

The Distribution of Tenascin-X Is Distinct and Often Reciprocal to That of Tenascin-C

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Abstract. We have isolated a cDNA encoding mouse tenascin-X (TN-X), a new member of the family of tenascin genes. The TN-X gene lies in the major histocompatibility complex (MHC) class III region, as it is the case for its human counterpart. On Northern blots we detected a TN-X mRNA of ~13 kb in most tissues analyzed, whereas in various mouse cell lines mRNAs of ~11 and 13 kb were detected, suggesting the possibility of alternative splicing of TN-X transcripts. We raised antibodies against mouse TN-X fragments expressed in bacteria and used these antibodies to identify the TN-X protein in heart cell extracts and in the conditioned medium of a renal carcinoma cell line. The subunit molecular size of TN-X is ~500 kD, suggesting that the protein may contain up to 40 fibronectin type III repeats, making it the largest tenascin family member known yet. TN-X in condi-

tioned medium, as well as the purified protein bind to heparin, but no binding to tenascin-C (TN-C), fibronectin, laminin or collagens could be detected. Thus the heparin-binding activity may be a common feature of the tenascins. The TN-X mRNA as well as the protein are predominantly expressed in heart and skeletal muscle, but the mRNA is found in most tissues at a low level. Immunostaining showed the protein to be associated with the extracellular matrix of the muscle tissues and with blood vessels in all of the tissues analyzed. Although the TN-X gene lies in the MHC class III locus, it is not expressed in the lymphoid organs analyzed, except for the staining around blood vessels. In skin and tissues of the digestive tract often a reciprocal distribution of TN-X and TN-C was observed.

THE tenascins are a growing family of extracellular matrix proteins. The first member termed tenascin attracted attention due to its prominent expression during tissue interactions in embryogenesis and its overexpression in many tumors (12; for reviews see 11, 20). Many important functions have been proposed for tenascin based on its tissue distribution and on experimental data from cell culture experiments. Thus, tenascin was suggested to be important in regulating cell-extracellular matrix interactions in a way to promote cell rounding, migration and/or differentiation. Examples are the development of the central and peripheral nervous system (for reviews see 6, 8) as well as many instances of tissue interactions during organogenesis and cell movements in embryogenesis (for reviews see 7-9, 18), and in adult life during wound healing (30, 50) or epithelial cell shedding in the gut (39).

All of these proposed functions have recently been questioned by the apparent well-being of mice lacking a func-

tional tenascin gene (42). Therefore, either tenascin is not required for the development of a mouse, or the absence of tenascin is compensated for. Since it is assumed that proteins which are expressed must have a function somewhere (4), it has recently been argued that other tenascin-like molecules could make up for the loss of tenascin in these knockout mice (3, 15, 19, 42). We therefore decided to investigate more closely other members of the family of tenascin-like genes, which are characterized by being built up of the same types of structural domains, such as EGF-like repeats, fibronectin type III repeats and a globular part homologous to fibrinogens. One of these family members codes for a protein specifically expressed in the central and peripheral nervous system, termed restrictin in the chicken (37, 40) and J1-160/180 in the rat (21). After the discovery of a third member, a tenascin-like gene present in the human major histocompatibility complex (MHC) class III locus (3, 23, 32, 33, 54), Erickson (19) and Bristow et al. (3) suggested

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1. *Abbreviations used in this paper:* CYP21, human steroid 21-hydroxylase; MHC, major histocompatibility complex; TN-C, tenascin-C; TN-R, tenascin-R; TN-X, tenascin-X.

to use a coherent nomenclature for this protein family: tenascin-C (TN-C; C for cytactin; 29) for the original tenascin, tenascin-R (TN-R) for restrictin and tenascin-X (TN-X) for the tenascin-like gene of the MHC class III locus, originally described as human gene X (35). Recently, evidence was provided for the existence of a fourth and probably more family members yet to be characterized (15).

Up to now the only knowledge about TN-X was from a partial human cDNA sequence (35) and from extensive sequencing of the genomic region encoding the human TN-X gene (3, 23, 32, 33). From the combined data it could be predicted that human TN-X is built up of an NH₂-terminal domain, followed by four heptad repeats, 18.5 tenascin-type EGF-like repeats, at least 29 fibronectin type III domains and a carboxy-terminal fibrinogen domain. Judged from Northern blot and RNase protection data TN-X mRNA was reported to be widely expressed with the highest expression in muscle and testis (3, 23).

Since nothing is known about the predicted protein encoded by the TN-X gene, we decided to study the TN-X protein in the mouse. We raised antibodies against recombinant mouse TN-X fragments, which were used to purify the authentic TN-X protein and to study its tissue distribution in comparison to other extracellular matrix proteins.

