Mouse Ten-m/Odz Is a New Family of Dimeric Type II Transmembrane Proteins Expressed in Many Tissues

Toshitaka Oohashi,‡§ Xiao-Hong Zhou,§ Kang Feng,§ Brigitta Richter,⁎ Matthias Mörgelin,§ Maria Theresia Perez,§ Wei-Dong Su,§ Ruth Cliquet-Ehrismann,† Uwe Rauch,† and Reinhard Fässler*§

*Max Planck Institute for Biochemistry, 82152 Martinsried, Germany; †Department of Molecular Biology and Biochemistry, Okayama University, Medical School, Okayama 700, Japan; ‡Department of Ophthalmology, Lund University, S-221 85 Lund, Sweden; §Department of Experimental Pathology and †Department of Experimental Pathology and ‡Department of Molecular Biology and Biochemistry, Lund University, S-221 85 Lund, Sweden. Tel.: 46-46-158202. E-mail: reinhard.fassler@pat.lu.se

Abstract. The Drosophila gene ten-m/odz is the only pair rule gene identified to date which is not a transcription factor. In an attempt to analyze the structure and the function of ten-m/odz in mouse, we isolated four murine ten-m cDNAs which code for proteins of 2,700–2,800 amino acids. All four proteins (Ten-m1–4) lack signal peptides at the NH2 terminus, but contain a short hydrophobic domain characteristic of transmembrane proteins, 300–400 amino acids after the NH2 terminus. About 200 amino acids COOH-terminal to this hydrophobic region are eight consecutive EGF-like domains.

The establishment of periodic patterns during the development of the Drosophila embryo is controlled by genes that act in a hierarchical manner (Nüsslein-Volhard and Wieschaus, 1980; Ingham 1988; St. Johnston and Nüsslein-Volhard, 1992). Maternal activities induce the expression of transcription factors, encoded by gap genes, which regulate the expression of other transcription factors encoded by pair rule genes. Pair rule genes are expressed in seven stripes along the anterior–posterior axis of Drosophila melanogaster. Their expression is crucial for the consecutive expression of segment polarity genes and the establishment of the segmental pattern of Drosophila embryos. Mutations in pair rule genes result in deletions of cuticle segments which appear in a reiterative manner along the body axis of the hatched larva.

All known pair rule genes code for transcription factors, except for a gene identified independently in two laboratories and designated ten-m (Baumgartner et al., 1994) and odz (Levine et al., 1994). ten-m and odz are identical genes and mutations lead to a pair rule phenotype (Baumgartner et al., 1994; Levine et al., 1994) similar to odd-paired in which every other segment is missing (Nüsslein-Volhard et al., 1995). Despite the fact that both reports showed identical sequences, Ten-m was described as a secreted Drosophila tenascin-like molecule (Baumgartner et al., 1994) and Odz as a type I transmembrane receptor (Levine et al., 1994). Tenascins are a family of extracellular matrix proteins with a modular structure composed of fibronectin type III (FNIII) repeats, EGF-like repeats, and a COOH-terminal fibrinogen-like repeat (Erickson, 1993). Biochemical studies using a Drosophila cell line indicated that

© The Rockefeller University Press, 0021-9525/99/05/563/15 $2.00
The Journal of Cell Biology, Volume 145, Number 3, May 3, 1999 563–577
http://www.jcb.org
Ten-m is a large secreted proteoglycan with chondroitinase ABC-sensitive chondroitin sulfate and/or dermatan sulfate side chains. The core protein was reported to contain EGF-like and FNIII repeats, but to lack the fibrinogen-like domain (Baumgartner et al., 1994). Odz was isolated as a novel phosphorysine-containing protein (Levine et al., 1994). A transmembrane region was predicted COOH-terminal of the EGF repeats, followed by the cytoplasmic domain containing several tyrosine kinase phosphorylation consensus sites (Levine et al., 1994). More recently, Wang et al. (1998) described a mammalian orthologue of Ten-m/Odz, termed DOC4 (downstream of chop), which is induced by the stress-induced transcription factor CHOP. The open reading frame of DOC4 shares 31% sequence identity and 50% sequence similarity with Ten-m/Odz. Furthermore, DOC4 contains a short stretch of hydrophobic amino acids ~400 amino acids COOH-terminal of the putative start codon. This together with the cell surface localization led to the suggestion that DOC4 may constitute a type II transmembrane molecule (Wang et al., 1998).

Ten-m/Odz, as well as DOC4, contains a stretch of eight consecutive EGF-like modules which are most similar to the EGF repeats of tenascins. EGF modules are structural units of proteins or parts of protein, located extracellularly. They can occur as isolated modules such as in reelin (D’Arcangelo et al., 1995) and in selectins (Whelan, 1996), or in arrays like in notch (Fleming et al., 1997) and tenascins (Spring et al., 1989). A conserved feature of the EGF domain in Ten-m/Odz, DOC4, and Ten-a, a Drosophila molecule related to Ten-m/Odz (Baumgartner and Chiquet-Ehrismann, 1993), is the substitution of a cysteine residue with an aromatic amino acid in two of the eight EGF-like modules. This leaves two cysteines with no intramodular partner. The importance of the integrity of the cysteine patterns in EGF-like modules is exemplified by the functional impairment of notch 3, which has been observed in patients with an autosomal dominant disorder causing stroke (Joutel et al., 1997). The molecular basis of this disease is predominantly the substitution of cysteines with other amino acids in the EGF modules of notch 3. The observation that the EGF-like modules of Ten-m/Odz with five cysteines are ontogenetically conserved indicates that they are able to fold into a structure which might be important for the function of the protein.

Many genes that control pattern formation are expressed at several different periods during development to function in a variety of processes both during embryogenesis and postnatal life. After the initial expression in seven stripes at the cellular blastoderm stage, ten-m/odz is down-regulated and appears at later stages in the central nervous system (CNS), dorsal vessel, trachea, and the eye and discs giving rise to the cephalic (antenna), ventral (wing), and dorsal (legs) thoracic appendages (Baumgartner et al., 1994; Levine et al., 1994). The highest level of Ten-m/Odz expression is observed in the CNS where the protein is deposited on the surface of axons (Levine et al., 1994; Levine et al., 1997). The Drosophila eye disk is another location where high levels of Ten-m/Odz are found in very distinct sites including the morphogenetic furrow, photoreceptor-like cells, and nonepithelial cells of the eye disc (Levine et al., 1997). The expression pattern of DOC4 in mammals is not well characterized but the presence of the mRNA has been demonstrated in the developing mouse brain (Wang et al., 1998).

Several mutations in the ten-m/odz gene have been identified, all resulting in embryonic lethality (Baumgartner et al., 1994; Levine et al., 1994). Due to the lack of viable hypomorphic mutations, it is not clear whether the protein executes an important function in all sites where it is expressed. One possible function for Ten-m/Odz comes from studies with DOC4 which has been isolated in search of GADD153/CHOP (growth arrest and DNA damage/C/EBP homology protein)-induced mRNA. GADD153/CHOP is responsive to many forms of stress, including alkylating agents, UV light, and conditions that trigger an ER stress response. For example, ER stress which occurs during ischemia alters proliferation of cells, induces cell death, and the expression of GADD153/CHOP (Zinszner et al., 1998). Recent studies have shown that GADD153/CHOP exerts at least part of its function via the induction of DOC4 and other proteins (Wang et al., 1998).

As an initial step to obtain insight into the function of Ten-m/Odz-related proteins in mammals, we have cloned several cDNAs for mouse orthologues of Ten-m/Odz. We report here that at least four different cDNAs with similarity to the Drosophila ten-m/odz cDNA are expressed in mice. One of them, ten-m4, is identical to the DOC4 cDNA. The alignment of the four deduced mouse protein sequences indicated a strong conservation of the characteristic features for type II transmembrane molecules, which was also recognized for DOC4 (Wang et al., 1998). This predicted topological orientation was experimentally confirmed by expression studies of recombinant mouse Ten-m1 fusion proteins in mammalian cells. In addition, the recombinant production of the putative extracellular domain of Ten-m1 revealed the formation of dimeric structures. We provide experimental evidence which shows that the dimerization of Ten-m1 is mediated via the single cysteine residues in the EGF modules that lack their intramodular partners. Furthermore, we show that Ten-m1 is able to make homophilic interactions.

