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

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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 NH_2 terminus, but contain a short hydrophobic domain characteristic of transmembrane proteins, 300–400 amino acids after the NH_2 terminus. About 200 amino acids COOH-terminal to this hydrophobic region are eight consecutive EGF-like domains.

Cell transfection, biochemical, and electronmicroscopic studies suggest that Ten-m1 is a dimeric type II transmembrane protein. Expression of fusion proteins composed of the NH_2-terminal and hydrophobic domain of ten-m1 attached to the alkaline phosphatase reporter gene resulted in membrane-associated staining of the alkaline phosphatase. Electronmicroscopic and electrophoretic analysis of a secreted form of the extracellular domain of Ten-m1 showed that Ten-m1 is a disulfide-linked dimer and that the dimerization is mediated by EGF-like modules 2 and 5 which contain an odd number of cysteines.

Northern blot and immunohistochemical analyses revealed widespread expression of mouse *ten-m* genes, with most prominent expression in brain. All four *ten-m* genes can be expressed in variously spliced mRNA isoforms. The extracellular domain of Ten-m1 fused to an alkaline phosphatase reporter bound to specific regions in many tissues which were partially overlapping with the Ten-m1 immunostaining. Far Western assays and electronmicroscopy demonstrated that Ten-m1 can bind to itself.

Key words: *ten-m/odz* • transmembrane protein • pair rule • epidermal growth factor

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 Wieschhaus, 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 larvae.

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

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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 phosphotyrosine-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 downregulated and appears at later stages in the central nervous system (CNS),[1] 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 ^{32}P-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 (Rauch et al., 1997). The 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). Using the insert of DT1 the brain library was rescreened to obtain cDNAs extending in the 3′ and 5′ direction. This rescreening was repeated until the entire coding region was cloned. All clones were 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-

1. *Abbreviations used in this paper:* AP, alkaline phosphatase; CNS, central nervous system.

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 polytron 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 Econo column (Pharmacia). After three additional washings of the oligo-dT with high salt buffer, polyadenylated RNA was eluted with 1 ml of RNase-free water. For Northern analysis, 4 μg of polyadenylated RNA was electrophoretically separated, blotted onto Hybond membrane (Amersham), UV cross-linked, and probed in Church buffer at 65°C. Filters were washed twice in 0.2× SSC/1% SDS at 65°C and exposed to x-ray film for 1 or 4 d at −80°C.

The following oligolabeled probes were used: for *ten-m1* a fragment ranging from nucleotide 75 to 1833, for *ten-m2* a fragment ranging from nucleotide 1 to 2006, for *ten-m3* an EcoRI fragment ranging from nucleotide 762 to 1408; for *ten-m4* a fragment ranging from nucleotide 1 to 1108. The RNA loading was controlled by probing blots with a GAPDH cDNA probe.

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 region was followed by amino acid E526 of the Ten-m1 sequence. The constructs for the expression of the alkaline phosphatase (AP) fused to either three or eight EGF domains or the entire extracellular domain (AP-ten-m1), respectively, started with an APLVGSSG sequence, followed by I23 of the human placental AP sequence, which terminated before the hydrophobic glypiation signal through a stop codon encoded by an HpaI site (Flanagan and Leder, 1990). To express both fusion proteins the NH_2-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 *ten-m1* cDNA fragment encoding the EGF domains or extracellular domain, respectively. In these fusion proteins the AP domain was linked to the Ten-m1 fragment via an SSGG sequence to the G359 of the Ten-m1 sequence. The fusion proteins were terminated by the introduction of stop codons after E624 for the AP-3EGF recombinant protein and after I796 for the AP-8EGF recombinant protein, respectively. To express the intracellular and transmembrane domain fused to the AP the first 370 amino acids of Ten-m1 were connected via the linker sequence IKLAYVRSSG 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 ring cloning. Positive clones were identified 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-Sephacel 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, pooled, and concentrated to <1 ml by centrifugation in 2- or 3.5-ml concentrators with membranes with a cutoff of 10 kD (Amicon or Pall-Filtron). 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 PMSF. Ten-m1sec–containing fractions were dialyzed against 50 mM $NaHCO_3$, 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 antiserum 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 antiserum 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 PMSF on ice using a Dounce homogenizer. After 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. Sucrose extracts were supplemented with 3× SDS-PAGE buffer, whereas Triton X-100 extracts were precipitated with acetone 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, also 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 (20 mM Tris-HCl, pH 7.6, 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 (Amersham) 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 antiserum (Sigma Chemical Co.), and developed with AP-conjugated secondary antibodies.

Triton X-114 Phase Partition

HEK 293 cells transfected with Ten-m1ap 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 phase (~30 μl) was precipitated with acetone and dissolved in 100 μl SDS-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 SDS-PAGE sample

buffer. 15-μl aliquots were used for protein stainings with Coomassie blue and for Western blots.

Far Western Blotting

Far Western blotting was performed as described (Denda et al., 1998) with minor modifications. Recombinant Ten-m1sec, neurocan, and BSA were separated by electrophoresis on SDS-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 AP-ten-m1 or AP, respectively, in the TBST-CM buffer containing 1% BSA for 3 h at room temperature, respectively. Afterwards 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 AP activity.

Immunofluorescence

Immunofluorescence analyses were carried out using affinity-purified rabbit antibodies against Ten-m1. 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 (Zeiss) cryostat. Sections were fixed in 4% paraformaldehyde for 5 min at room temperature and washed in PBS. Affinity-purified antibodies against Ten-m1 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 β-phenylenediamine. Microscopy was carried out with an Axiophot fluorescence microscope (Zeiss).