Materials and Methods

Cell Lines

The mouse tumor cells, 203 glioma, Ren-Ca (renal carcinoma), RSV-M (glioma), and Colon (colon carcinoma), were generously provided by Dr. Yagita (Jyutendou University, Japan), and two tumor cells, NFS-Y83 (fibrosarcoma) and Sq-1979 (planoepithelial carcinoma) were kindly provided by Dr. Ando (Natl. Inst. Radiol. Sci., Japan) through the RIKEN Cell Bank (Tsukuba, Japan). The other cells were obtained directly from the RIKEN Cell Bank (Tsukuba, Japan). 2H6GR is a mammary tumor cell line established in our laboratory (42). The culture medium for all cells was Dulbecco's medium (DME; GIBCO BRL, Gaithersburg, MD) containing 10% fetal calf serum (FCS; GIBCO BRL) and the cells were grown at 37°C in a humidified atmosphere and 6% CO₂.

cDNA Library Screening and DNA Sequencing

For the cloning of a short fragment encoding mouse TN-X, reverse transcriptase/PCR (RT/PCR) reactions were performed using 10 µg of total RNA from thymus of postnatal day 32 (P32) C3H/HeN mice (Charles River, Yokohama, Japan). The RT/PCR primers corresponded to sequences within the fibrinogen domain of the human TN-X gene. The 5' primer consisted of the nucleotides at position 1849 to 1875 and the 3' primer from 2406 to 2430 according to the sequence presented by (35). The corresponding sequences of the mouse TN-X are marked in Fig. 1 at nucleotide positions 1969 and 2550. The resulting 550-bp fragment was cloned into EcoRV digested plasmid vector pBluescript II pKS(-) (Stratagene, La Jolla, CA), and the nucleotide sequence was determined with the Sequenase Version 2.0 Kit (United States Biochemical Corp., Cleveland, OH). This clone was termed pG40. Subsequently, a λt11 cDNA library from adult Balb/c mouse heart (Clontech, Palo Alto, CA) was screened with digoxigenin-dUTP (Boehringer Mannheim, Mannheim, Germany) labeled pG40. Several cDNA clones encompassing the carboxy terminus of mouse TN-X were recovered and their nucleotide sequences were determined. DNA sequence data were analyzed by the Genetics Computer Group (GCG) program version 7.2. Searches for sequence homology were performed through Genbank data base (release 76.0, 1993). Sequence comparisons were performed on a UNIX computer using the program align in FLAT.

Northern Blot Analysis

Total RNA from various tissues of embryonic day 17 (E17), postnatal day 6 (P6) and adult mice, as well as from various tumor cells was isolated as

described (16). 10 µg of total RNA was separated on a 1% agarose/2.2 M formaldehyde-denaturing gel and transferred to Gene Screen membranes (Dupont NEN, Boston, MA). The mouse TN-X pG40 and a 2.5-kb TN-C probe were used for hybridization (41). DNA probes were labeled with random primers by the DNA polymerase Klenow fragment (Amersham International, Buckinghamshire, England).

Production of Fusion Proteins in *E. coli* and Generation of Polyclonal Antibodies

Two different constructs containing portions of mouse TN-X were made by subcloning PCR-generated fragments into the pGEX-1 or -2 vectors (Pharmacia, Piscataway, NJ) in correct translational reading frame (46). The vector-insertion junctions were sequenced to confirm correct expression of the glutathione S-transferase fusion proteins. After induction and lysis of the bacteria, fusion proteins were extracted using a series of rising urea concentration (52). The fusion proteins are designated as follows. The GST-GE800 fusion protein is derived from pGE800 and spans three fibronectin type III repeats at nucleotides 266-1062 (see Figs. 1 and 2). The GST-G40 fusion protein is derived from pG40 and spans the fibrinogen domain at nucleotides 1969-2550. The pGE800 clone was in addition expressed as a fusion protein with a truncated β-galactosidase as described (52) by modification of the commercially available pEX-1 (Genofit) (48) resulting in the fusion protein β-gal-GE800.

Polyclonal antibodies were prepared in chickens against the murine TN-X fusion proteins. The fusion proteins (200 µg per chicken) emulsified in complete Freund's adjuvant were injected into the breast muscles. After 3 wk, the chickens were boosted with 200 µg of TN-X fusion proteins emulsified in incomplete Freund's adjuvant. Antibodies were isolated from the egg yolk using sequential polyethylene glycol precipitations (22) resulting in the antibody preparations pAb40 and pAb800. Antibody pAb800 (raised against GST-GE800) was affinity purified using β-gal-GE800 coupled to CNBr-activated Sepharose 4B (Pharmacia). The monospecific antibodies were eluted using 0.1 M glycine/HCl, pH 2.6, and the eluate was immediately neutralized.