Materials and Methods

Isolation of cDNA Encoding Mouse Ten-m1, Ten-m2, Ten-m3, and Ten-m4

A random and oligo-dt–primed mouse adult brain cDNA library (ML1042a; Clontech) was screened with a 32P-labeled chicken ten-m cDNA (cten22, 1.8 kb; Chiquet-Ehrismann, R., manuscript in preparation) which encodes EGF-like repeats and part of the COOH-terminal cysteine-rich sequence. Filters were hybridized and washed as previously described (Ruch et al., 1997). The chicken tten-m4 cDNA insert of clone DT1 was subcloned into pBluescriptSK (Stratagene) and both DNA strands were sequenced using the ABI Prism Dye Terminator kit (Applied Biosystems). The sequences were analyzed using a 373A automatic sequencer (Applied Biosystems). Several mutations in the ten-m/odz gene have been identified (Rauch et al., 1997). The cDNA insert of clone DT1 was subcloned and both DNA strands were sequenced. The ten-m2, ten-m3, and ten-m4 cDNAs were isolated by screening the brain cDNA library with the PstI/EcoRI fragment of DT19 (731–1,833 bp) at low stringency as previously described (Oohashi et al., 1994). Full-
length cDNA fragments were isolated and characterized from the same mouse brain cDNA library. Sequence alignments were performed using the BLAST program (Altschul et al., 1994) and PileUp from the GCG (Genetics Computer Group) package.

**Northern Blot Analysis**
Poly(A)+ RNA was isolated from brain, heart, liver, kidney, skeletal muscle, testes, and spleen of 7-wk-old and thymus of 3-wk-old 129/Sv mice. 20 ml of proteinase K buffer (20 mM Tris-HCl, pH 7.4, 0.5% SDS, 0.1 M NaCl, 1 mM EDTA, 200 mg/ml proteinase K) was added to cell pellets and DNA was sheared by treating cells with a polystyron for 1 min. 1 ml of oligo-dt cellulose (Pharmacia) suspended (vol/vol) and equilibrated for 30 min in high salt buffer (10 mM Tris-HCl, pH 7.4, 0.4 M NaCl, 1 mM EDTA, 0.1% SDS) and 1.25 ml 5 M NaCl (0.4 M final concentration) were added to the cell lysate and incubated on a rocking platform for 1 h at room temperature. Polyadenylated RNA bound to the oligo-dt cellulose was washed three times in high salt buffer and subsequently put on an E. coli column (Pharmacia). A fraction of three additional washings of the oligo-dt with high salt buffer, polyadenylated RNA was eluted with 1 ml of RNase-free water.

**Expression of Recombinant Ten-m1 and Ten-m1 Fusion Proteins**
To obtain secretion of the recombinant proteins, all cDNA fragments were linked to the BM-40 signal peptide via an NheI site in the BM-40 sequence (Mayer et al., 1993). To express the entire extracellular domain of Ten-m1, the tetrapeptide APLA derived from the BM-40 signal peptide was linked to the N-terminal AP domain with the BM-40 signal peptide was cleaved at the HpaI site (which destroyed the stop codon) and ligated via a linker segment to the mature AP domain, which terminated before the hydrophobic glycation signal through a stop codon encoded by an Hpal site (Flanagan and Leder, 1990). To express both fusion proteins the N-hemterminal A P domain with the BM-40 signal peptide was cleaved at the Hpal site (which destroyed the stop codon) and ligated via a linker segment to the ten-m1 cDNA fragment encoding the EGF domains or extracellular domain, respectively. In these fusion proteins the A P domain was linked to the Ten-m1 fragment via an SSSG sequence to the G39 of the Ten-m1 sequence. The fusion proteins were terminated by the introduction of stop codons after E624 for the A P-3EG recombinant protein and after I796 for the A P-8EG recombinant protein, respectively. To express the intracellular and transmembrane domain fused to the A P the first 370 amino acids of Ten-m1 were connected via the linker sequence K L A Y V R S S G to I23 of the AP sequence.

Human embryonic kidney cells (HEK 293 cells; American Type Culture Collection) were transfected with the constructs in an eukaryotic expression vector containing a CMV promoter (pRC/CMV; Invitrogen) and a puromycin resistance gene. Puromycin-resistant clones were isolated by SDS-PAGE and Coomassie blue staining according to standard protocols and maintained as described (Retzler et al., 1996). The AP expression was determined as described by Flanagan and Leder (1990). For determining whether the intracellular and transmembrane domain localized the AP module on the outer cell surface, cells were treated with trypsin for 30 min at 37°C and then allowed to adhere on gelatinized glass coverslips for 30 min, washed, fixed, and stained for AP activity.

**Protein and Antibody Purification**
Conditioned medium containing the entire extracellular domain of Ten-m1 (Ten-m1sec) was dialyzed against TBS and applied to 5% of the volume of DEAE-Sephalen equilibrated with the same buffer. The proteins were eluted by a gradient from 0 to 500 mM NaCl in TBS. Ten-m1sec-containing fractions were identified by SDS-PAGE and Coomassie blue staining and pooled, and concentrated by centrifugation in 2- or 3 ml concentrators with membranes with a cutoff of 10 kDa (Amicon or Pall-Filter). The concentrated protein was applied to a Superose 6 column equilibrated with 50 mM Tris-HCl, pH 7.6, 500 mM NaCl, 2 mM EDTA, 0.5 mM NEM, and 0.5 mM PM SF. Ten-m1sec-containing fractions were dialyzed against 50 mM NaHCO3, 150 mM NaCl and stored at −80°C. 2 mg purified Ten-m1sec was coupled to 2 ml CNBr-activated Sepharose (Pharmacia) according to the manufacturer’s protocol. The remaining activated groups were blocked with 1 M ethanolamine, pH 8. The affinity matrix was incubated with 10 ml of anti-Ten-m1sec antisum and washed with PBS (50 mM Na-phosphate, pH 7.4, 150 mM NaCl), with PBS containing 500 mM NaCl, and was prepared for elution with PBS containing 10 mM Na-phosphate, pH 8, 150 mM NaCl. Antibodies were eluted with 50 mM diethylamine, pH 11.5, and immediately neutralized with 1/10 volume of 1 M Na-phosphate, pH 6.8. The eluted and neutralized antibodies were supplemented with 1/10 volume of 5% BSA and dialyzed against PBS. The purified antibodies represented ~1% of the immunoglobulin fraction of the unpurified antisum and, according to enzyme-linked immunosorbent assays, exhibited ~10% of their antigen-binding capacity.

**Western Blot Analysis of Tissue Extracts and Recombinant Proteins**
Brain of 8-wk-old mice was homogenized in 5 vol of 20 mM Tris-HCl, pH 8, containing 11% sucrose, protease inhibitors (5 mM EDTA, 5 mM N-ethylmaleimide, 5 mM benzamidine), and freshly added 1 mM PM SF on ice using a Dounce homogenizer. A filter centrifugation at 16,000 g the insoluble material was washed once with the same buffer, then washed with a buffer containing 150 mM NaCl instead of sucrose, and extracted with 20 mM Tris-HCl, pH 8, 150 mM NaCl, protease inhibitors, and 3% Triton X-100. Suroce extracts were supplemented with 3× SDS-PAGE buffer, whereas Triton X-100 extracts were precipitated with aceton and the pellet was dissolved in 1× SDS-PAGE buffer (2% SDS, 62.5 mM Tris-HCl, pH 6.8, 10% glycerol). A critical variable for the proper solubilization and migration of Ten-m1 was the reducing agent in the sample buffer. In initial experiments SDS-PAGE buffer with 5% mercaptoethanol was used. Under these conditions recombinant Ten-m1sec barely entered the stacking gel. In further studies with tissue extracts and recombinant Ten-m1sec, mercaptoethanol was replaced by 10 mM dithioerythrol which improved the migration behavior of Ten-m1sec and discrete bands could be observed. However, under these conditions the recombinant Ten-m1sec molecules were not always separated into monomeric structures. Western blots of tissue extracts were performed by transfer of proteins separated by SDS-PAGE to PVDF membranes (Hybond-P; Amersham) in Tris-glycine buffer containing 10% methanol for 1 h with 100 V with the Bio-Rad mini gel system. The membranes were blocked with 5% nonfat dry milk in TBST (0.1% Triton X-100, 150 mM NaCl, 0.1% Tween 20), incubated with affinity-purified polyclonal antibody against Ten-m1sec in TBST, and developed with horseradish peroxidase-conjugated secondary antibody in TBST containing 5% nonfat dry milk and the ECL+ detection system (Amerham) according to the manufacturer’s protocol.