Staining of Tissue Sections with the AP-ten-m1 Fusion Protein

Frozen 8-μm-thick tissue sections were prefixed in 4% paraformaldehyde for 5 min. Staining with AP-ten-m1 or AP 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 AP-ten-m1 or AP, respectively, in HBSS buffer including 1% BSA at room temperature overnight. Sections were washed five times with HBSS buffer, fixed with 60% acetone, 3% formaldehyde, 20 mM Hepes (pH 7.5) for 30 s, washed with 150 mM NaCl, 20 mM Hepes (pH 7.5; HBS) three times, heated at 65°C to inactivate endogenous AP for 15 min in HBS 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). Afterwards 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-m1sec 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 rotary shadowed 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 5 μl of purified Ten-m1sec 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. Evaluation of the data from electron micrographs was done as described previously (Engel and Furthmayr, 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 NH_2-terminal 300–400 amino acids serve as cytosolic domain. They showed on average the lowest level of identity between the four mouse ten-m sequences, ranging between 34 and 46%. The extracellular part of ten-m molecules would consist of a linker domain of ~200 amino acids, a region with eight EGF-like do-

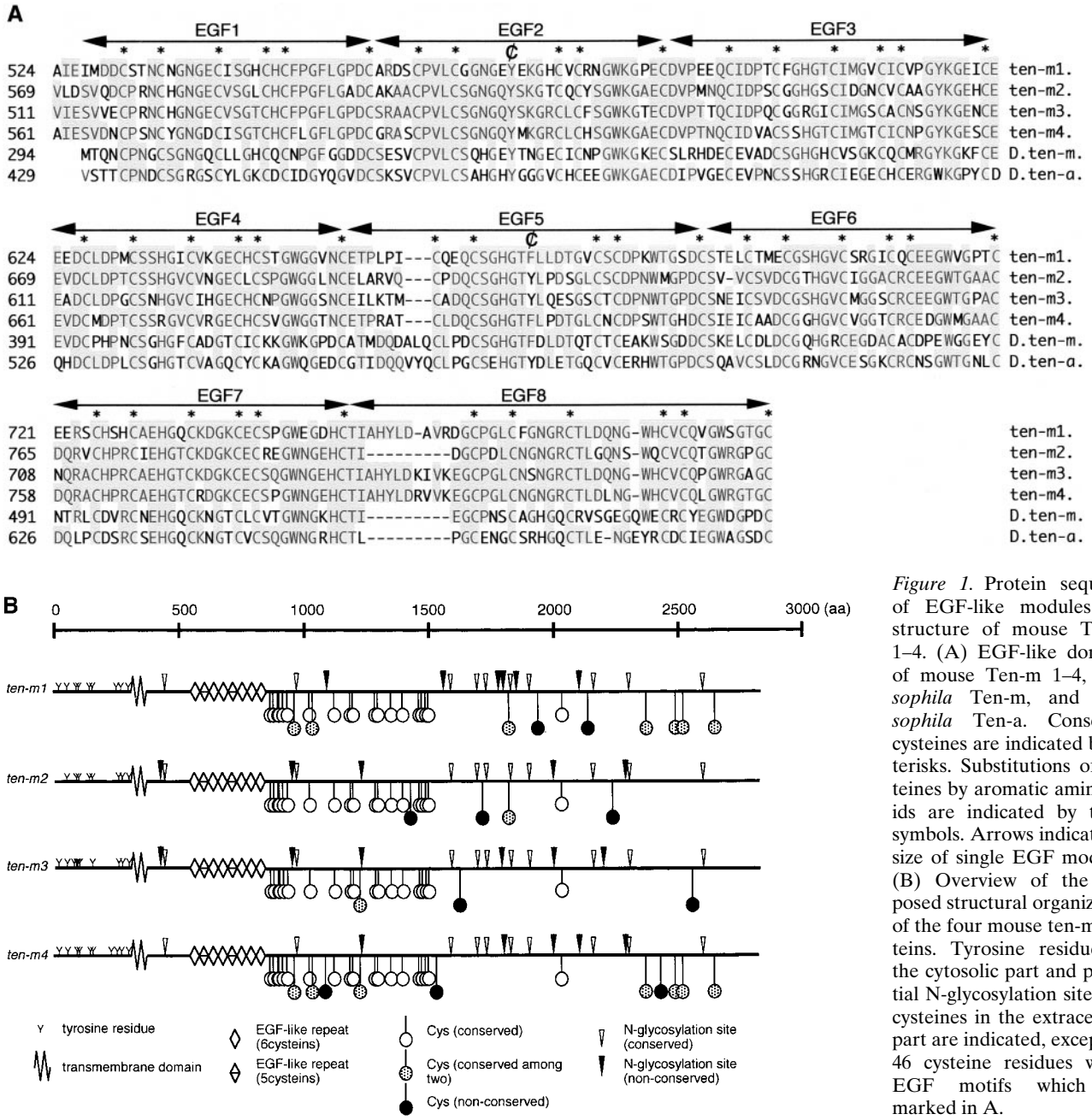

Figure 1. Protein sequence of EGF-like modules and structure of mouse Ten-m 1–4. (A) EGF-like domains of mouse Ten-m 1–4, *Drosophila* Ten-m, and *Drosophila* Ten-a. Conserved cysteines are indicated by asterisks. Substitutions of cysteines by aromatic amino acids are indicated by the ¢ symbols. Arrows 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.

mains of ~250 amino acids, and a COOH-terminal domain of ~2,000 amino acids (Fig. 1 B). 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 (Supplemental 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 NH_2-terminal part of the human γ-heregulin, identified in the breast cancer cell line MDA-MB-175 (Schäfer et al., 1997), had 95% identity to the NH_2-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).

Ten-m1 Is a Type II Transmembrane Molecule

To obtain evidence for the proposed model in which ten-m 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). The supernatant of the transfected clones showed no AP activity.

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 NH_2-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 NH_2-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.

