to dishes coated with poly-L-ornithine (Sigma). For immunofluorescence, primary cultures were prepared on coverslips coated with poly-L-ornithine and cell staining was performed as described (Harlow and Lane, 1988; Stamm et al., 1993). For the preabsorption experiments, the affinity purified N-Tmod antibody was diluted and incubated with 1.5 μg of His-tagged E- or N-Tmod at 4°C overnight, and the absorbed antibody was removed by binding to 20 μl (1:1) of Ni-NTA resin. The supernatant was used undiluted for immunofluorescence.

RESULTS

Identification of TMBr3-binding proteins using a blot overlay assay

Blot overlay assays have been used successfully to identify novel binding proteins, including a TM binding protein, tropomodulin (Crawford and Beckerle, 1994; Fowler, 1987; Gershoni, 1987). To identify TMBr3 binding proteins in brain, we employed a variation of this technique in which antibody was used to detect the probe binding. A total extract from brain was separated on SDS-PAGE, transferred to a nitrocellulose membrane, and probed with recombinant tropomyosin containing a HA-tag. Binding of the probe was subsequently detected by immunoblotting using an anti-HA antibody. We used as probes two tropomyosin isoforms, TM5a and TMBr3, alternatively produced from the TM α gene. The control assay using no probe was carried out in parallel in order to distinguish the probe binding from cross-reactivity of anti-HA antibody to brain proteins (Fig. 2, lane 3).

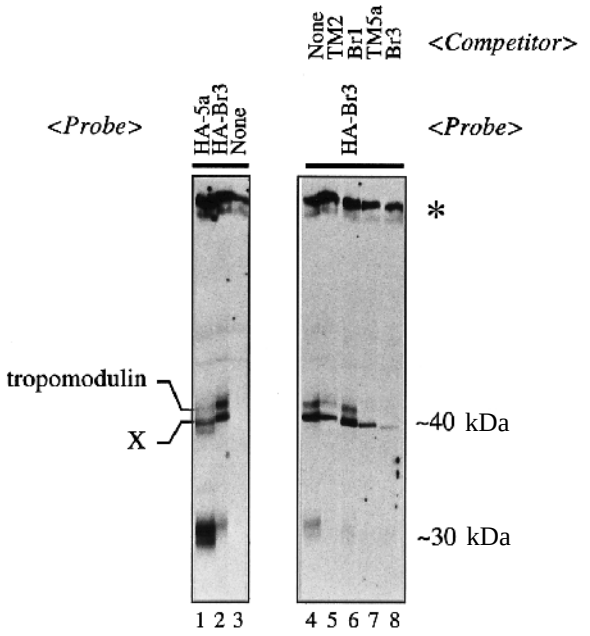

Fig. 2. Blot overlay assay to detect TM binding proteins in brain extracts. The blot overlay assay was carried out with the HA-tagged probes shown at the top of the panel. In lane 3, a control assay was carried out with no HA-tagged probe. In lanes 4-8, competition experiments were carried out with untagged TM isoforms. The membrane was first incubated overnight with a fivefold molar excess of various untagged competitors shown above the panel and HA-tagged TMBr3 was added and incubated for additional 2.5 hours before detection by anti-HA antibody. The band labeled * is the result of a cross reaction of the HA-antibody and appeared also in the absence of TM probes. The upper band of the 40 kDa doublet comigrated with tropomodulin. The upper and lower band of the 30 kDa doublet comigrated with TM4 and TMBr3, respectively. The lower 40 kDa band that was neither tropomodulin nor TM is indicated by X.

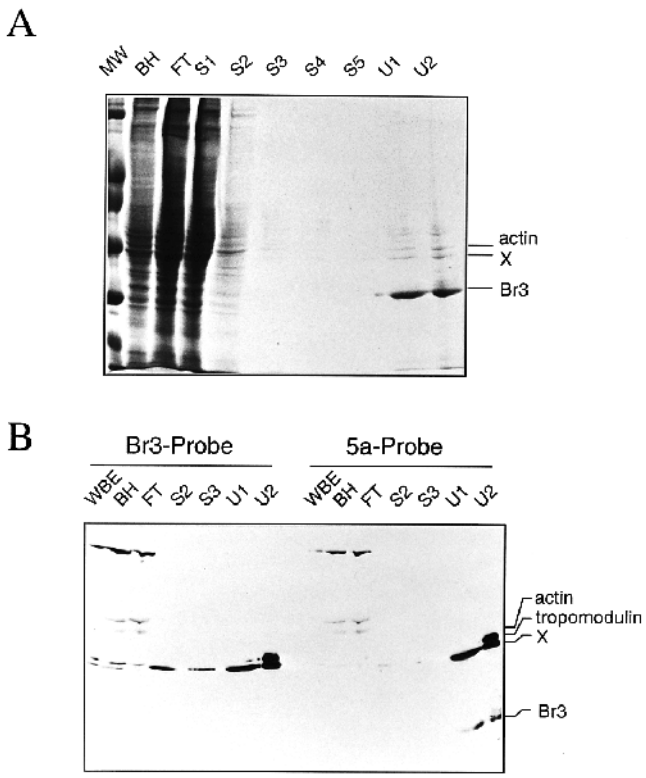

Fig. 3. Affinity purification of TMBr3 binding proteins from brain extracts. (A) Coomassie staining of purified fractions. Brain homogenate (BH) was mixed with TMBr3-conjugated Sepharose and the flow-through fraction was recovered (FT). The Sepharose beads were then washed with 1 M NaCl (S2-S5) and the bound proteins were eluted with 8 M urea (U1-U2); 10 μl of each sample was mixed with SDS-gel loading buffer, run on SDS-PAGE and stained with Coomassie blue. A prominent band labeled as Br3 indicates the position of recombinant TMBr3 eluted from the affinity resin. The upper band of around 40 kDa proved to be actin by microsequencing. X indicates the position of the lower 40 kDa band identified in the overlay assay. (B) Overlay assay of purified fractions. The samples described above were subjected to an overlay assay with either the HA-TMBr3 probe or the HA-TM5a probe.