Western blots for detecting recombinant proteins were performed by transferring proteins separated by SDS-PAGE to supported nitrocellulose (Bio-Rad) in Tris-glycine buffer containing 10% methanol for 1 h with 100 V with the Bio-Rad mini gel system. The blots were blocked with 1% BSA, incubated with an anti-calf intestine AP antisum (Sigma Chemical Co.), and developed with AP-conjugated secondary antibodies.

**Triton X-114 Phase Partition**
HEK 293 cells transfected with Ten-m1sec were washed with PBS, lysed at 4°C in 2 ml TBS (20 mM Tris-HCl, pH 7.4, 150 mM NaCl) supplemented with 0.7% Triton X-114, and centrifuged at 4°C at 5,000 rpm (2,000 RCF). 550 μl supernatant was layered on top of 800 μl 6% sucrose in TBS, incubated for 3 min at 30°C, and centrifuged in a swing out rotor with 30°C warm buckets for 3 min at 3,000 rpm (1,000 RCF). The Triton X-114-rich bottom fraction (~30 μl) was precipitated with acetone and dissolved in 100 μl SD-S-PAGE sample buffer. From the Triton X-114-depleted upper phase 500 μl was recovered, cooled to 4°C, mixed with 30 μl 14% Triton X-114 in TBS, and layered on top of another 800 μl 6% sucrose in TBS, incubated 3 min at 30°C, and centrifuged. 400 μl of this second upper phase was precipitated with 110 μl 55% TCA solution. The precipitate was washed with acetone and dissolved in 100 μl SD-S-PAGE sample buffer.
Far Western Blotting

Far Western blotting was performed as described (Denda et al., 1998) with minor modifications. Recombinant Ten-m-1sec, neurocan, and BSA were separated by electrophoresis on SD-PAGE gels and transferred to nitrocellulose membranes. Membranes were blocked with 1% BSA in 10 mM Tris-HCl (pH 8) buffer (containing 150 mM NaCl, 0.05% Tween 20, 2 mM CaCl$_2$, and 2 mM MgCl$_2$; TBST-CM buffer) at 4°C overnight, washed five times with TBST-CM buffer and then incubated with purified 30 nM A-P-tet-m1 or A-P, respectively, in the TBST-CM buffer containing 1% BSA for 3 h at room temperature, respectively. A further membranes were washed with TBST-CM buffer five times and stained for 15 min in 0.1 M Tris-HCl, pH 9.5, buffer containing 0.1 M NaCl, 0.05 M MgCl$_2$, 1.75 μg/ml BCIP, and 4.5 μg/ml NBT (Boehringer Mannheim) for A-P activity.

Immunofluorescence analyses were carried out using affinity-purified rabbit antibodies against Ten-m-1. All tissue specimens (testes, lung, brain, eyes, and kidneys) were derived from 6-wk-old mice, frozen on dry ice, and sectioned (6 μm) on a Microm Zeiss cryostat. Sections were fixed in 4% paraformaldehyde for 5 min at room temperature and washed in PBS. A affinity-purified antibodies against Ten-m-1 were diluted 1:25 in PBS containing 2.5% ovalbumin. Normal rabbit serum (1:150) served as the negative control. Fluorescence labeling was performed with Cy3-conjugated goat anti-rabbit immunoglobulins (Sigma Chemical Co.). Fluorescent specimens were mounted in 90% glycerol containing 1 mg/ml 4,6-diamino-2-phenylindole. Microscopy was carried out with an Axiophot fluorescence microscope (Zeiss).

Staining of Tissue Sections with the A-P-tet-m1 Fusion Protein

Frozen 8-μm-thick tissue sections were prefixed in 4% paraformaldehyde for 5 min. Staining with A-P-tet-m1 or A-P was performed as described (Cheng and Flanagan, 1994) with some minor modifications. In brief, sections were blocked with 1% BSA in HBSS for 1 h at room temperature and then incubated with either 30, 60, 120, or 240 nM A-P-tet-m1 or A-P, respectively, in HBSS buffer including 1% BSA at room temperature overnight. Sections were washed five times with HBSS buffer, fixed with 30% acetone, 3% formaldehyde, 20 mM Hepes (pH 7.5) for 30 s, washed with 150 mM NaCl, 20 mM Hepes (pH 7.5; HB3) three times, heated at 65°C to inactivate endogenous A-P for 15 min in HB3 buffer; rinsed with 100 mM Tris-HCl (pH 9.5), 100 mM NaCl, 5 mM MgCl$_2$, and stained at room temperature for 5 h in the same buffer containing 10 mM l-homoarginine, 0.17 mg/ml BCIP, and 0.33 mg/ml NBT (Boehringer Mannheim). A further sections were dehydrated, cover-slipped, and photographed.

Electron Microscopy

Samples were stored in TBS (150 mM NaCl, 50 mM Tris-HCl, pH 7.4) at concentrations of ~1 mg/ml and diluted to 0.2 M ammonium hydrogen carbonate, pH 7.9, shortly before use (final concentration 5–10 μg/ml). The diluted Ten-m-1sec solution was subsequently mixed with an equal volume of 80% glycerol immediately before spraying on to freshly cleaved mica pieces. They were dried in a high vacuum and rotated slowly with 2 nm platinum/carbon at a 9° angle by means of electron bombardment heating, followed by coating with 10 nm carbon from above (Fowler and Erickson, 1979; Shotton et al., 1979; Tyler and Branton, 1980). For negative staining 3 μl of purified Ten-m-1sec in TBS (typical concentration 3 μg/ml) was adsorbed onto 400 mesh carbon-coated grids for 1 min, washed with two drops of water, and stained with two drops of 0.75% uranyl formate. The grids were rendered hydrophilic by glow discharge at low pressure in air. Specimens were observed in a Jeol 1200 EX transmission electron microscope operated at 60 kV accelerating voltage. Images were recorded on Kodak SO-163 plates without preirradiation at a dose of typically 2,000 electrons/nm$^2$. Evaluation of the data from electron micrographs was done as described previously (Engel and Furthmayer, 1987).

Online Supplemental Material

Figure 1: Proposed cytosolic, transmembrane (framed), and linker domain of Ten-m 1–4. Conserved amino acid residues are in shadowed boxes. The dibasic motif conserved in all four mouse Ten-m proteins, in Drosophila Ten-m and Drosophila Ten-a is indicated by asterisks in a box below the sequence. Other amino acids shown in this box are conserved in these six molecules.

Figure 2: COOH-terminal domain of mouse Ten-m 1–4. Conserved amino acid residues are in shadowed boxes.

Results

Mouse Ten-m Is a Member of a New Protein Family

To obtain the primary structure of ten-m/odz in mouse, a cDNA library derived from an adult mouse brain mRNA was screened using an radiolabeled chicken ten-m cDNA fragment (cten22, 1.8 kb; Chiquet-Ehrismann, R., unpublished observations) as a probe. The initial screening led to the isolation of one cDNA clone (DT1) which was purified and sequenced. The deduced amino acid sequence of DT1 showed a 35% homology with Drosophila Ten-m/Odz and contained three EGF-like repeats and part of the cysteine-rich domain also present in Drosophila Ten-m/Odz. Therefore, we designated this cDNA mouse homologue of Drosophila ten-m/odz and named the gene ten-m1. The full-length cDNA was obtained by isolating 10 overlapping cDNA clones. The deduced Ten-m1 polypeptide contained 2,731 amino acid residues.