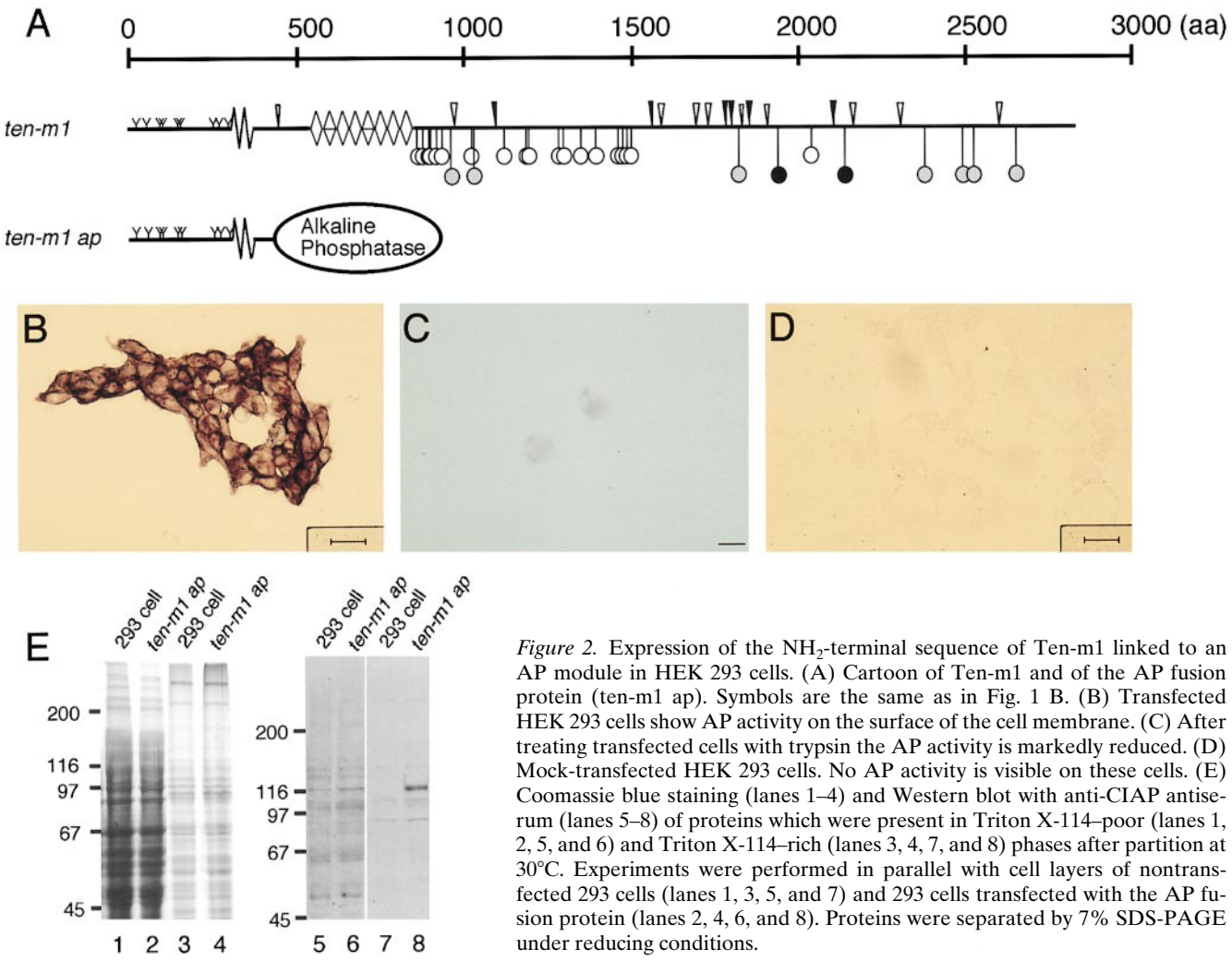

Figure 2. Expression of the NH_2-terminal sequence of Ten-m1 linked to an AP module in HEK 293 cells. (A) Cartoon of Ten-m1 and of the AP fusion protein (ten-m1 ap). Symbols are the same as in Fig. 1 B. (B) Transfected HEK 293 cells show AP activity on the surface of the cell membrane. (C) After treating 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 NH_2-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 d1 of 13.2 ± 1.3 nm and a short diameter d2 of 7.8 ± 1.3 nm. Values are corrected for overestimation of ~3 nm due to decoration with platinum. Negative staining revealed similar values (d1 12.7 ± 0.8 nm, d2 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