As shown in Fig. 2, several bands were detected by the overlay assay using either HA-TMBr3 or HA-TM5a probes. Of these bands, the 40 kDa doublet was detected more strongly with the HA-TMBr3 probe than with the HA-TM5a probe, while the 30 kDa doublet was stronger with the HA-TM5a probe (Fig. 2, compare lanes 1 and 2). These 40 kDa and 30 kDa doublets were not detected in the control lane that used no TM probe (Fig. 2, lane 3). To confirm the binding specificity of these bands to the TMBr3-probe, we carried out competition experiments using various TM isoforms without the HA-tag. These experiments showed that untagged TMBr3 and TM5a compete for binding to the 40 kDa doublet, whereas two high molecular mass TMs from the TM α gene, TM2 and TMBr1, do not (Fig. 2, lanes 4-8). Although TM-2 appears to partially compete for binding of HA-TMBr3 to the 40 kDa doublet in this figure, competition was not observed in a separate experiment and HA-tagged TM2 did not bind to the 40 kDa doublet in the overlay assay (data not shown). Thus the 40 kDa proteins in brain represent TM-binding proteins with different affinities for different TM isoforms.

Fowler (1987, 1990) identified tropomodulin as a TM binding protein using a blot overlay assay. We tested if either of the bands in the 40 kDa doublet correspond to tropomodulin by immunoblotting with a tropomodulin antibody. This experiment revealed that the upper band corresponded to the size of tropomodulin (data not shown). Since TM can bind to itself in the overlay assay (Fowler, 1987), we electrophoresed various TM isoforms together with brain extract and examined if any of the TM isoforms correspond to the size of the 40 or 30 kDa doublets. We found that the upper and lower bands in the 30 kDa doublet correspond to the size of TM4 and TMBr3, respectively (data not shown). The mobility of the 40 kDa doublet did not correspond to any known TM isoforms. Thus, the lower 40 kDa band (labeled X in Fig. 2) is not TM nor tropomodulin, and most likely represented a novel TM-binding protein.

Purification and peptide sequencing of TMBr3 binding proteins

To purify the lower 40 kDa TMBr3 binding protein, extracts from adult rat brains were applied to a TMBr3-affinity column. After binding, the column was washed with high salt buffer, and then the proteins remaining bound to the column were

eluted with urea. As shown in Fig. 3A, the urea-eluted fractions (U1, U2) after 5 column volume washes with the high-salt buffer (S1-S5) contain only several major polypeptides when visualized by Coomassie staining. The most prominent band around 30 kDa is recombinant TMBr3 eluted from the column. Notably, we detected two major bands around 40 kDa and a minor band between them (weakly detectable in lane U2). Of these bands, the upper 40 kDa band was found to be actin by microsequencing (data not shown).

To determine if any band detected by Coomassie staining corresponds to the lower 40 kDa protein, an overlay assay was carried out using HA-tagged TMBr3 and TM5a probes (Fig. 3B). Comparing the binding pattern in the brain extract (lane BH) and the flow through (lane FT) from the affinity column, demonstrates that both bands in the 40 kDa doublet, especially the lower 40 kDa protein (labeled X) were adsorbed to the column. In the high salt wash fractions, S2 and S3, considerable amounts of lower 40 kDa protein were eluted. However, most of this protein remained bound to the column and eluted only after the addition of 8 M urea (Fig. 3B, lanes U1 and U2), suggesting a strong interaction with TMBr3. Direct comparison of Ponceau S staining and the overlay pattern of the same membrane showed that the lower 40 kDa Coomassie stained band is identical to the lower 40 kDa band detected by the overlay assay (labeled X in Fig. 3A and B). The upper 40 kDa band in the overlay assay that co-migrated with tropomodulin was found to migrate between this band and the actin band.

Using the highly purified protein preparations described above, we were able to gel-purify and obtain several peptide sequences of the lower 40 kDa band as well as a minor band that has the same mobility as tropomodulin (Table 1). Interestingly, a search of the database showed that K24 and K28 were homologous to tropomodulin. We also obtained peptide sequence (K8) from the minor band. This sequence matched that of tropomodulin, confirming its identity as tropomodulin. Thus, it is most likely that brain contains two distinct isoforms of tropomodulin: one corresponding to the isoform first identified by Fowler, and a second isoform present in brain.

cDNA cloning of two isoforms of rat tropomodulin

To further characterize and establish the identity of the putative novel tropomodulin isoform, it was necessary to determine the entire coding sequence of this protein. For this purpose, degenerate PCR primers were designed based on the peptide sequence of K32 and K24, which were obtained from the purified protein. RT-PCR with these primers were carried out using rat adult brain RNA. The fragment generated by RT-PCR was used as the probe to screen a rat adult brain cDNA library. The deduced amino acid sequence of the clone obtained is shown in Fig. 4A. The predicted protein sequence contains all