Western Blot Analysis

One adult mouse heart was homogenized in 5 ml of 10 mM Tris-HCl, pH 7.5 containing leupeptin (0.5 µg/ml), pepstatin (1 µg/ml), aprotinin (5 U/µl), and 0.25 mM phenylmethylsulfonyl fluoride (Sigma Chemical Co., St. Louis, MO) as protease inhibitors. Homogenates were shaken gently for 2 h at 4°C, then centrifuged for 15 min at 8,000 rpm, the pellet resuspended in 20 mM 3-cyclohexylamino-l-propanesulfonic acid (CAPS), pH 11.0, 150 mM NaCl, 10 mM EDTA containing protease inhibitors. The suspension was extracted for 2 h at 4°C with shaking and centrifuged for 15 min at 8,000 rpm and the pellet and supernatant were subject to 5% SDS-PAGE under reducing conditions. Proteins were transferred to Immobilon-P transfer membranes (Millipore Corp., Bedford, MA) and immunoblotting was performed essentially as described (26), with the exception that a chemiluminescence detection kit (ECL, Amersham) was used to develop the filter. As secondary antibody, a rabbit anti-chicken horseradish peroxidase-labeled antibody (Cappel Laboratories, Malvern, PA) was used.

Immunoprecipitation

Ren-Ca cells were grown to 80% confluence in DME/10% FCS. The conditioned medium was removed and replaced with 10% FCS containing DME without methionine and cysteine containing 100 µCi/ml Trans ³⁵S-label (ICN, Costa Mesa, CA). Cells were incubated for 24 h at 37°C, before harvesting the conditioned medium. Subsequently, immunoprecipitations of the conditioned medium using 2 µl of anti-mouse TN-X antibody (pAb800) per 150 µl of medium were performed and analyzed as described (26) using rabbit anti-chicken IgG (Cappel Laboratories) as secondary antibody.

Purification of Murine TN-X from Ren-Ca Cells and Binding Studies

Affinity purified anti-mouse TN-X antibody pAb800 (1.5 mg) was coupled to 0.5 g of CNBr-activated Sepharose 4B (Pharmacia) according to the manufacturer's instructions. Conditioned medium from 1 l of confluent Ren-Ca cells was precipitated with 50% ammonium sulfate by stirring for 1 h at 4°C. After centrifugation for 20 min at 10,000 rpm, the pellet was dissolved in 20 ml of PBS and dialyzed overnight against PBS at 4°C. The concentrated sample was centrifuged and passed over a gelatin-agarose

column (Sigma Chemical Co.). The flow-through was subsequently adsorbed to the pAb800-sepharose column. The pAb800 affinity column was washed extensively with PBS before eluting the mouse TN-X with 50 mM triethylamine, pH 11.0. The proteins (mostly fibronectin) bound to the gelatin-agarose column were eluted with 4 M urea. The fractions containing the 500-kD TN-X were analyzed by 5% SDS-PAGE under reducing conditions and visualized by silver staining (53).

The binding of TN-X to other extracellular matrix proteins was done using solid phase binding assays. ELISA plates were coated with the following proteins at 100 µg/ml: fibronectin (purified from bovine serum by gelatin affinity chromatography), laminin (GIBCO BRL) and acid soluble collagens type I and IV (Sigma Chemical Co.) in PBS. After blocking and washing the coated plates, they were incubated with Ren-Ca cell-conditioned medium. The binding assay was performed as described for the binding of TN-C in conditioned medium to fibronectin coated plates (14). For the detection of bound TN-X pAb800 was used.

For the heparin-binding studies, a column of 0.5 ml of heparin-agarose (Sigma Chemical Co.) was used. The column was equilibrated in PBS and either 500 µl of ReCa cell conditioned medium or 100 µl of purified TN-X in PBS (5 µg/ml) was loaded. The column was subsequently washed five times with 500 µl of PBS and then eluted with three times 500 µl of 1 M NaCl in PBS. 500-µl fractions were always collected and dilution series of each fraction were coated on ELISA plates to quantitate the TN-X present in each fraction using the same procedure as we previously described for the quantitation of TN-C (38) with the exception of using pAb800 for the detection of TN-X. The absorbances measured and presented in Fig. 8 represent relative quantities of TN-X found in the fractions analyzed.