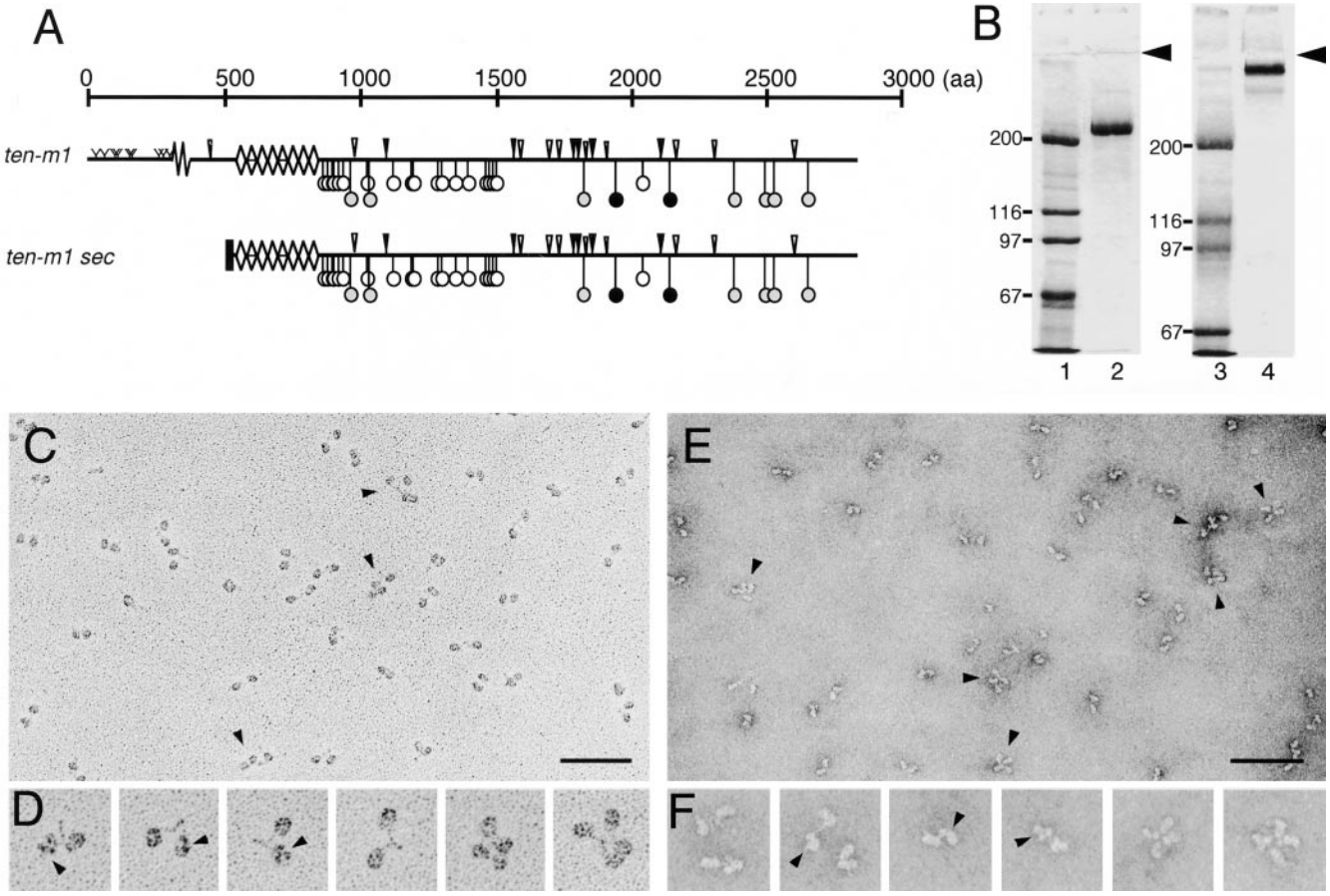

Figure 3. Expression and electronmicroscopic analysis of the extracellular domain of Ten-m1. (A) Cartoon of Ten-m1 and the secreted COOH-terminal part of Ten-m1 (Ten-m1sec). (B) Coomassie blue staining of molecular mass standards (lanes 1 and 3) and purified Ten-m1sec (lanes 2 and 4) separated by 5% SDS-PAGE under reducing (lanes 1 and 2) and nonreducing (lanes 3 and 4) conditions. The arrowhead indicates the beginning of the separating gel. (C–F) Electron micrographs after glycerol spraying/rotary shadowing (C and D) and negative staining (E and F) of Ten-m1sec. Representative fields of molecules (C and E) and selected species of the same material (D and F) are shown. Pairs of spherical domains, connected by thin elongated rods, are visible. Some of the spheres are resolved into three globular subdomains as indicated by arrowheads (D and F). Arrowheads (C and E) also indicate pairs of Ten-m1sec dimers interacting with each other. Bars, 50 nm.

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 NH_2 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 AP-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 AP-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

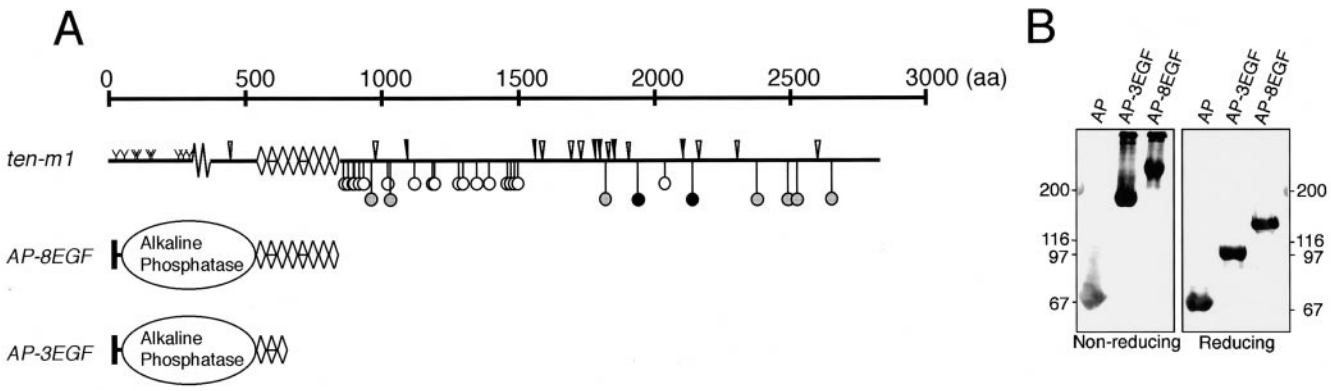

Figure 4. Expression of EGF-like domains derived from Ten-m1 and fused to an AP module. (A) Cartoon of Ten-m1 and the AP fusion proteins. In AP-3EGF the AP module is linked to the first three EGF-like domains and AP-8EGF to the eight EGF-like domains of Ten-m1. Symbols are the same as in Fig. 1 B. (B) 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.