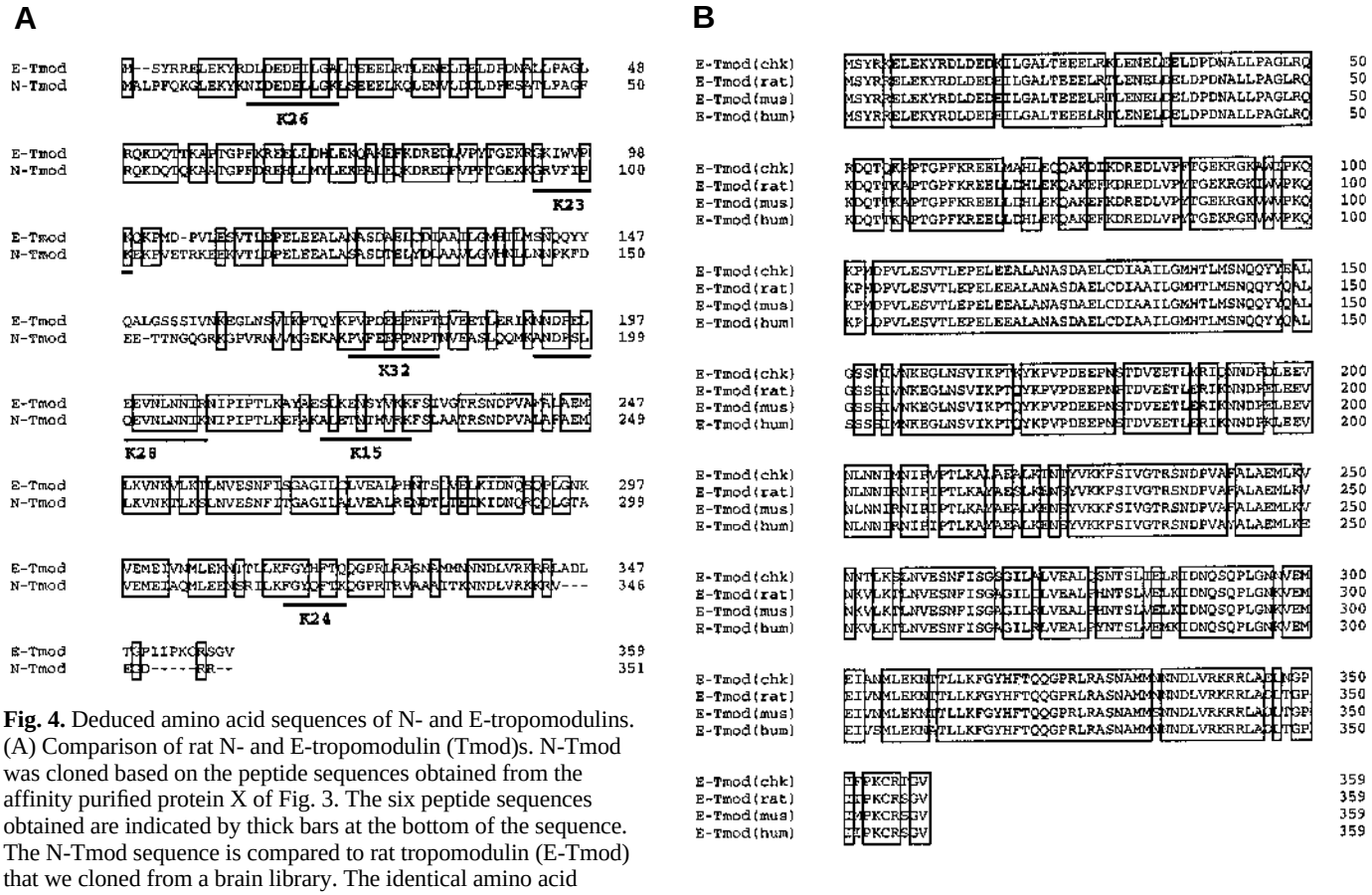

Fig. 4. Deduced amino acid sequences of N- and E-tropomodulins. (A) Comparison of rat N- and E-tropomodulin (Tmod)s. N-Tmod was cloned based on the peptide sequences obtained from the affinity purified protein X of Fig. 3. The six peptide sequences obtained are indicated by thick bars at the bottom of the sequence. The N-Tmod sequence is compared to rat tropomodulin (E-Tmod) that we cloned from a brain library. The identical amino acid sequences between the two isoforms are indicated by boxes. The nucleotide sequences of the rat N- and E-Tmod cDNA are available from GenBank under accession numbers U59240 and U59241, respectively. (B) Comparison of E-Tmods of various species. The amino acid sequence of rat E-Tmod is compared to E-Tmod of human (hum: Sung et al., 1992), chick (chk: Babcock and Fowler, 1994), and mouse (mus: Ito et al., 1995). The sequences identical in all the four sequences are indicated by boxes. The partial clone for rat E-Tmod was previously cloned from a cardiac library (Sussman et al., 1994). Its nucleotide sequence was essentially identical to our clone except for several discrepancies.

of the 6 peptide sequences obtained from affinity-purified 40 kDa protein (indicated by thick bars under the sequence). The calculated molecular mass is 39.5 kDa, and corresponds to the expected size for this protein. Comparison of the amino acid sequence of this protein with that of tropomodulin demonstrated that the clone we obtained encodes a novel isoform of tropomodulin, which is the product of a distinct gene. We designate this isoform N-tropomodulin (N-Tmod) since, as we later describe, this isoform is predominantly expressed in neurons. To distinguish this isoform from the original tropomodulin isoform, we refer to the latter as E-tropomodulin (E-Tmod), based on its first identification in erythrocytes (Fowler, 1987, 1990).

In a parallel screening, we also obtained a cDNA clone for the rat homologue of E-Tmod. The identity between the deduced amino acid sequence of the rat clone and the mouse, human, and chick counterpart was 98%, 96% and 88%, respectively, showing a high degree of sequence conservation between species (Fig. 4B). In contrast, the amino acid sequences of the two tropomodulin isoforms from rat showed only 59% identity overall, with regions of high homology or divergence. Most notably, the central region of 29 amino acids (aa 141-170) contains only 4 identical amino acids to E-Tmod. The presence of high and low conserved segments suggests that these regions may actually correspond to functional domains. Interestingly, Fowler and co-workers previously assigned the region between residues 6 and 94 in E-Tmod as the skeletal-TM binding domain and residues 90-184 as erythrocyte-TM binding domain (Babcock and Fowler, 1994). It was also shown that the antibody recognizing the C-terminal half (aa 190-359) can inhibit the pointed-end capping activity of tropomodulin (Gregorio et al., 1995).

Binding of recombinant N-tropomodulin to tropomyosin

To confirm the binding of N-Tmod to TMBr3, recombinant N-Tmod produced in *E. coli* was purified and used in overlay assays with HA-TMBr3 probe as the probe. As shown in Fig. 5A, the recombinant N-Tmod did bind to TMBr3 in this overlay assay (lanes 2 and 3). Moreover, the mobility of N-Tmod corresponded exactly to that of the lower 40 kDa protein in the brain extract (Fig. 5A, lanes 1-3). This binding is specific since the presence of five molar excess of untagged TMBr3 competes out the signal to approximately one fifth (Fig. 5A, lanes 4-6).

We also performed binding experiments under native conditions, because denaturation of the protein during the overlay assay may affect the binding properties of Tmods to TMs. Recombinant N- and E-Tmod containing Hisx6-tag were produced in *E. coli* and purified. Since previous reports have located the TM-binding domain in the N-terminal region in E-Tmod (Babcock and Fowler, 1994), the Hisx6 tag was fused to the C-terminal ends of N- and E-Tmod. These His-tagged Tmods were incubated with various TM isoforms at room temperature for 1 hour and the Tmods were recovered by Ni-NTA resin together with bound TMs. The bound (Fig. 5B, lanes B) and unbound (lanes U) fractions were separated on SDS-PAGE and visualized by Coomassie staining. Under the assay conditions used, all the His-tagged Tmods were recovered by the resin, while no non-specific protein binding was observed in the absence of His-tagged Tmod (data not shown).