Immunofluorescence

Immunofluorescence was carried out as follows. E15.5 embryos, and various tissues of P0, and adult (P32) mice were embedded in Tissue-Tek O.C.T. compound (Miles Inc., Kankakee, IL) and frozen. Sections of 12 µm were cut at -20°C and collected on gelatin-coated slides. Sections were incubated for 2 h at room temperature with anti-TN-X (pAb800), anti-TN-C (12), anti-fibronectin (17), or anti-laminin (GIBCO BRL), diluted 200-fold in PBS containing 0.1% bovine serum albumin (BSA). The sections were washed with PBS/0.1% BSA and then incubated for 2 h with rabbit anti-chicken FITC-labeled IgG (Cappel Laboratories), rabbit anti-chicken RITC-labeled IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA), or goat anti-rabbit RITC-labeled IgG (Cappel Laboratories) diluted 500-fold in PBS/0.1% BSA.

Results

Cloning of a Mouse TN-X cDNA

Previously, human TN-X has been shown to be located within ~70 kb centromeric of human steroid 21-hydroxylase (CYP21) in the MHC class III region (3, 23, 32, 33, 35). Since many genes located in MHC loci are known to have important roles in the immune response, we considered that murine TN-X might be expressed in lymphoid organs. Using total RNA from adult murine thymus, we performed RT/PCR reactions with two primers derived from the human TN-X fibrinogen domain. This resulted in a major product of ~550 bp, the size expected from the known human TN-X sequence. This fragment was subcloned and sequenced. The sequence of this clone named pG40 (see Fig. 1) corresponds to nucleotide position 1969–2550 in Fig. 2.

To obtain longer cDNAs of murine TN-X, pG40 was used as probe to screen an adult mouse heart cDNA library. Three overlapping clones encoding the 860 carboxy-terminal amino acids and the 3' untranslated region of murine TN-X were identified. A schematic representation of the TN-X cDNA clones obtained is shown in Fig. 1 and the nucleotide sequence and the predicted amino acid sequence is presented in Fig. 2. The DNA sequence encodes 7 fibronectin type III repeats followed by a fibrinogen domain. A poly (A) addition

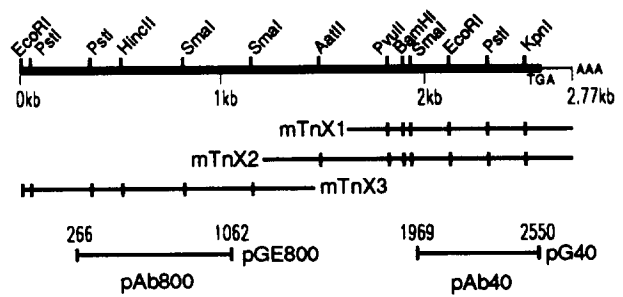


Figure 1. Schematic representation of the TN-X cDNA clones. On the top the restriction enzyme map of the 2.77 kb of cDNA sequences covered by the cDNA clones mTnX1, mTnX2, and mTnX3 (shown below) is drawn. The coding sequence ends at the stop codon TGA as indicated and is followed by a short 3' noncoding region terminating in a stretch of poly A (AAA). The cDNA regions subcloned to produce recombinant fusion proteins are indicated (pGE800 and pG40). They were used to produce the respective antibody preparations pAb800 and pAb40.

signal (AATAAA) was found at nucleotide 2737–2742 followed by a short poly (A) tail at position 2771. This indicates that the clones may cover the complete 3' untranslated region of the TN-X transcript. The comparison of this mouse sequence with the sequence of human TN-X (3) revealed an 87.1% similarity at the amino acid level (Fig. 3), indicating that our cDNAs indeed encode the murine counterpart of human TN-X.

The homology of human and mouse TN-X was further substantiated by the homologous genomic location of their genes. In humans, the TN-X gene lies on the opposite strand of the steroid 21-hydroxylase (CYP21) gene, and its last exon overlaps the 3' untranslated region of CYP21 with opposite polarity (3, 32, 33). In the vicinity of CYP21, there is a pseudogene for CYP21 known as CYP21P (51) as well as two genes for complement C4. The gene order is CYP21 (active)–C4B–CYP21P(pseudogene)–C4A, arranged from centromere towards telomere (47). The 3' untranslated region of CYP21P also contains a sequence which is complementary to the 3' portion of a truncated TN-X gene referred to as XA (23). In the murine MHC class III region (S region of the H-2 complex, locus site 18.7–18.9 in chromosome 17), there is also an active and a pseudogene for the steroid 21-hydroxylase (Cyp21), but the relative position of the active and pseudogenes is different from that of human (36). The order is Cyp21ps (pseudogene)–C4–Cyp21 (active)–Slp (sex-limited protein variant of C4). To determine whether the mouse TN-X cDNA also arises from the opposite strand of the Cyp21ps or Cyp21 gene analogous to the human TN-X, we compared the 3' sequence of mouse TN-X with the known Cyp21ps and Cyp21 gene sequences (Fig. 4). The 3' region of murine TN-X shows high similarity to cCyp21ps (complementary sequence of the 3' untranslated region of murine Cyp21ps) with 96.8% identity at the nucleotide level, and it contains a Cyp21ps-specific 30-bp insertion at nucleotide position 2614–2643 of TN-X. In contrast the same region of mouse TN-X shows only 93.6% identity to cCyp21 (complementary sequence of the 3' untranslated region of murine Cyp21). Thus, the mouse TN-X gene also appears to be located in the MHC class III locus. At present, we can