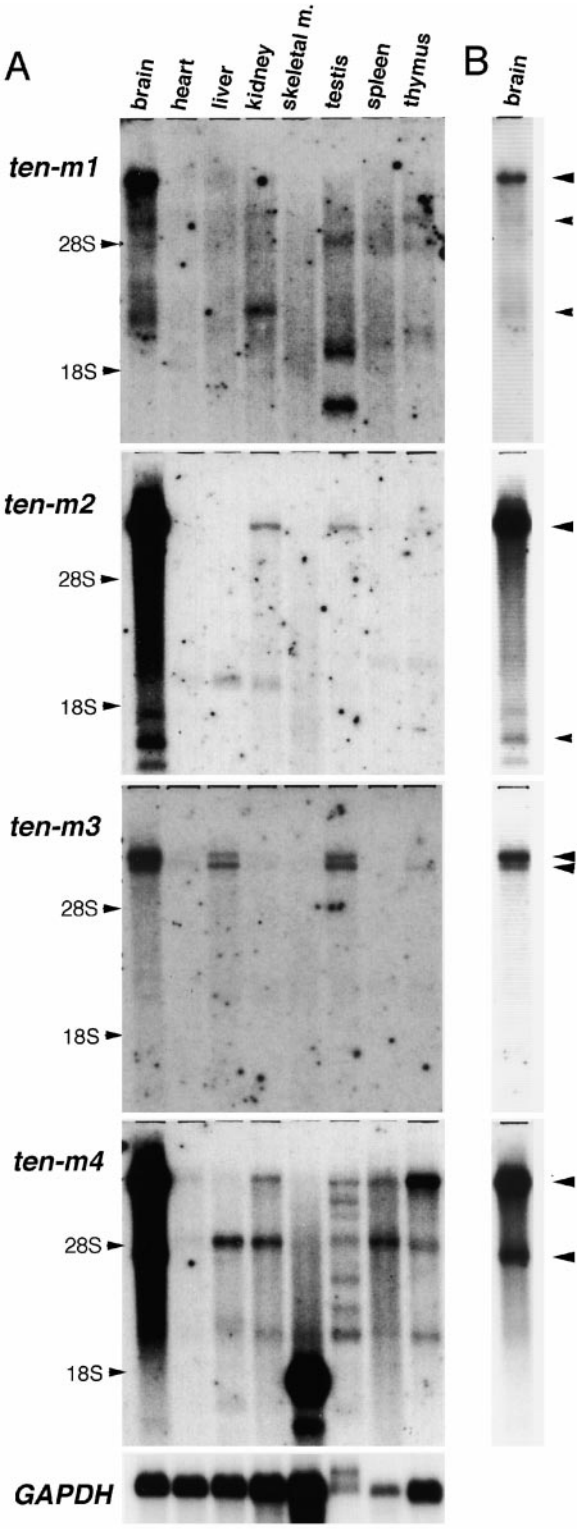

Figure 5. Northern blot of tissues using *ten-m* cDNAs as probes. Poly(A)$^+$ RNA was isolated from the tissues indicated and probed with *ten-m1*, *2*, *3*, and *4* cDNAs coding for the cytoplasmic and transmembrane domain and exposed for 4 d (A) or 24 h (B). Arrowheads in B show the alternatively spliced mRNAs present in brain tissue.

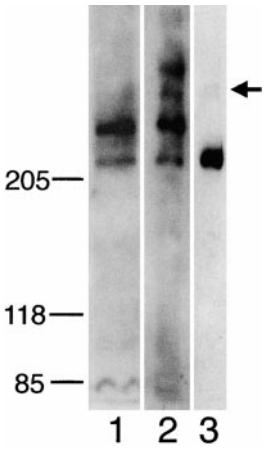

Figure 6. Western blot of brain extracts with affinity-purified antibodies against Ten-m1. 3% Triton X-100 extracts of mouse brain (lanes 1 and 2) were precipitated with acetone. Dried precipitates were dissolved in sample buffer, either for 30 min at 70°C (lane 1) or at room temperature (lane 2). Both samples were reduced for 5 min at 95°C before electrophoresis. Lane 3 shows recombinant Ten-m1sec which served as a positive control and size marker. The arrow indicates the position of a second band occasionally occurring in the recombinant Ten-m1sec lane (also faintly visible here), probably reflecting incompletely reduced Ten-m1sec dimers.

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 medullar region (not shown). Immunostaining of adult testes revealed Ten-m1 expression around sper-