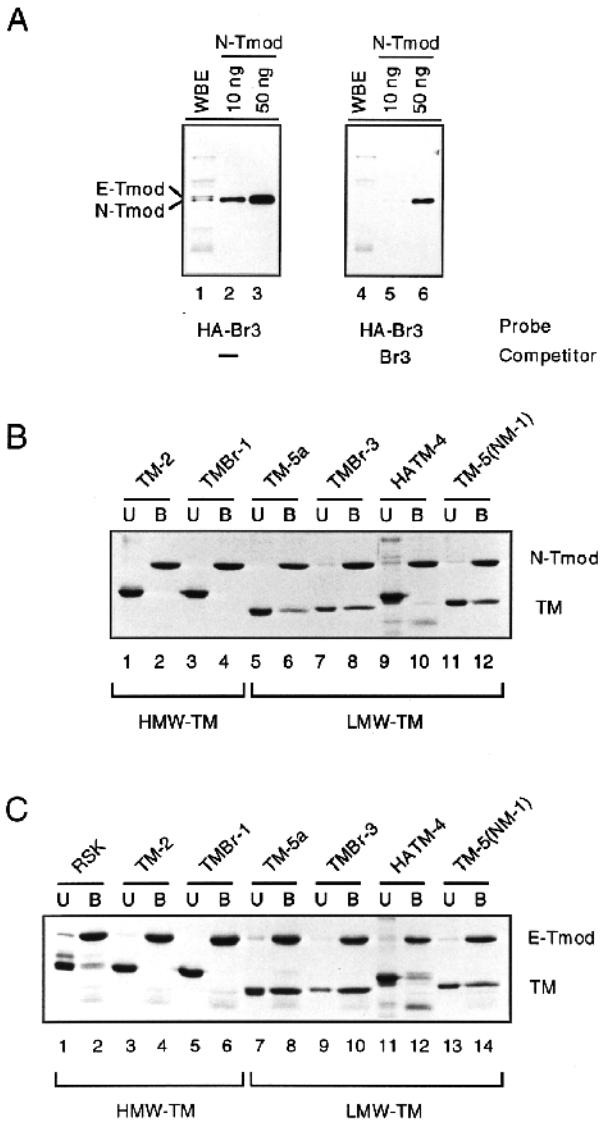

Fig. 5. Binding of recombinant Tmods to TMs. (A) Overlay assay of recombinant N-Tmod. Indicated amounts of the recombinant N-Tmod were separated on SDS-PAGE along with the whole brain extract (WBE) and subjected to an overlay assay using HA-TMBr3 as a probe (lanes 1-3). To demonstrate the specificity of binding, a parallel experiment was done in the presence of a fivefold excess of untagged TMBr3 competitor (right panel). (B) Binding of Hisx6 tagged N-Tmod to TMs. Recombinant N-Tmod carrying Hisx6 tag was incubated with various TMs and bound proteins were recovered using Ni-NTA resin. Note that the His-tag is fused to the C terminus to avoid interference with the potential TM-binding domains in the N-terminal half. The same amounts of unbound (U) and bound (B) fractions were separated by SDS-PAGE. HMW-TM, high molecular mass tropomyosin; LMW-TM, low molecular mass tropomyosin. (C) Binding of Hisx6 tagged E-Tmod to TMs. The same as in B except E-Tmod was used for the experiments. RSK: rabbit skeletal muscle TM. RSK consists of a heterodimer of TMα and TMβ skeletal muscle isoforms.

Consistent with the overlay assay, TM2 and TMBr1 did not bind to N-Tmod in this assay (Fig. 5B, lanes 1-4). In contrast, TM5a and TMBr3 both bound to N-Tmod (lanes 6 and 8). We also tested the binding of HA-tagged TM4 and TM5 (NM-1),

two low-molecular mass TMs produced from different genes (see Fig. 1A). While HA-TM4 did not bind to N-Tmod, TM5 (NM-1) bound to N-Tmod as effectively as TMBr3. Since HA-tagged TMBr3 binds to N-Tmod as well as does untagged TMBr3 (data not shown), the relative weak binding of HA-TM4 is not due to the presence of the HA-tag.

The same binding assay was performed with His-tagged E-Tmod for comparison (Fig. 5C). E-Tmod showed a similar binding pattern as N-Tmod: E-Tmod did not bind to TM2, or TMBr1, while it bound to TM5a, TMBr3 and TM5 (NM-1). We detected weak binding of E-Tmod to HA-TM4. We also found that E-Tmod binds to rabbit skeletal muscle TM (RSK) in our native binding assay. However, the ratio of bound to unbound TM with HA-TM4 and rabbit skeletal muscle TM was much lower than that of TMBr3 and TM5 (NM-1). When we compared the binding of a certain TM isoform to N- and E-Tmod, more TM generally bound to E-Tmod than to N-Tmod.

N-tropomodulin is predominantly expressed in brain

In order to determine the expression pattern of N-Tmod in various tissues, we carried out northern blot and RNase protection analyses. Fig. 6 shows the northern blot analysis of adult and embryo brain, heart, thymus and uterus RNA using probes to actin, N-Tmod and E-Tmod. As expected, N-Tmod is expressed in adult brain (lane Ba). We also observed N-Tmod expression in the brain of E19 embryo (lane Be). However, N-Tmod expression was not detected in other tissues tested (lanes H, T and U). In contrast, E-Tmod was expressed strongly in heart (lane H) in addition to adult brain (lane Ba). Interestingly, E-Tmod expression was not detected in embryonic brain (lane Be). These results are consistent with

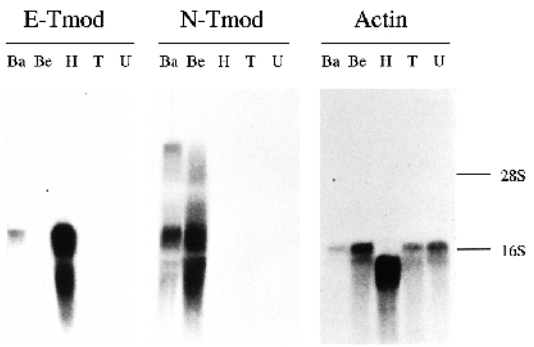

Fig. 6. Northern blotting of tropomodulin. Total RNA (15 μg) of various tissues was separated by 1.2% formaldehyde-agarose gel, transferred to nylon membranes and probed with the ^{32}P-labeled probes of E-Tmod, N-Tmod and actin. The positions of 28 S and 16 S ribosomal RNAs are indicated on the right of the panels. Ba, adult brain; Be, E19 embryonic brain; H, adult heart; T, adult thymus; U, adult uterus.