When the ten-m1 cDNA fragment DT19 (731–1833 bp) was hybridized to the brain cDNA library at low stringent condition, several clones showing a weak hybridization signal were isolated and sequenced. The deduced amino acid sequence of three clones contained the eight EGF-like repeats and part of the cysteine-rich domain of ten-m molecules. Despite the high homology of 63, 51, and 52% to Ten-m1, respectively, the three cDNA clones were clearly different from ten-m1 and from each other. To obtain full-length cDNAs, overlapping cDNA clones were isolated from the same library. The cDNA sequences were designated ten-m2, ten-m3, and ten-m4, which had open reading frames of 2,764, 2,715, and 2,771 amino acid residues, respectively, and the translated amino acid sequences showed an overall similarity between 56 and 70%.

None of the four ten-m sequences contained a signal peptide. Approximately 300–400 residues after the start codon, all four sequences showed a continuous stretch of 34 amino acids lacking charged residues, which could serve as transmembrane domain (Supplemental Figure 1). About 200 amino acids COOH-terminal of the hydrophobic amino acids were eight consecutive EGF modules (Fig. 1 A) followed by a large COOH-terminal sequence which was rich in cysteines and devoid of any known modular motifs (Supplemental Figure 2). The absence of a signal peptide and the presence of a stretch of hydrophobic amino acids suggested that the family of ten-m proteins may be expressed as type II transmembrane molecules. According to this model, the NH2-terminal domain of 200 amino acids, a region with eight EGF-like do-
mains of ~250 amino acids, and a COOH-terminal domain of ~2,000 amino acids (Fig. 1B). The linker domain of all mouse ten-m proteins contained several dibasic amino acid residues which could serve as potential sites for proteolytic processing of the molecules. One of these sites is conserved in all four mouse ten-m sequences and the Drosophila Ten-m/Odz and Drosophila Ten-a (Supplementary Figure 1). The Drosophila Ten-m/Odz shows higher identity to the mouse ten-m sequences in the linker domain (26–29%) than in the cytosolic domain (19–21%). Between the mouse ten-m sequences, the similarity of the cysteine-free linker domain ranged from 43 to 48%, the EGF domains ranged from 65 to 72%, and the large cysteine-rich COOH-terminal domain ranged from 58 to 68%. The similarity to Drosophila Ten-m/Odz over the entire COOH-terminal domain ranged from 30 to 33%.

When the ten-m protein sequences were compared with the cDNA and protein data bank, DOC4 (Wang et al., 1998) was identical to Ten-m4. In addition, the NH2-terminal part of the human g heregulin, identified in the breast cancer cell line MDA-MB-175 (Schäfer et al., 1997), had 95% identity to the NH2-terminal fragment of mouse Ten-m4/DOC4. The COOH terminus of the ten-m proteins contained several amino acid repeats which showed similarities with the rearrangement hot spot elements (rhs) of Escherichia coli (Feulner et al., 1990) and with a wall-associated protein (WAP) of Bacillus subtilis (Foster, 1993).

---

**Figure 1.** Protein sequence of EGF-like modules and structure of mouse Ten-m 1–4. (A) EGF-like domains of mouse Ten-m 1–4, Drosophila Ten-m, and Drosophila Ten-a. Conserved cysteines are indicated by asterisks. Substitutions of cysteines by aromatic amino acids are indicated by the ø symbols. Arrows indicate the size of single EGF modules. (B) Overview of the proposed structural organization of the four mouse ten-m proteins. Tyrosine residues in the cytosolic part and potential N-glycosylation sites and cysteines in the extracellular part are indicated, except the 46 cysteine residues within EGF motifs which are marked in A.
Ten-m1 Is a Type II Transmembrane Molecule

To obtain evidence for the proposed model in which ten-m1 proteins are expressed as type II transmembrane molecules, a fusion cDNA was constructed in which the entire putative extracellular part of Ten-m1 was replaced by an AP module (Fig. 2 A). The AP module was derived from the GPI-linked placental AP by introducing a stop codon in front of the hydrophobic COOH-terminal GPI sequence (Flanagan and Leder, 1990). The fusion cDNA was cloned into an expression vector containing a puromycin resistance gene and transfected into HEK 293 cells. 24 puromycin-resistant cell clones were isolated, grown on glass coverslips, fixed, and stained for AP activity. Whereas untransfected HEK 293 cells showed no AP expression, all transfected clones tested stained strongly for AP at the plasma membrane (Fig. 2, B and D). Recombinant cells treated with trypsin for 30 min and then cultured for 30 min to allow adhesion showed significantly reduced AP activity (Fig. 2 C). Due to the extensive trypsin treatment and short incubation time for adhesion, only a few cells were attached on the gelatinized glass coverslips which showed no or very poor cell spreading (Fig. 2 C).

To obtain biochemical evidence for the membrane localization of the fusion protein, the expressing cells were analyzed by Triton X-114 phase partition experiments. Aqueous solutions of 0.7% Triton X-114 separate into two phases when warmed from 4 to 30°C. The dense Triton X-114 containing detergent-rich phase has been shown to be enriched in integral membrane and membrane-anchored proteins, whereas the Triton X-114-depleted phase is enriched in soluble proteins (Justice et al., 1995). In such experiments the fusion protein of 116 kD, which is the expected size for a protein consisting of the NH2-terminal 370 amino acids of Ten-m1 and the 67-kD AP module, was distributed preferentially to the detergent-rich phase (Fig. 2 E, lane 8). The majority of the proteins released from the cell layer at 4°C with 0.7% Triton X-114 remained at 30°C in the detergent-depleted phase (Fig. 2 E, lanes 1 and 2). These data show that the NH2-terminal and the hydrophobic sequence of Ten-m1 can direct the AP to the plasma membrane. This, together with the finding that the AP activity can be found on the cell surface, suggests that Ten-m1 is a type II transmembrane protein.

![Figure 2](image_url)

Figure 2. Expression of the NH2-terminal sequence of Ten-m1 linked to an AP module in HEK 293 cells. (A) Cartoon of Ten-m1 and of the AP fusion protein (ten-m1 ap). Symbols are the same as in Fig. 1 B. (B) Transfected HEK 293 cells show AP activity on the surface of the cell membrane. (C) After treating transfected cells with trypsin the AP activity is markedly reduced. (D) Mock-transfected HEK 293 cells. No AP activity is visible on these cells. (E) Coomassie blue staining (lanes 1–4) and Western blot with anti-CIAP antiserum (lanes 5–8) of proteins which were present in Triton X-114–poor (lanes 1, 2, 5, and 6) and Triton X-114–rich (lanes 3, 4, 7, and 8) phases after partition at 30°C. Experiments were performed in parallel with cell layers of nontransfected 293 cells (lanes 1, 3, 5, and 7) and 293 cells transfected with the AP fusion protein (lanes 2, 4, 6, and 8). Proteins were separated by 7% SDS-PAGE under reducing conditions.
Ten-m1 Is Expressed in a Dimeric Form

To analyze the structure of the extracellular domain of Ten-m1, the cDNA sequence starting with the first EGF module was linked to the BM-40 signal peptide cDNA (Fig. 3 A), cloned into an expression vector, and transfected into HEK 293 cells. 96 samples of serum-free conditioned culture medium derived from individual cell clones were collected and separated by SDS-PAGE under reducing conditions. Three recombinant cell clones expressed a protein with an apparent molecular mass of 225 kD after reduction which was not present in untransfected or mock-transfected controls. The protein was purified by ion exchange and gel permeation chromatography (Fig. 3 B). Under nonreducing electrophoresis conditions, the recombinant protein barely entered the separating gel. To confirm the identity of the recombinant Ten-m1, the purified protein was subjected to NH₂-terminal sequencing. The amino acid sequence obtained was APLAEIMD which represents the last four amino acids of the fused BM-40 signal peptide and the first four amino acids (526–529) of the fused Ten-m1 protein.