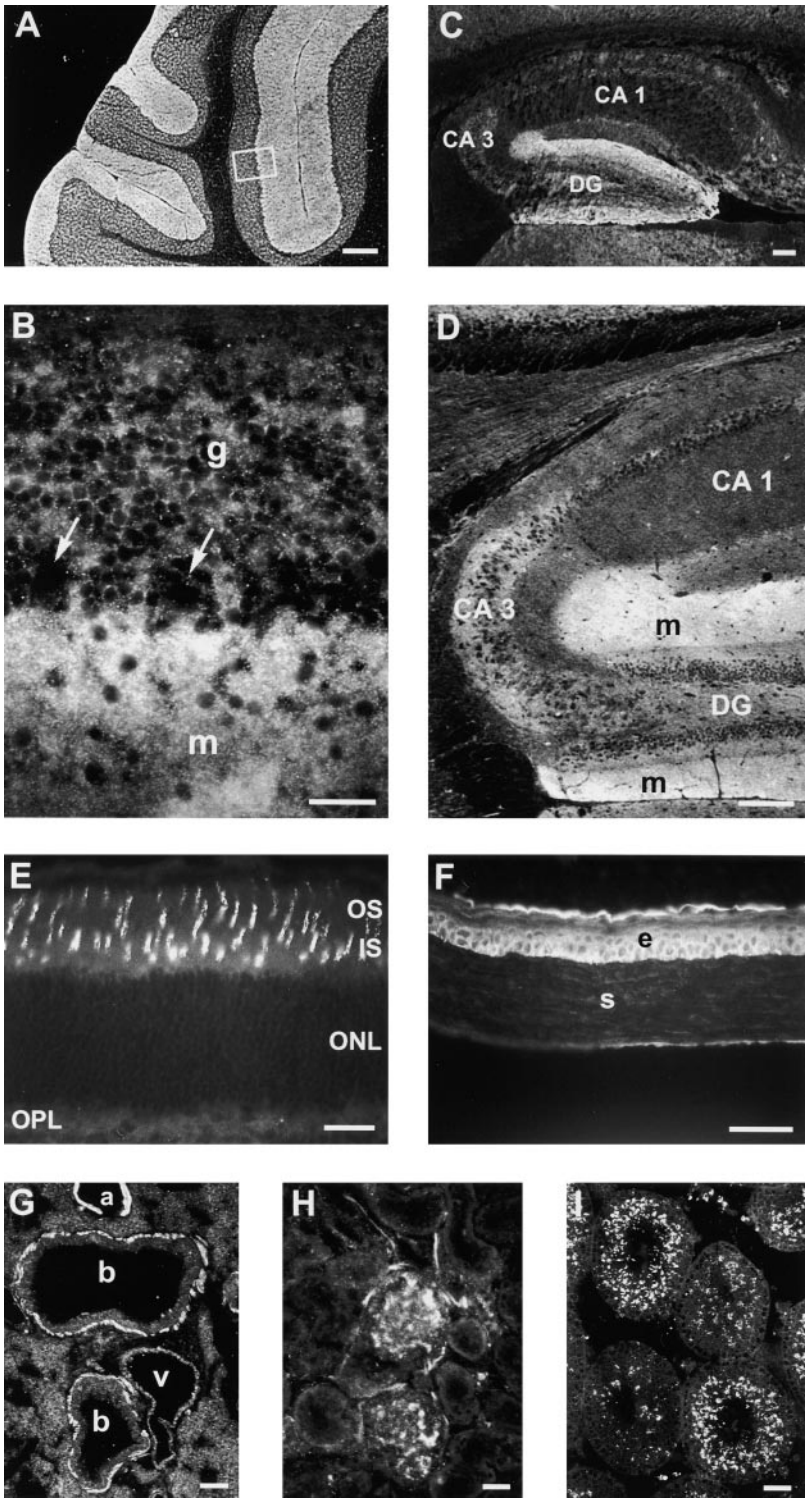

Figure 7. Immunolocalization of Ten-m1 in tissue sections. (A and B) Immunostaining of cerebellum. Strong staining is seen in the molecular layer (m) and weaker staining around cells in the granular cell layer (g). Purkinje cells (arrow in B) show no expression of Ten-m1. The boxed area in A is shown at higher magnification in B. (C and D) Immunostaining in the hippocampus. The staining is very strong in the molecular layer (m) of the CA3 region and weaker around neuronal cells in the CA3 region and dentate gyrus. (E and F) Immunostaining in retina and cornea. Strong staining is seen over the photoreceptor inner (IS) and outer (OS) segment. Very faint staining could be detected in the outer plexiform layer (OPL) and no staining could be detected in the outer nuclear layer (ONL) of the retina. (F) The basal cell layer of the corneal epithelium stained strongly for Ten-m1. Linear staining was also observed on the superficial layer of the corneal epithelium. (G) Ten-m1 staining of a lung section. The expression was high in the smooth muscle cell layer of arteries (a), bronchi (b), and veins (v). Low expression could be also observed on alveolar cells. (H) Ten-m1 expression in glomeruli and (I) on spermatides present in the seminiferous tubules of the testes. The signal was absent from spermatogonia, mature sperm (center of the tubules), Sertoli cells, and Leydig cells. Bars, 150 μm in A, C, and D; 30 μm in B, E, F, and H; 60 μm in G and I.

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

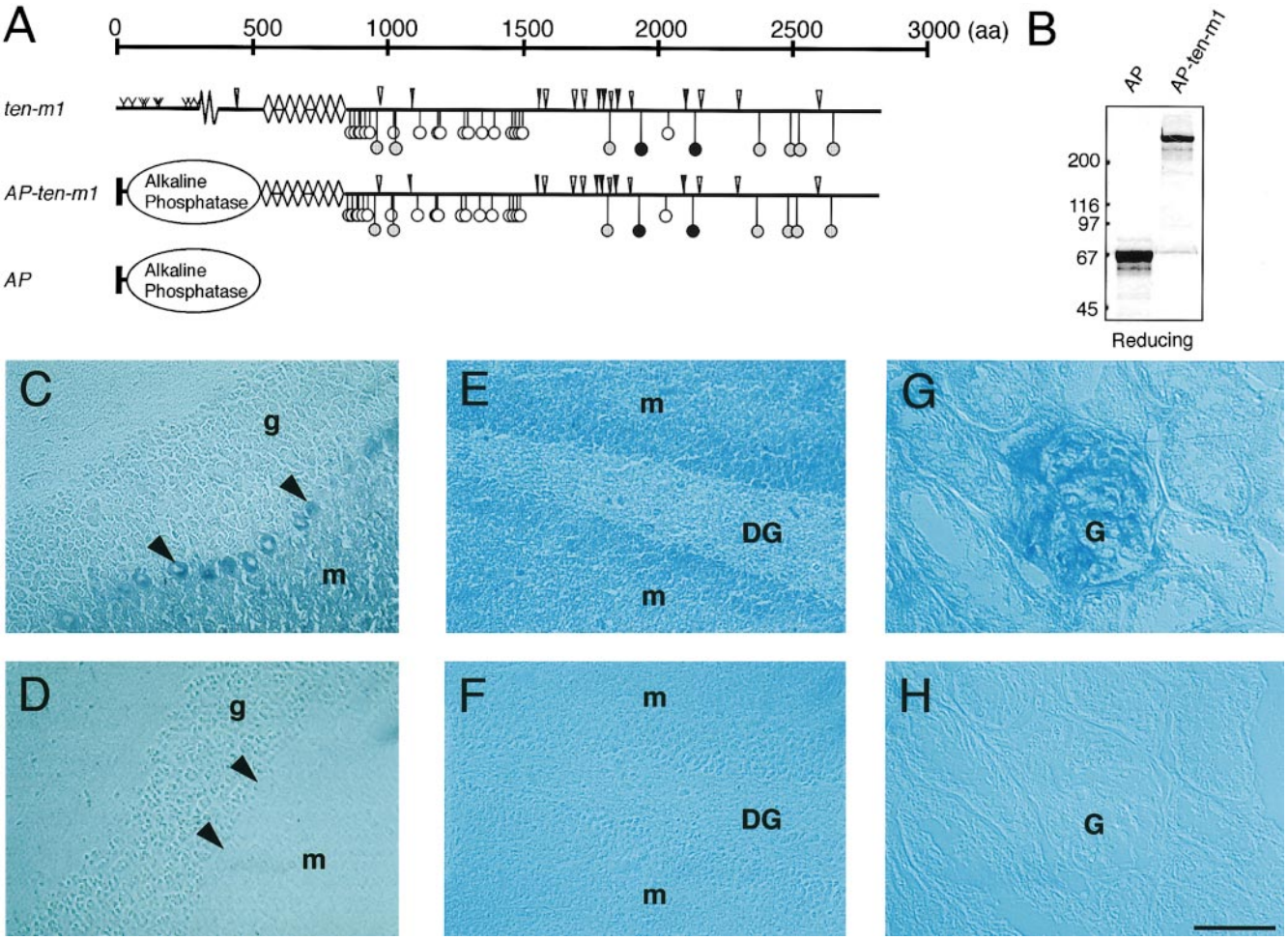

Figure 8. Binding pattern of AP-ten-m1 in tissue sections. (A) Cartoon of Ten-m1, the fusion protein composed of AP-ten-m1 and AP. Symbols are the same as in Fig. 1 B. (B) Coomassie blue staining of the purified AP-ten-m1 and AP separated by 7% SDS-PAGE under reducing conditions. (C–F) Frozen sections derived from cerebellum (C), hippocampus (E), and kidney (G) were incubated with AP-ten-m1. Control sections (D, F, and H) were incubated with AP. Arrowheads indicate Purkinje cells. g, granular layer; m, molecular layer; DG, dentate gyrus; G, glomerulus. Bars, 50 μm in C, D, G, and H; 25 μm in E and F.