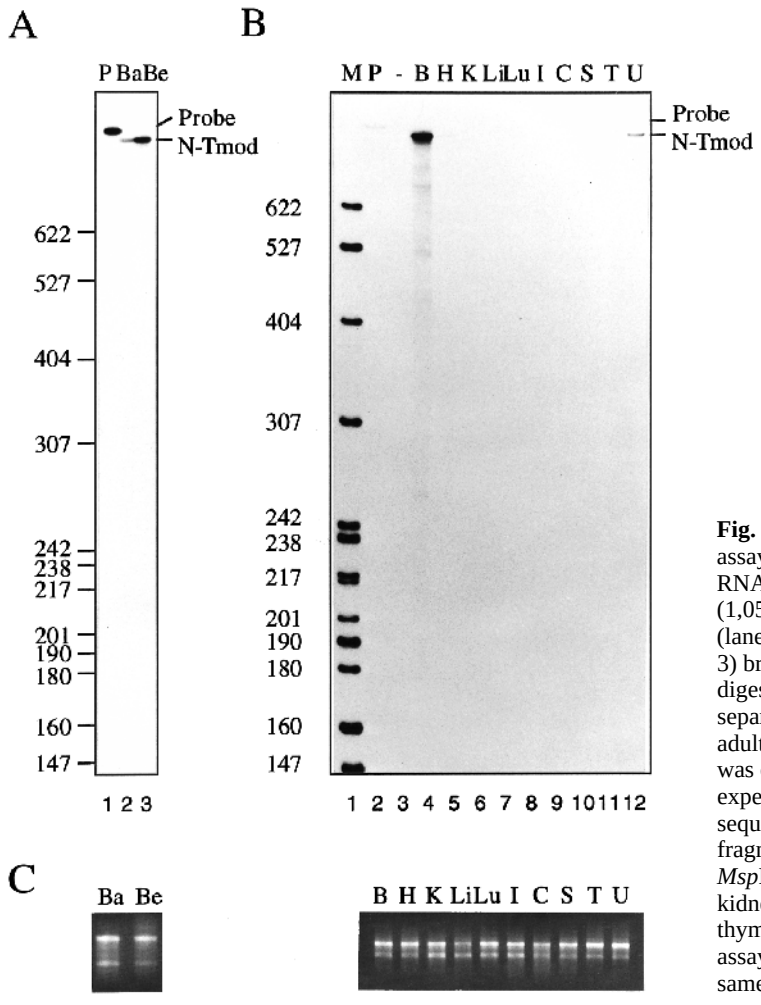

Fig. 7. RNase protection analysis of N-Tmod. (A) RNase protection assay was carried out using the antisense N-Tmod RNA. The probe RNA contained the full length of the N-Tmod coding sequence (1,056 nt) as well as the leader sequence derived from the vector (lane 1). Total RNA (10 μg) of adult (lane 2) and E19 embryo (lane 3) brains were hybridized with the antisense N-Tmod probe and digested with RNase A and T1. The protected fragments were separated by 4% PAGE containing 8 M urea. P, probe RNA; Ba, adult brain; Be, embryonic brain (E19). (B) RNase protection assay was carried out using total RNA (10 μg) of various tissues. In this experiment, shorter antisense RNA containing 952 nt of coding sequence was used. When 10 μg of yeast tRNA was used, no fragments were detected (lane 3). M, pBR322 DNA digested with *Msp*I; P, probe RNA as a position marker; B, brain; H, heart; K, kidney; Li, liver; Lu, lung; I, small intestine; C, colon; S, spleen; T, thymus; U, uterus. (C) Tissue RNA (0.5 μg) used for the protection assay was run on an agarose gel. Abbreviations for the tissues are the same as in B.

previous reports that E-Tmod is expressed in heart and adult brain (Fowler, 1990; Ito et al., 1995), but not in embryonic brain (Sussman et al., 1994). Thus, the two isoforms of Tmod exhibit a distinct pattern of expression.

We also carried out RNase protection analysis to determine if there is any alternatively-spliced region within the coding sequence. We synthesized an antisense RNA containing the full coding region of N-Tmod (Fig. 7A, lane 1). The probe RNA was hybridized to 10 μg of either adult brain RNA or 19-day embryonic brain RNA and the RNase-protected fragments were analyzed by electrophoresis. As shown in Fig. 7A, lanes 2 and 3, we detected the band corresponding to full protection of the coding region. We could not detect any other major bands, indicating that there is only one type of N-Tmod coding sequence. Using a sensitive RNase protection assay, we also further characterized the N-Tmod expression in various tissues. The total RNA from various tissues of adult rat was used for the RNase protection assay with a slightly shorter antisense RNA probe (Fig. 7B). This experiment demonstrated that N-Tmod is predominantly expressed in brain. However, we also observed low level expression in other tissues, such as uterus (lane 12).

Immunoblot analysis of N-Tmod expression

A GST-N-Tmod fusion protein was used as an antigen to immunize rabbits and the antisera were affinity-purified. To test the specificity of the antibody, recombinant N-Tmod was run on SDS-PAGE adjacent to the brain extract and immunoblotting was performed. As shown in Fig. 8A, the antibody detected the recombinant N-Tmod band (lanes 2-4), as well as a single band of the same size in the brain extract (lane 1). We did not observe cross-reactivity to E-Tmod in brain. This experiment showed that the antibody specifically recognizes N-Tmod.