Purified recombinant molecules were visualized by electron microscopy after spraying from glycerol spraying/buffer mixtures (Fig. 3 C and D) and after adsorption to carbon films and negative staining (Fig. 3, E and F). By both techniques mainly particles with a structure compatible with dimeric molecules were visible. They consisted of two elongated globular domains connected by a thin extended rod. 24% of these dimeric particles were, in addition, visible in close proximity to each other. The size distribution of the spherical moiety exhibited Gaussian profiles with a long diameter $d_1$ of 13.2 ± 1.3 nm and a short diameter $d_2$ of 7.8 ± 1.3 nm. Values are corrected for overestimation of ~3 nm due to decoration with platinum. Negative staining revealed similar values ($d_1$ 12.7 ± 0.8 nm, $d_2$ 6.9 ± 0.7 nm), but in addition, each globular domain was resolved into three globular subdomains with similar sizes of 5.4 ± 0.9 nm (Fig. 3, E and F). The extended rod...
connecting the spherical domains had a total length of 5.9 ± 2.9 nm after negative staining. This rod appeared more variable in structure after rotary shadowing with a total length of 17.9 ± 4.9 nm where the middle part occasionally formed a short branch of 8.8 ± 2.8 nm extending sideways.

### Ten-m1 Is Dimerized via EGF Domains

Tandem arrays of multiple EGF-like modules often appear as rod-like structures (Sasaki et al., 1995). Therefore, the short extended rods in the Ten-m1 extracellular domain most likely represent the EGF-like modules, which are located at the NH₂ termini of the recombinantly expressed Ten-m1 monomers. This hypothesis was further supported by the fact that the second and fifth EGF-like domains had an odd number of cysteines which might enable the formation of intermolecular disulfide bonds. To test this biochemically, the AP cDNA was fused to the BM-40 signal peptide and to either the first three or all eight EGF-like domains of ten-m1 (Fig. 4 A). The EGF-like modules ended COOH-terminally at glutamic acid 624 and isoleucine 796, respectively. The fusion constructs were cloned into an expression vector and stably transfected into HEK 293 cells. Control cells were transfected with an expression construct containing the AP cDNA only fused to the signal peptide. Individual clones which showed AP activity in the supernatant were expanded. Serum-free conditioned media from several clones were analyzed by Western blot assay using AP specific antibodies. Fig. 4 B shows that cells transfected with AP only express a protein of ~67 kD under nonreducing as well as reducing conditions. Cells transfected with the A-P-3EGF repeat construct secreted a recombinant protein of apparent molecular mass of 110 kD when the proteins were separated under reducing conditions (Fig. 4 B). When the A-P-8EGF repeat construct was transfected the secreted protein had an apparent molecular mass of 150 kD under reducing electrophoresis conditions (Fig. 4 B). Under nonreducing conditions both molecules showed approximately twice these sizes (Fig. 4 B). Untransfected or mock-transfected cells did not secrete immunoreactive AP.

These data suggest that the EGF-like modules of Ten-m1 with an odd number of cysteines can form intermolecular disulfide bonds, leading to the homodimerization of Ten-m1.

### Expression of the ten-m Genes in Mouse Tissues

Northern blot analyses using the cytoplasmic and transmembrane part of the cDNAs as probes revealed widespread expression of ten-m1, 2, 3, and 4 genes in various mouse tissues (Fig. 5 A). All four ten-m genes showed tissue-specific expression of variously spliced mRNAs. Alternative splicing was most prominent in the testes. The highest levels of all four mouse ten-m mRNAs were observed in brain. Fig. 5 B shows a short exposure of Northern blots of brain mRNA. All four ten-m genes are expressed in alternatively spliced forms.

To assess protein expression of Ten-m1, a specific antiserum was produced by immunizing rabbits with the recombinant extracellular domain of Ten-m1. The antiserum was further purified by affinity chromatography with covalently immobilized immunogen. The results obtained by the specific antibodies in Western blots depended strongly on the reducing agent in the sample buffer and the treatment of the samples (see Materials and Methods). After exhaustive reduction of detergent extracts from mouse brain with dithiothreitol, two major protein bands with apparent molecular masses of 270 and 225 kD were observed (Fig. 6, lane 1). Often also higher molecular mass proteins, probably representing incompletely reduced protein dimers, were recognized by the purified antibody (Fig. 6, lane 2), occasionally even after prolonged incubations in the reducing sample buffer. Whereas the 270-kD form might correspond to the complete Ten-m1 molecule, the 225-kD form might represent an alternatively spliced or proteolytically processed molecule. It is also possible that the 225-kD form is a soluble form of the molecule, since it migrated at a similar position as the recombinant extracellular domain of Ten-m1 (Fig. 6, lane 3). A clear pattern of protein bands recognized by the purified antiserum could only be observed in brain extracts, but not in extracts of

---

**Figure 4.** Expression of EGF-like domains derived from Ten-m1 and fused to an AP module. (A) Cartoon of Ten-m1 and the AP fusion proteins. In AP-3EGF the AP module is linked to the first three EGF-like domains and AP-8EGF to the eight EGF-like domains of Ten-m1. Symbols are the same as in Fig. 1 B. (B) Western blot of AP fusion proteins. Supernatants of 293 cells secreting soluble AP alone, AP-3EGF, or AP-8EGF were precipitated with TCA. The precipitated proteins were separated by 6% SDS-PAGE under nonreducing or reducing conditions, blotted, and detected with anti-CIAP antiserum.

---

The Journal of Cell Biology, Volume 145, 1999

570
other tissue such as thymus, lung, and kidney (not shown). A lower abundance of Ten-m1 protein in tissues other than brain would be in agreement with the results of the Northern blot assay.

To localize Ten-m1 protein expression in mice, immunofluorescence assays were performed on a variety of tissue sections. Strong signals were observed in many regions of the mouse brain (Fig. 7, A–D). The cerebellum showed intensive staining in the molecular layer and weaker staining around cells in the granular layer. The staining around the cells appeared membrane associated and sometimes speckled. This localization strengthens the notion that Ten-m1 is a type II transmembrane protein with the COOH terminus on the extracellular side (antibodies were raised against the extracellular domain), as already suggested by sequence analysis and results obtained with recombinant fusion proteins. Purkinje cells and white matter were negative for Ten-m1 expression (Fig. 7, A and B). The hippocampus had strong staining in the molecular layer and the neuronal layer of the CA3 region. Weak staining was observed in the CA1 region and dentate gyrus (Fig. 7, C and D). Several other regions of the brain including the deep cerebellar nuclei, thalamic nuclei, cortex, and brain stem showed strong staining for Ten-m1 (not shown). Double immunostaining using anti–β3 tubulin antibodies which specifically label neurons revealed colocalization in many areas (not shown).

Expression of Ten-m1 was also seen in the outer and inner segments of the retina where rods and cones showed very strong staining (Fig. 7 E) and around the basal epithelial cells of the cornea (Fig. 7 F). The corneal surface showed a filamentous, strong staining (Fig. 7 F) which either could identify soluble Ten-m1 present in the tear film moistening the corneal surface or could be an artifact. Lung showed high expression in smooth muscle cell layers of bronchi, veins, and arteries and low expression on alveolar epithelial cells (Fig. 7 G). In kidney, the staining for Ten-m1 was restricted to the glomeruli (Fig. 7 H) and vessels in the medullary region (not shown). Immunostaining of adult testes revealed Ten-m1 expression around sper-
matides but no expression was detected in spermatogonia and mature sperms (Fig. 7 I). Staining of control sections with a nonimmune serum was negative (data not shown).