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

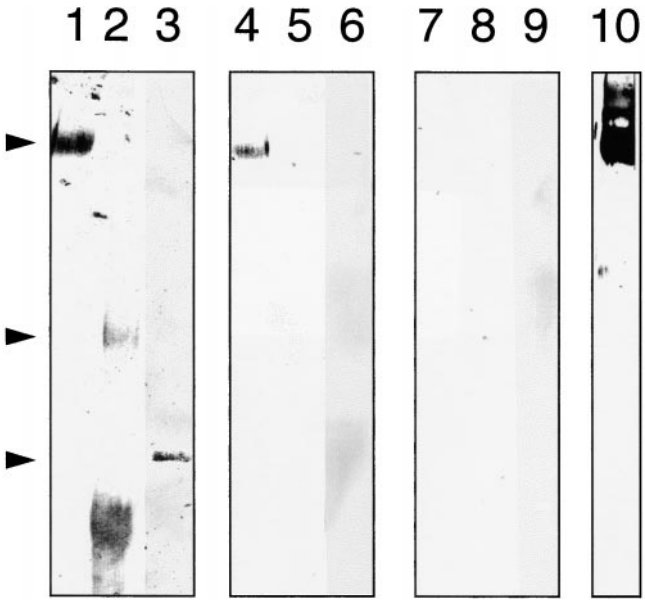

Figure 9. Homophilic interaction of Ten-m1. Western blot of the recombinant extracellular domain of Ten-m1 (lanes 1, 4, 7, and 10), recombinant neurocan core protein (lanes 2, 5, and 8), and BSA (lanes 3, 6, and 9), separated on a 5% (Ten-m1 and neurocan) or on a 7% (BSA) SDS-PAGE performed under nonreducing conditions. Lanes 1–3 were stained with amido black. Arrowheads indicate the 400-kD Ten-m1sec dimer, the 250-kD neurocan core protein, and the 67-kD BSA band. Lanes 4–6 were incubated with purified AP-ten-m1 fusion protein, and lanes 7–9 with purified AP alone. Lane 10 was incubated with the purified anti–Ten-m1 antiserum and AP-conjugated secondary antibodies.

was recognized by AP-ten-m1 fusion protein (Fig. 9, lanes 5 and 6). AP 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. 1 B). Sequence comparison with the data bank revealed homologies with several genes including *Drosophila ten-m/odz*, *Drosophila ten-a*, human γ-heregulin, and mouse *DOC4*. *Drosophila* Ten-a (Baumgartner and Chiquet-Ehrismann, 1993) shares homologies with the NH_2-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 NH_2-terminal part of γ-heregulin, a protein produced by the human breast cancer cell line MDA-MB-175 (Schäfer et al., 1997), shows 95% identity to the 562 NH_2-terminal amino acids of mouse Ten-m4. Since the COOH-terminal part of γ-heregulin is identical to heregulin β3, the expression of γ-heregulin is very likely the result of a gene rearrangement between the 5′ region of the *ten-m4* gene and the *heregulin* 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).

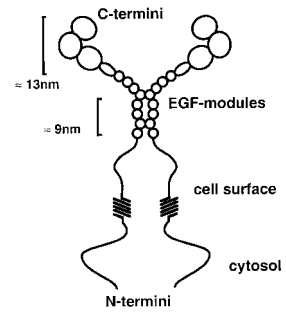

Figure 10. Model of mouse Ten-m1. Two Ten-m1 protein chains are inserted into the plasma membrane as a type II transmembrane molecule. EGF-like modules 2 and 5 are engaged in intermolecular bonds. The COOH terminus is divided into three globular domains.

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 NH_2-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 four 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 represent the EGF repeat domains. The EGF repeat domain of all ten-m proteins is composed of eight EGF-like domains which lack Ca^{2+} 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 loose 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. 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 NH_2 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

events (Weiss and Schlessinger, 1998). 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 specific splice variants 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 (Ruoslahti 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 proline-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-heregulin in the supernatant of the breast cancer cell line MDA-MB-175 (Schäfer et al., 1996). Since γ-heregulin and mouse Ten-m4 share marked homologies in their NH_2 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 antiserum 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.

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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 endoproteases. *Cell.* 66:1–3.

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

Baumgartner, S., D. Martin, C. Hagios, and R. Chiquet-Ehrismann. 1994. *Ten^m*, a *Drosophila* gene related to tenascin, 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 Mek4 and Sek receptor tyrosine kinases. *Cell.* 79:157–168.

D'Arcangelo, G., G.G. Miao, S.C. Chen, H.D. Soares, J.I. 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 α8β1 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? *FEBS Lett.* 251:1–7.

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.

Erickson, H.P. 1993. Tenascin-C, tenascin-R, tenascin-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. Albrecht. 1995. Lack of β1 integrin gene in embryonic stem cells affects morphology, adhesion, and migration but not integration into the inner cell mass of blastocysts. *J. Cell Biol.* 128:979–988.

Feulner, G., J.A. Gray, J.A. Kirschman, A.F. Lehner, A.B. Sadosky, D.A. Vlazny, J. Zhang, S. Zhao, and C.W. Hill. 1990. Structure of the rhsA locus from *Escherichia coli* K-12 and comparison of rhsA with other members of the rhs multigene family. *J. Bacteriol.* 172:446–456.

Flanagan, J.G., and P. Leder. 1990. The kit ligand: a cell surface molecule altered in steel mutant fibroblasts. *Cell.* 63:185–194.

Fleming, R.J., Y. Gu, and N.A. Hukriede. 1997. Serrate-mediated activation of Notch is specifically blocked by the product of the gene *fringe* in the dorsal compartment of the *Drosophila* wing imaginal disc. *Development.* 124:2973–2981.

Foster, S.J. 1993. Molecular analysis of three major wall-associated proteins of *Bacillus subtilis* 168: evidence for processing of the product of a gene encoding a 258 kD precursor two-domain ligand-binding protein. *Mol. Microbiol.* 8:299–310.