Using this antibody, brain extracts of various developmental stages, as well as extracts from various tissues were tested for N-Tmod expression. As shown in Fig. 8B, N-Tmod was detected in the brain as early as embryonic day 14 (E14). N-Tmod expression reaches maximum levels before birth (E16, E19) and is maintained in the adult brain. The immunoblot of different tissues basically confirmed the result of the RNase protection assay showing that N-Tmod was expressed predominantly in brain. The longer exposure of the film, however, showed a low level expression in other tissues such as uterus, kidney, and lung (Fig. 8B, lanes 8, 11, 13).

We also performed immunoblotting using two kinds of antibodies against TM, one that recognizes TMBr1 and 3 (Stamm et al., 1993) and one that recognizes high molecular mass TMs (HMW-TM). As shown in Fig. 8B, TMBr3 was detected at E19 and increased to the adult level. TMBr1 or 3 was not detected in any other tissues. These results show that TMBr1 and 3 expression in brain begins much later than N-Tmod expression. In contrast to TMBr3, high molecular mass TMs were not abundant in brain compared to other tissues. It was also observed that different tissues contain different amounts of high molecular mass TMs. These two blots illustrate the diversity of TM expression.

N-Tmod is neuron-specific

To determine if N-Tmod expression is restricted to neurons or expressed in other cell types in brain, primary cell cultures of

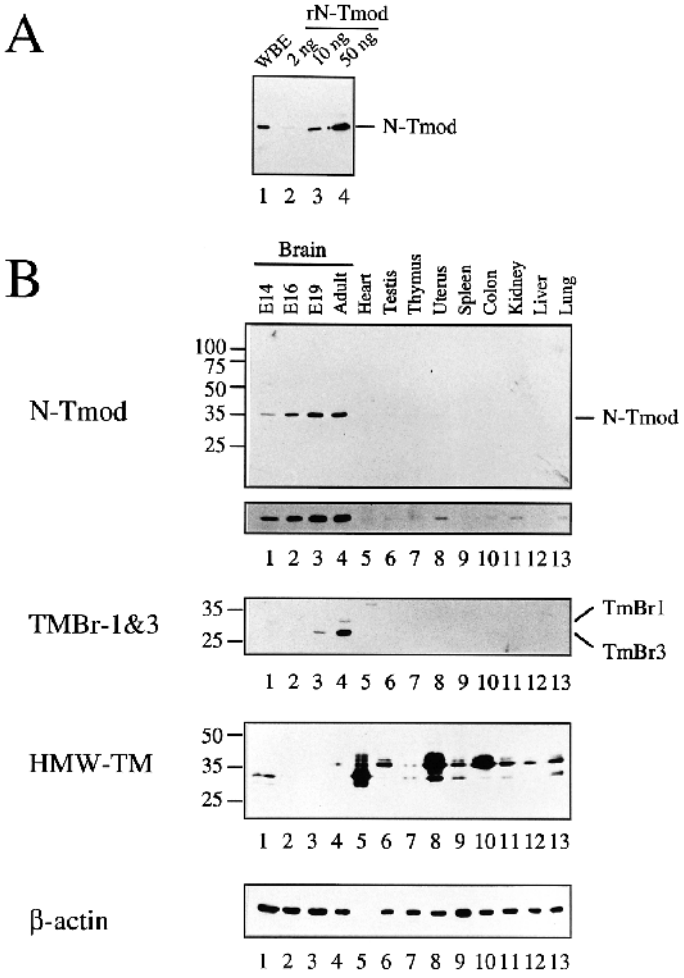

Fig. 8. Detection of tropomodulin protein in various tissues. (A) Immunoblotting of recombinant N-Tmod. Recombinant N-Tmod was separated on SDS-PAGE along with whole brain extract (WBE) and the immunoblotting was carried out using anti-N-Tmod antibody. (B) Immunoblotting of various tissue extracts. Whole cell extracts of various tissues (10 μg each) were separated by SDS-PAGE and the immunoblotting was carried out using either anti-N-Tmod antibody, anti-exon 9c antibody, anti-high molecular mass TM antibody (HMW-TM), and anti-β-actin antibody. The anti-exon 9c antibody recognizes both TMBr1 and TMBr3. Anti-high molecular mass TM antibody detects various TM isoforms including skeletal α and β TM, smooth muscle α TM, TM1, 2, 3 and TMBr1. Lane 1, E14 embryonic brain; lane 2, E16 embryonic brain; lane 3, E19 embryonic brain; lane 4, adult brain; lane 5, heart; lane 6, testis; lane 7, thymus; lane 8, uterus; lane 9, spleen; lane 10, colon; lane 11, kidney; lane 12, liver; lane 13, lung.

E16 rat frontal cortex were stained with affinity purified N-Tmod antibody. As shown in Fig. 9a and e, N-Tmod immunofluorescence was diffusely distributed throughout the neurons, including growth cones (white arrowhead) and neurites. In some neurons, N-Tmod staining seemed to be concentrated at discrete spots (Fig. 9e, white arrows) which co-localized with F-actin (Fig. 9d, white arrows). This staining pattern is specific, since preabsorption of the antibody to recombinant His-tagged N-Tmod (Fig. 9c), but not to E-Tmod (Fig. 9b), abolished staining. The primary cultures contain fibroblasts as well as glial cells in addition to neurons. As shown in Fig. 10a-c,