**Localization of Ten-m1 Binding in Mouse Tissues**

Transmembrane proteins often have the capability to bind to molecules outside the cell and thereby act as receptors. To search for ligands that bind to Ten-m1, the cDNA encoding the entire extracellular domain was linked to an AP module equipped with a signal peptide resulting in the fusion protein AP-ten-m1 (Fig. 8 A). Fig. 8 B shows that AP-ten-m1 and AP alone, which was used as a control, are secreted proteins with molecular masses of ~300 and 67 kD, respectively. As ten-m1 is expressed in many tissues (see Figs. 5 and 7), AP-ten-m1 fusion protein should be able to

![Image of tissue sections](https://example.com/image.png)
detect its ligand(s) directly in a variety of tissue sections. Cryosections were treated with AP-ten-m1, washed, and then tested for bound AP activity of the fusion protein using an AP substrate precipitating on the cells. Whereas each concentration of AP-ten-m1 produced a specific staining pattern in tissues (Fig. 8, C, E, and G), equimolar concentrations of unfused AP did not show any AP reaction products (Fig. 8, D, F, and H).

The binding of AP-ten-m1 in brain sections was high in the molecular layer of the cerebellum and around Purkinje cells. Little but significant staining was observed in the granular layer (Fig. 8 C). The hippocampus showed a clear signal in the molecular layer (Fig. 8 E) and a weak signal was visible in the CA3 region, CA1 region (not shown), and dentate gyrus (Fig. 8 E). Strong reactivity was also observed in many other regions of brain including cortex and several deep cerebellar nuclei (not shown). Kidney sections showed very intense staining in glomeruli (Fig. 8 G) and vessels of the medullar region (not shown). The lung sections had AP reactivity in the smooth muscle cell layers of bronchi, arteries, and veins (not shown). Incubation of tissues with the AP module did not reveal any staining reaction (Fig. 8, D, F, and H and data not shown).

**Ten-m1 Can Mediate Homophilic Interactions**

The similar staining pattern of the Ten-m1 antiserum and the AP-ten-m1 probe prompted us to test whether Ten-m1 may be able to bind to itself. To test this hypothesis, purified Ten-m1 extracellular domain was applied in a Far Western blot using AP-ten-m1 as a probe. Under nonreducing conditions the extracellular domain of Ten-m1 had an apparent molecular mass of ~400 kD as shown by amido black protein staining (Fig. 9, lane 1) and Western blot with purified anti-Ten-m1 antibodies (Fig. 9, lane 10). The AP-ten-m1 probe bound specifically the Ten-m1 on the blot as revealed by an AP catalyzed staining reaction (Fig. 9, lane 4). Neither recombinant neurocan nor BSA...
was recognized by a P-ten-m1 fusion protein (Fig. 9, lanes 5 and 6). A P alone did not bind to any of these proteins (Fig. 9, lanes 7–9).

Given the fact that Ten-m1 appears to interact in a homophilic fashion, it is noteworthy that by rotary shadowing and negative staining of purified Ten-m1 extracellular domain ~24% of the Ten-m1 particles showed a clear interaction with each other (see arrows in Fig. 3, C and E).

Discussion

Mouse Orthologues of Ten-m/Odz: Define a New Protein Family of Transmembrane Molecules

The *Drosophila* ten-m and odz genes are identical and define a new class of pair rule genes which do not function as transcription factors. In this paper we report the cloning and characterization of mouse ten-m/odz cDNAs. The mouse has at least four genes, named ten-m1–4, which are homologous to the *Drosophila* ten-m/odz gene. The primary sequence of all four ten-m proteins lacked a signal peptide, and contained a stretch of hydrophobic amino acid residues ~300–400 amino acids COOH-terminal of the start codon, eight EGF-like domains, and a large COOH terminus. Based on the cysteine and the putative N-linked glycosylation patterns, we divided the COOH terminus of the molecule tentatively into a cysteine-rich part, a strongly N-glycosylated part, and a part with four cysteines conserved in ten-m1 and 4, but not in ten-m2 and 3 (Fig. 1B). Sequence comparison with the data bank revealed homologies with several genes including *Drosophila* ten-m/odz, *Drosophila* ten-a, human γ-hercugulin, and mouse DOC4. *Drosophila* Ten-a (Baumgartner and Chiquet-Ehrismann, 1993) shares homologies with the NH2-terminal part of Ten-m/Odz and the mouse ten-m proteins but lacks the large COOH-terminal region. Ten-a is expressed in the CNS, muscle, and eye. Its function is not known (Baumgartner et al., 1993). The NH2-terminal part of γ-hercugulin, a protein produced by the human breast cancer cell line MDA-MB-175 (Schäfer et al., 1997), shows 95% identity to the 562 NH2-terminal amino acids of mouse Ten-m4. Since the COOH-terminal part of γ-hercugulin is identical to hercugulin β3, the expression of γ-hercugulin is very likely the result of a gene rearrangement between the 5’ region of the ten-m4 gene and the hercugulin β gene. The DOC4 gene is identical to ten-m4 and can be activated by cell stress–induced transcription factor CHOP (Wang et al., 1998). The COOH terminus of the ten-m proteins contained several amino acid repeats which showed similarities with the rearrangement hot spot elements of *E. coli* (Feulner et al., 1990) and with a wall-associated protein (WAP) of *B. subtilis* (Foster, 1993). Experimental evidence suggests that these proteins may be involved in sugar binding (Feulner et al., 1990; Foster, 1993).

The mRNAs for all four ten-m genes were present in all tissues analyzed so far. The highest levels of ten-m mRNAs were found in brain similar to the expression of Ten-m/Odz in *Drosophila* (Baumgartner et al., 1994; Levine et al., 1994). In many tissues the mRNA bands appeared as different sizes, suggesting that the ten-m genes are alternatively spliced. This was supported by the isolation of further ten-m cDNA clones corresponding to the different mRNA bands seen in Northern analysis (Oohashi, T., and R. Fässler, unpublished observations). The expression of Ten-m1 protein was further demonstrated by Western blotting and immunohistochemistry. Immunostaining for Ten-m1 confirmed the widespread expression. Moreover, Ten-m1 was expressed in a very restricted manner in the tissues analyzed. For example, in kidney Ten-m1 is only found in the glomeruli and in vessels of the medulla, in lung the expression is particularly high in the smooth muscle layers of the bronchi, veins, and arteries and in the testes Ten-m1 is present in spermatides but not in spermatogonia, mature sperms, or Sertoli cells. The brain sections revealed a distinct signal around neuronal cells (hippocampus, cerebellum, cortex) and a very strong signal in fiber tract regions. A similar staining pattern was also observed in the CNS of developing and adult flies.
Similarly to *Drosophila* ten-m/odz, ten-m1 mRNA is expressed in nerve cell bodies and the protein in axons, suggesting that Ten-m1 may also have an axonal localization signal (Zhou, X.-H., T. Oohashi, and R. Fässler, unpublished observations).

The lack of a signal peptide and the presence of a single stretch of hydrophobic amino acids COOH-terminal of the start codon suggested that the ten-m proteins in mouse are expressed as type II transmembrane proteins (Fig. 10).

**Structure of the ten-m Protein Family**

The recombinant expression of various parts of Ten-m1 alone or fused to the AP reporter provided strong evidence that Ten-m1 is a dimeric type II transmembrane molecule, in which the subunits are covalently linked to each other by cysteines in EGF-like domains 2 and 5 (Fig. 8). This finding is unexpected since Ten-m can be isolated as a soluble protein from the supernatant of *Drosophila* Schneider cells. It is also in contrast to a report describing the isolation of Odz as a type I phosphotyrosine-containing transmembrane receptor from the same cell line. Although we do not have direct evidence for secreted versions of the ten-m proteins, the presence of dibasic amino acids in the region which links the transmembrane domain to the first EGF-like domain (linker region, Supplemental Figure 1) suggests that ten-m proteins may be cleaved from the membrane. This could explain the presence of Ten-m/Odz in the supernatant of *Drosophila* Schneider cells (Baumgartner et al., 1994). Interestingly, we isolated splice variants of ten-m cDNAs which lacked the linker region (Oohashi, T., and R. Fässler, unpublished observations), suggesting that ten-m proteins may be expressed in a form which does not allow cleavage of the extracellular domain.