Fowler, W.E., and H.P. Erickson. 1979. Trinodular structure of fibrinogen. Confirmation by both shadowing and negative stain electron microscopy. *Mol. Biol.* 134:241–249.

Graner, M.W., T.A. Bunch, S. Baumgartner, A. Kerschen, and D. Brower. 1998. Splice variants of the *Drosophila* PS2 integrins differentially interact with the extracellular ligands Tiggrin, D-laminin α2 and Ten-m. *J. Biol. Chem.* 273:18235–18241.

Harrison, D.A., P.E. McCoon, R. Binari, M. Gilman, and N. Perrimon. 1998. *Drosophila* unpaired encodes a secreted protein that activates the JAK signaling pathway. *Genes Dev.* 12:3252–3263.

Hou, X.S., M.B. Melnick, and N. Perrimon. 1996. *marelle* acts downstream of the *Drosophila* HOP/JAK kinase 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.

Joutel, A., K. Vahedi, C. Corpechot, A. Troesch, H. Chabriat, 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, J.M., J.J. Murtagh, Jr., J. Moss, and M. Vaughan. 1995. Hydrophobicity and subunit interactions of rod outer segment proteins investigated using Triton X-114 phase partitioning. *J. Biol. Chem.* 270:17970–17976.

Levine, A., A. Bashan-Ahrend, O. Budai-Hadrian, D. Gartenberg, S. Menasherow, and R. Wides. 1994. *Odd Oz*: a novel *Drosophila* pair rule gene. *Cell.* 77:587–598.

Levine, A., C. Weiss, and R. Wides. 1997. Expression of the pair-rule gene odd Oz (odz) in imaginal tissues. *Dev. Dyn.* 209:1–14.

Mayer, B.J., and M.J. Eck. 1995. SH3 domains. Minding your p's and q's. *Curr. Biol.* 5:364–367.

Mayer, U., K. Mann, R. Timpl, and G. Murphy. 1993. Sites of nidogen cleavage by proteases involved in tissue homeostasis and remodelling. *Eur. J. Biochem.* 217:877–884.

Nüsslein-Volhard, C., and E. Wieschhaus. 1980. Mutations affecting segment number and polarity in *Drosophila*. *Nature.* 287:795–801.

Nüsslein-Volhard, C., H. Kluding, and G. Jürgens. 1995. Genes affecting the segmental subdivisions of the *Drosophila* embryo. *Cold Spring Harb. Symp. Quant. Biol.* 50:145–154.

Oohashi, T., M. Sugimoto, M.-G. Mattei, and Y. Ninomiya. 1994. Identification of a new collagen IV chain, a6(IV), by cDNA isolation and assignment of the gene to chromosome Xq22, which is the same locus for COL4A5. *J. Biol. Chem.* 269:7520–7526.

Pan, T.-C., M. Kluge, R.-Z. Zhang, U. Mayer, R. Timpl, and M.-L. Chu. 1993. Sequence of extracellular mouse protein BM-90/fibulin and its calcium-dependent binding to the other basement-membrane ligands. *Eur. J. Biochem.* 215:733–740.

Rauch, U., H. Meyer, C. Brakebusch, C. Seidenbecher, E.D. Gundelfinger, D.R. Beier, and R. Fässler. 1997. Sequence and chromosomal localization of the mouse brevican gene. *Genomics.* 44:15–21.

Retzler, C., H. Wiedemann, G. Kulbe, and U. Rauch. 1996. Structural and electron microscopic analysis of neurocan and recombinant neurocan fragments. *J. Biol. Chem.* 271:17107–17213.

Ruoslahti, E., and M.D. Pierschbacher. 1987. New perspectives in cell adhesion: RGD and integrins. *Science.* 238:491–497.

Sasaki, T., G. Kostka, W. Göhring, H. Wiedemann, K. Mann, M.L. Chu, and R. Timpl. 1995. Structural characterization of two variants of fibulin-1 that differ in nidogen affinity. *Mol. Biol.* 245:241–250.

Schäfer, G., V.D. Fitzpatrick, and M.X. Sliwkowski. 1997. γ-heregulin: a novel heregulin isoform that is an autocrine growth factor for the human breast cancer cell line, MDA-MB-175. *Oncogene.* 15:1385–1394.

Schwartz, M.A., M.D. Schaller, and M.H. Ginsberg. 1995. Integrins: emerging paradigms of signal transduction. *Annu. Rev. Cell Dev. Biol.* 11:549–599.

Shotton, D.M., B.E. Burke, and D. Branton. 1979. The molecular structure of human erythrocyte spectrin. Biophysical and electron microscopic studies. *J. Mol. Biol.* 131:303–429.

Spring, J., K. Beck, and R. Chiquet-Ehrismann. 1989. Two contrary functions of tenascin: dissection of the active sites by recombinant tenascin fragments. *Cell.* 59:325–334.

St. Johnston, D., and C. Nüsslein-Volhard. 1992. The origin of pattern and polarity in the *Drosophila* embryo. *Cell.* 68:201–219.

Tyler, J.M., and D. Branton. 1980. Rotary shadowing of extended molecules dried from glycerol. *J. Ultrastruct. Res.* 71:95–102.

Wang, X.Z., M. Kuroda, J. Sok, N. Batchvarova, R. Kimmel, P. Chung, H. Zinszner, and D. Ron. 1998. Identification of novel stress-induced genes downstream of *chop*. *EMBO (Eur. Mol. Biol. Organ.) J.* 17:3619–3630.

Weiss, A., and J. Schlessinger. 1998. Switching signals on or off by receptor dimerization. *Cell.* 94:277–280.

Whelan, J. 1996. Selectin synthesis and inflammation. *Trends Biochem. Sci.* 21: 65–69.

Yan, R., S. Small, C. Desplan, C.R. Dearolf, and J.E. Darnell, Jr. 1996. Identification of a Stat gene that functions in *Drosophila* development. *Cell.* 84: 421–430.

Zinszner, H., M. Kuroda, X. Wang, N. Batchvarova, R.T. Lightfoot, H. Remotti, J.L. Stevens, and D. Ron. 1998. CHOP is implicated in programmed cell death in response to impaired function of the endoplasmic reticulum. *Genes Dev.* 12:982–995.