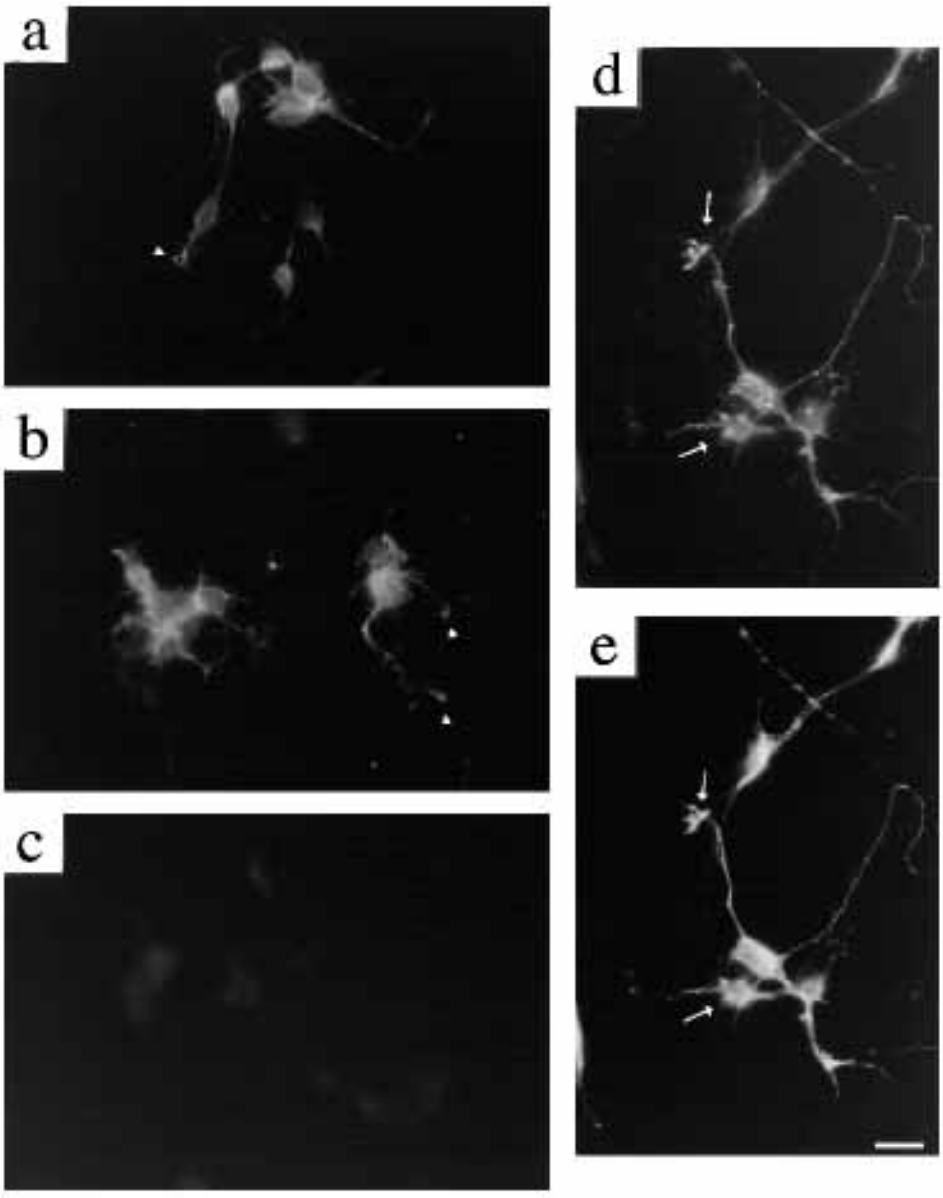

Fig. 9. Immunofluorescence of N-Tmod in neurons. Immunofluorescence analyses of N-Tmod (a-c, e) and F-actin (d). Neurons were prepared from E16 rat frontal cortex and cultured for 1 day (a-c) or for 5 days (d and e). In preabsorption experiments, N-Tmod antibody was incubated with recombinant His-tagged E-Tmod (b) or His-tagged N-Tmod (c) and the absorbed antibody was removed by using Ni-NTA resin. (a-c) These were exposed for the same length of time to compare the staining intensity. Note that N-Tmod is distributed to the tip of neurites (shown by white arrowhead). Neurons were double-stained with N-Tmod antibody and phalloidin in d and e. The concentrated staining of N-Tmod corresponds to the concentrated staining of F-actin (shown by white arrows). Bar, 30 μm.

double-staining with N-Tmod antibody (rhodamine) and phalloidin (FITC) showed that N-Tmod is not expressed in fibroblasts which are identified by intense staining of stress fibers. The antibody against GFAP (glial fibrillary acidic protein) recognizes glial cells (both type 1 and type 2 astroglia). Double-staining with N-Tmod (rhodamine) and GFAP antibody (FITC) revealed that N-Tmod is not expressed in glial cells, either (Fig. 10d-f). Collectively, these data show that N-Tmod is specifically expressed in neurons.

DISCUSSION

We report here the identification of two brain proteins that bind to a neuron-specific TM isoform, TMBr3. Protein purification and cDNA cloning clarified that these two proteins are two tropomodulin isoforms expressed from different genes. Tropomodulin was first identified in erythrocytes as a binding protein to erythrocyte TM (Fowler, 1987). Its cDNA was later cloned from various tissues of various species (Babcock and Fowler, 1994; Ito et al., 1995; Sung et al., 1992; Sussman et al., 1994) including our own cloning from adult rat brain. The sequence data indicate that these tropomodulin cDNAs belong to the same isoform type. The novel isoform of tropomodulin that we identified in brain clearly differs from this type. The overall identity of the two tropomodulins is only 59% at the amino acid level and their expression patterns are distinct. Because of its predominant expression in neurons, we have designated the novel isoform N-tropomodulin (N-Tmod). To distinguish, we propose to call the original isoform E-tropomodulin (E-Tmod) based on its first identification in erythrocytes.

One of the key questions that arise from isoform diversity of TMs and Tmods is their binding specificity. Our binding assay showed that both N- and E-Tmod have similar specificity for TM-binding. Both Tmods could bind low molecular mass-TMs, TM5a, TMBr3 and TM5 (NM-1), while we could not