Our finding that the NH₂-terminal part of Ten-m1 including the short hydrophobic domain has the ability to locate an AP reporter protein at the outside of the cell membrane suggests that this region of the protein contains all the information necessary to insert the protein into membranes as a type II transmembrane molecule. These results are different from the sequence interpretation of Odz (Levine et al., 1994) which described a transmembrane domain COOH-terminal of the EGF repeat domain. However, this sequence is interrupted by charged amino acids and, therefore, is not entirely hydrophobic, which is unusual for transmembrane domains. Furthermore, the transmembrane domain of Odz is poorly conserved in the mouse ten-m proteins. In addition to charged amino acids, all mouse ten-m proteins contain a conserved potential N-glycosylation site in this region. Finally, the secreted form of the recombinant mouse Ten-m1 contained this stretch of amino acids but we never observed integration of the recombinant protein into the cell membrane of transfected HEK 293 cells. Therefore, we believe that even in *Drosophila* Odz this region cannot be used as a transmembrane domain for a type I insertion.

The ultrastructural analysis of the extracellular domain of Ten-m1 and the expression of recombinant fusion proteins composed of an AP reporter and either the first three or all eight EGF-like domains revealed that ten-m proteins are expressed as dimers covalently linked by a pair of free cysteine residues in EGF repeats 2 and 5. Negative staining of the extracellular domain in the EM revealed that the large COOH terminus of Ten-m1 is divided into three globular domains preceded by a fine rod which represents the EGF repeat domains. The EGF repeat domain of all ten-m proteins is composed of eight EGF-like domains which lack Ca²⁺ binding consensus motifs (Pan et al., 1993) and are most similar to the EGF-like repeats of the ECM molecule tenascin-C (Spring et al., 1989). The predicted length of the complete EGF repeat domain is 16 nm and of the first five EGF-like repeats 10 nm (Engel, 1989). This is in agreement with the EM observation that the extended rods which connect the two monomers are ~9 nm long. In EGF-like domains 2 and 5 the third cysteine is replaced by either a tyrosine or phenylalanine residue. This cysteine substitution is conserved in all four mouse ten-m proteins and in the *Drosophila* Ten-m/Odz. In a normal EGF repeat, the first cysteine would make a disulfide link to this third cysteine residue. Since it cannot be engaged in such a link in EGF-like domains 2 and 5 of the Ten-m proteins, it conceivably can form an intermolecular disulfide bond with another ten-m molecule. To our knowledge it has never been reported that an EGF repeat domain can covalently interact with another EGF domain. However, it cannot be excluded that an EGF-like domain with only five cysteine residues will lose the typical EGF-like folding pattern when it is engaged in intermolecular covalent interactions. Clearly, more structural studies are needed to resolve the folding pattern of the EGF-like repeats in its dimerized form.

The only other molecule which shares this incomplete cysteine pattern in modules within an EGF repeat array is *Drosophila* Ten-a (Baumgartner et al., 1993), which appears to have a topography very similar to ten-m molecules, but terminates shortly after the EGF repeat domain. The EGF repeat domain of Ten-m/Odz and Ten-a are more similar to each other than to any of the four mouse ten-m sequences (Oohashi, T., and R. Fässler, unpublished observations). Therefore, in addition to the ten-m family it is possible that a ten-a family of mammalian orthologues exists.

**Possible Functions of the ten-m Protein Family**

The finding that ten-m proteins are expressed as type II transmembrane molecules and the immunolocalization of ten-m1 and DOC4/Ten-m4 (Wang et al., 1998) on cells suggest that they function as cellular signal transducers. A additional support for a signaling function of the ten-m proteins comes from the fact that Odz was isolated in *Drosophila* as a phosphotyrosine-containing transmembrane protein (Levine et al., 1994). The high structural similarity of *Drosophila* Ten-m/Odz together with our experimental findings with the recombinant expression of the NH₂ terminus of mouse Ten-m1 would suggest that Ten-m/Odz is also expressed as a type II rather than a type I transmembrane protein. The presence of several tyrosines in the putative intracellular domain of the mouse ten-m molecules as well as of the *Drosophila* Ten-m/Odz would allow phosphorylation to occur. Tyrosine phosphorylation of intracellular domains of transmembrane proteins is often associated with intracellular signaling pathways.
The potential intracellular domains of the mouse ten-m molecules contain between 8 and 10 tyrosine residues, respectively.

To be able to visualize potential Ten-m1 ligand(s) in tissue sections and show binding on Far Western blots, we expressed and purified a recombinant fusion protein in which the AP module was fused to the extracellular domain of Ten-m1. This fusion protein bound strongly to specific regions in several tissues which, especially in the nervous tissue, overlapped with the immunostaining. Far Western blots and electron micrographs indicated that like other membrane molecules in the nervous system, for example NCAM and NgCAM/L1 (Brümmendorf and Rathjen, 1996), Ten-m1 is able to interact homophilically. Such interactions could influence a wide variety of processes including cell migration, neurite extension, and fasciculation. However, since the staining patterns of immunohistochemistry and affinity histochemistry were not completely overlapping, heterophilic interactions of Ten-m1 with other, so far unidentified ligands are likely to occur also. Additional heterophilic interactions have been observed as well for NCAM and especially for NgCAM/L1 which is able to interact with several other molecules including laminin, proteoglycans, other neural cell adhesion molecules, and integrins (Brümmendorf and Rathjen, 1996).

The presence of an RGD containing motif in Drosophila Ten-m/Odz together with results from cell adhesion and cell spreading assays using Ten-m/Odz–derived peptides suggested an interaction with a specific splice variant of the Drosophila PS2 integrins (Graner et al., 1998). We could not observe an altered adhesion or spreading behavior when we did adhesion assays with a β1 integrin–deficient cell line (Fässler et al., 1995) and recombinant mouse Ten-m1 (Fässler, R., unpublished observations). Furthermore, none of the mouse ten-m proteins contains an RGD motif which is frequently used for integrin engagement (Ruoslahiti and Pierschbacher, 1987).

The mechanism by which ten-m molecules may transduce signals from the extracellular environment into the cell is not obvious. The activation of many signaling receptors is mediated by ligand-induced dimerization. Since ten-m molecules are already dimerized before ligand binding, an alternative activation could be associated with a conformational change induced by ligand binding which allows intracellular protein interactions and phosphorylation. A similar signaling cascade has been suggested for integrins (Schwartz et al., 1995). Furthermore, the cytoplasmic domains of the ten-m proteins contain several prolinc-rich regions which could serve as docking domains for intracellular SH3-containing proteins (Mayer and Eck, 1995).

Another way to transmit signals into the interior of the cell could occur via proteolytic cleavage of the ten-m molecules in the linker domain close to the cell surface. All mouse ten-m proteins contain several dibasic sites in the linker domain and one of them is conserved in all sequences as well as in the Drosophila Ten-m/Odz and Ten-a. Dibasic sites have been observed frequently as recognition sites for a family of mammalian proteases with similarity to bacterial subtilisin (Barr, 1991). A proteolytic cleavage in this domain would release the cytosolic part of the molecule from a dimeric into a monomeric state, which might alter its ability to interact with other molecules or its accessibility for kinases and phosphatases. Evidence for a proteolytic cleavage was reported for Drosophila Ten-m by Baumgartner et al. (1994). Another observation which indicates proteolytic processing events in the linker domain is the occurrence of a soluble form of γ-hereregulin in the supernatant of the breast cancer cell line MDA-MB-175 (Schäfer et al., 1996). Since γ-hereregulin and mouse Ten-m4 share marked homologies in their NH2 termini, including their linker regions, the potential for shedding of the extracellular domain may be a characteristic of all the ten-m family members.