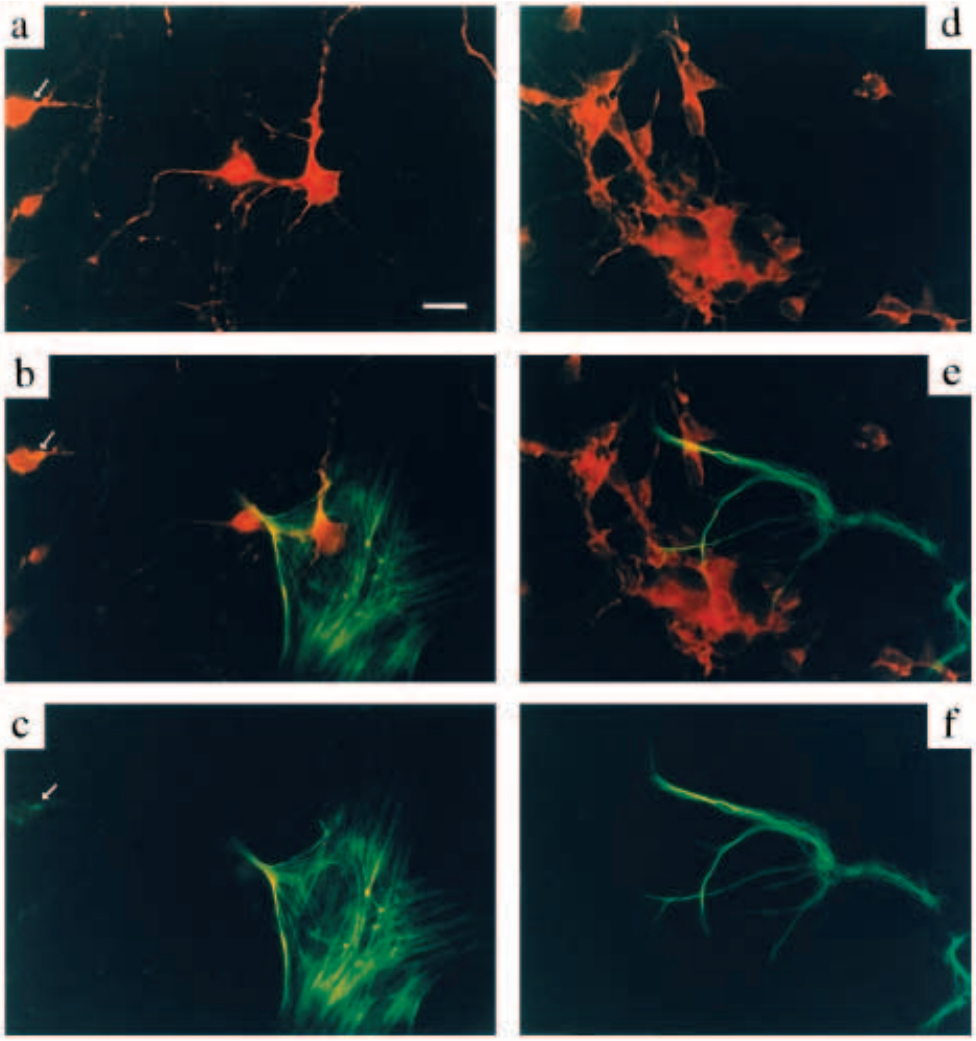

Fig. 10. Specificity of neuronal expression of N-Tmod. The neurons were prepared from E16 rat frontal cortex and cultured for 5 days. The rhodamine staining shows the distribution of N-Tmod (a,b,d,e). FITC-staining shows the F-actin distribution in b and c, and GFAP (glial fibrillary acidic protein) distribution in e and f; a-c and d-e, respectively, show the same fields with different filter sets. Note that neither fibroblasts (revealed by intense stress-fiber staining in b and c) nor glial cells (e and f) are stained by N-Tmod antibody (see a and d). F-actin staining in neurons in c (white arrow) correspond to the concentrated staining of N-Tmod in b. Bar, 30 μm.

detect binding of either of the high molecular mass-TMs, TM2 or TMBr1 (Fig. 5). The low molecular mass-TMs and high molecular mass-TMs can be classified by the usage of the first exon (see Fig. 1A) and the N-terminal amino acid sequences of each isoform is conserved within the category. Therefore it is most likely that both Tmod isoforms mainly recognize the structure of the N terminus which is conserved between low molecular mass-TMs of the α gene and TM5 (NM-1). Sun et al. (Sung and Lin, 1994) previously carried out an overlay assay using TM3, TM5 (NM-1) and a chimeric TM construct and concluded that E-Tmod binds to the N terminus of TM5 (NM-1). This result agrees well with our work.

It should be noted, however, that E-Tmod can also bind skeletal muscle TM at a low efficiency, which is a heterodimer of high molecular mass-TMs generated from α and β TM genes. Using various truncation mutants of E-Tmod, Babcock and Fowler (1994) showed that E-Tmod has overlapping but distinct binding sites for skeletal TM and erythrocyte TM. This structural feature may explain why E-Tmod can recognize two different structures of TM molecules. It remains to be determined if N-Tmod has a similar domain structure. It is also necessary to precisely determine the binding affinity and stoichiometry of Tmods to TM isoforms. Whereas previous work (Sussman and Fowler, 1992) and ours both suggest inefficient binding of skeletal TM to E-Tmod compared with low molecular mass TM, another study using a solid-phase binding assay suggests similar affinities of skeletal TM and erythrocyte TM for E-Tmod (Babcock and Fowler, 1994). This discrepancy may be due to their assay method, in which Tmod is partially denatured by immobilization on the nitrocellulose filter. Alternatively, the stoichiometry of E-Tmod versus skeletal TM and low molecular mass TM may be different and this may result in the apparently inefficient binding of skeletal TM to E-Tmod.

Although N- and E-Tmod show similar binding characteristic for TM isoforms, their expression patterns are strikingly different. N-Tmod is almost exclusively expressed in brain (neurons), while E-Tmod is expressed in a variety of tissues. In brain, N-Tmod is strongly expressed in the prenatal period, during which neurons of the cerebrum and cerebellum generate, migrate and extend axons to form a neuronal circuit, while E-Tmod expression in brain starts after most of these events are over (about 15 days after birth) (Sussman et al., 1994). Considering such developmental expression patterns, N-Tmod may be involved in the dynamic organizations of actin-filaments in developing and mature neurons, while E-Tmod may serve to stabilize long-term actin cytoskeleton assemblies. Further studies on differential expression, local-

ization and biochemical properties of these two Tmod isoforms will provide a greater understanding of their functions, as well as the function of TM isoform diversity in neurons.

We are grateful to Dr Mario Gimona for valuable comments on the manuscript. We thank Drs Mary K. Woo and Velia M. Fowler for providing us with tropomodulin antibody. We thank Lisa Bianco, Charlene DePoto and Nora Poppito for technical assistance and Phil Renna, Jim Duffy and Michael Ockler for photographic assistance. A.W. received a Human Frontier Science Program fellowship (LT-239/93). R.K. is supported by NIH grant CA45508. D.M.H. is supported by National Cancer Institute Grant CA58607 and is an Established Investigator of the American Heart Association.

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(Received 13 March 1996 - Accepted 24 June 1996)