Do signaling cascades also modulate pair rule gene activity in Drosophila? During the syncytial blastoderm stage gap genes and pair rule genes are directly regulated by the spatial and temporal expression of upstream transcription factors. In the cellular blastoderm stage, when cell membranes form and cellular boundaries are established, the regulation of gene expression may become dependent on signal transduction. In elegant genetic and biochemical experiments it was shown that several pair rule genes including even-skipped, fushi tarazu, and runt are regulated by the soluble ligand unpaired which activates a Janus kinase (JAK; hopscotch)/signal transducer and activator of transcription (STAT; Stat92E) signaling pathway (Binari and Perrimon, 1994; Hou et al., 1996; Yan et al., 1996; Harrison et al., 1998). Although current models of the JAK/STAT signaling pathway propose that the activation of JAK, phosphorylation of STAT, and finally regulation of gene transcription (Darnell, 1997) are preceded by ligand-induced dimerization or multimerization of transmembrane receptors, experimental evidence for this speculation is lacking, and other signal transduction mechanism, like conformational changes in transmembrane molecules, cannot be ruled out.

In Drosophila, pair rule genes including ten-m/odz initiate a genetic program which is essential for the normal segmentation. The expression pattern of Ten-m/Odz in adult flies suggests further functions in many organs which have not yet been elucidated. Our data using immunostaining of various tissues derived from adult mice also indicate that the ten-m family has a widespread expression. At present, however, we are unable to exclude that the antisem raised against the extracellular domain of Ten-m1 does not cross-react with other family members.

A possible function for Ten-m4 has been reported by Wang et al. (1998) who searched for stress response gene which are dependent on the activity of the transcription factor CHOP. Using representational difference analysis, they isolated the DOC4 cDNA which is identical to the ten-m4 cDNA. Although DOC4/Ten-m4 expression is induced by cellular stress, it is not clear which function is propagated by this protein. To obtain direct evidence for possible roles of ten-m proteins during development and disease we have isolated the mouse ten-m genes and have generated mutant mice. The analysis of these mice will provide further insights into the function(s) of these interesting molecules.

We thank Dr. Karl-Heinz Mann for amino acid sequencing, Hanna Wiedemann for electron microscopy, Dr. J. Flanagan for the AP module, Drs. Stefan Baumgartner, Anders Björklund, John Bateman, Ray Boot-
References

Altschul, S.F., M.S. Boguski, W. Gish, and J.C. Wootton. 1994. Issues in searching molecular sequence databases. Nat. Genet. 6:119–129.

Barr, P.J. 1991. Mammalian subtilisins: the long-sought dibasic processing endopeptidases. Cell 66:1–3.

Baumgartner, S., and R. Chiquet-Ehrismann. 1993. Tenm, a Drosophila gene related to tenasin, shows selective transcript localization. Mech. Dev. 40:165–176.

Baumgartner, S., D. Martin, C. Hagios, and R. Chiquet-Ehrismann. 1994. Tenm, a Drosophila gene related to tenasin, is a new pair-rule gene. EMBO (Eur. Mol. Biol. Organ.) J. 13:3728–3740.

Binari, R., and N. Perrimon. 1994. Stripe-specific regulation of pair-rule genes by hopscotch, a putative Jak family tyrosine kinase in Drosophila. Genes Dev. 8:300–312.

Brümmendorf, T., and F.G. Rathjen. 1996. Structure/function relationships of axon-associated adhesion receptors of the immunoglobulin superfamily. Curr. Opin. Neurobiol. 6:584–593.

Cheng, H.-J., and J.G. Flanagan. 1994. Identification and cloning of ELF-1, a developmentally expressed ligand for the Meck and Seck receptor tyrosine kinases. Cell. 79:157–168.

D’Arcangelo, G., G.G. Miao, S.C. Chen, H.D. Soares, J.J. Morgan, and T. Curran. 1995. A protein related to extracellular matrix proteins deleted in the mouse mutant reeler. Nature. 374:719–723.

Darnell, J.E., Jr. 1997. STATs and gene regulation. Science. 277:1630–1635.

Denda, S., L.F. Reichardt, and U. Müller. 1998. Identification of osteopontin as a novel ligand for the integrin αβ1 and potential roles for this integrin-ligand interaction in kidney morphogenesis. Mol. Biol. Cell. 9:1425–1435.

Engel, J. 1989. EGF-like domains in extracellular matrix proteins: localized signals for growth and differentiation? FERS Lett. 25:1–7.

Engel, J., and H. Furthmayr. 1987. Electron microscopy and other physical methods for the characterization of extracellular matrix components: laminin, fibronectin, collagen IV, collagen VI, and proteoglycans. Methods Enzymol. 145:1–47.

Ericksen, H.P. 1993. Tenasin-C, tenasin-R, tenasin-X: a family of talented proteins in search of functions. Curr. Opin. Cell Biol. 5:869–876.

Fässler, R., M. Pfaff, J. Murphy, A.A. Noegel, S. Johansson, R. Timpl, and R. Engel, J., and H. Furthmayr. 1987. Electron microscopy and other physical methods for the characterization of extracellular matrix components: laminin, fibronectin, collagen IV, collagen VI, and proteoglycans. Methods Enzymol. 145:1–47.

Fleming, R.J., Y. Gu, and N.A. Hukriede. 1997. Serrate-mediated activation of cadherins. Trends Biochem. Sci. 22:428–430.

Fässler, R., M. Pfaff, J. Murphy, A.A. Noegel, S. Johansson, R. Timpl, and R. Engel. 1987. Electron microscopy and other physical methods for the characterization of extracellular matrix components: laminin, fibronectin, collagen IV, collagen VI, and proteoglycans. Methods Enzymol. 145:1–47.

Engel, J., and H. Furthmayr. 1987. Electron microscopy and other physical methods for the characterization of extracellular matrix components: laminin, fibronectin, collagen IV, collagen VI, and proteoglycans. Methods Enzymol. 145:1–47.

Heinrich, C. 1987. A Molecular and Functional Analysis of Tenascins. Ph.D. Thesis, University of California, San Francisco.

Hou, X.S., M.B. Melnick, and N. Perrimon. 1996. Marelle acts downstream of the Drosophila hopscotch gene and encodes a protein similar to the mammalian STATs. Cell. 84:411–419.

Ingham, P.W. 1988. The molecular genetics of embryonic pattern formation in Drosophila. Nature. 335:25–34.

Jostel, A., K. Vahedi, C. Corcoran, A. Troesch, H. Chabrait, C. Vayssiere, C. Cruaud, J. Maciazek, J. Weissenbach, M.G. Bousser, J.F. Bach, and E. Tournier-Lasserve. 1997. Strong clustering and stereotyped nature of Notch3 mutations in CADASIL patients. Lancet. 350:1511–1515.

Justice, I.M., J.J. Murtagh, Jr., J. Moss, and M. Vaughan. 1995. Hydropathy and subunit interactions of rod outer segment proteins investigated using Triton X-114 phase partitioning. J. Biol. Chem. 270:17970–17976.

Kjærnsli, H., and G. Dreyer. 1985. The origin of pattern and polarity in Drosophila. Nature. 319:757–760.

Kjærnsli, H., and G. Dreyer. 1985. The origin of pattern and polarity in Drosophila. Nature. 319:757–760.

Kjærnsli, H., and G. Dreyer. 1985. The origin of pattern and polarity in Drosophila. Nature. 319:757–760.

Kjærnsli, H., and G. Dreyer. 1985. The origin of pattern and polarity in Drosophila. Nature. 319:757–760.

Kjærnsli, H., and G. Dreyer. 1985. The origin of pattern and polarity in Drosophila. Nature. 319:757–760.

Kjærnsli, H., and G. Dreyer. 1985. The origin of pattern and polarity in Drosophila. Nature. 319:757–760.

Kjærnsli, H., and G. Dreyer. 1985. The origin of pattern and polarity in Drosophila. Nature. 319:757–760.

Kjærnsli, H., and G. Dreyer. 1985. The origin of pattern and polarity in Drosophila. Nature. 319:757–760.

Kjærnsli, H., and G. Dreyer. 1985. The origin of pattern and polarity in Drosophila. Nature. 319:757–760.