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1 EFDSFLVQYKNRGGQVQVVAADQREVTTISSEPNRKYRFLLPGLIGLKRGLPVSVBGTTPAVKTPQPRGLGELSVTEETSMSHLSWSVAQGFDFSL
2764 EFDSFVVQYKDKDGRQLQVVAANQREVTVQALEPSRKYRFLLYGLSGRKRGLPISADSTTAPLEKELPPLHGLTVABETSSSLRLSWTVAQGFDFSL

101 VQYKDRDQGLQTVSLAADQREVTTISSEPNRKYRFLLPGLIGLKRGLPVSVBGTTPAVKTPQPRGLGELSVTEETSMSHLSWSVAQGFDFSL
2864 VQYKDRDQGLQTVSLAADQREVTVQALEPSRKYRFLLYGLSGRKRGLPISADSTTAPLEKELPPLHGLTVABETSSSLRLSWTVAQGFDFSL

197 GPFDSFVVQYKDRDQGLQTVSLAADQREVTTISSEPNRKYRFLLPGLIGLKRGLPVSVBGTTPAVKTPQPRGLGELSVTEETSMSHLSWSVAQGFDFSL
2964 GPFDSFVVQYKDRDQGLQTVSLAADQREVTVQALEPSRKYRFLLYGLSGRKRGLPISADSTTAPLEKELPPLHGLTVABETSSSLRLSWTVAQGFDFSL

297 DFDSFLVQYKNRGGQVQVVAADQREVTTISSEPNRKYRFLLPGLIGLKRGLPVSVBGTTPAVKTPQPRGLGELSVTEETSMSHLSWSVAQGFDFSL
3064 DFDSFLVQYKNRGGQVQVVAADQREVTVQALEPSRKYRFLLYGLSGRKRGLPISADSTTAPLEKELPPLHGLTVABETSSSLRLSWTVAQGFDFSL

397 SRVDSFKISYQLADGGEPQSVQVQDGTQTQILQGLIPDTRIEYTVVSVRGEFEESEPLTGFLTTPVDPGPTQLRALNLTGSGALLHWKPPHKFVDRKYDVEVE
3164 SRVDSFKISYQLADGGEPQSVQVQDGTQTQILQGLIPDTRIEYTVVSVRGEFEESEPLTGFLTTPVDPGPTQLRALNLTGSGALLHWKPPHKFVDRKYDVEVE

497 SPGAPPLQASAPGSAVDYPLTDLALDNTYATVVRGLRGNFTSPASITFTTGLKPPQDLEAKEVTPRTALLTWTEPEVPTGYLLSFDTPGGQIQEILLP
3264 SPGAPPLQASAPGSAVDYPLTDLALDNTYATVVRGLRGNFTSPASITFTTGLKPPQDLEAKEVTPRTALLTWTEPEVPTGYLLSFDTPGGQIQEILLP

597 AGTTSRLLRLFPSTFYSAQLRAIWGESLTPPVLTSFTTGGQRIFFPRDCGEEELKNGPASKTTTIFLNGNRERPLDVFCDMETDGGGMLVFPQRMDGQT
3364 AGTTSRLLRLFPSTFYSAQLRAIWGESLTPPVLTSFTTGGQRIFFPRDCGEEELKNGPASKTTTIFLNGNRERPLDVFCDMETDGGGMLVFPQRMDGQT

697 DFWRDWEYAHGFGNISGEFPLGNEALHSLTQAGDYSIRVLDLRAGDRAVFAQYDFFRVDSSAKENYRHLHLEGYHGTAGDSMSYHSGSVFSARDRDPNLLI
3464 DFWRDWEYAHGFGNISGEFPLGNEALHSLTQAGDYSIRVLDLRAGDRAVFAQYDFFRVDSSAKENYRHLHLEGYHGTAGDSMSYHSGSVFSARDRDPNLLI

797 SCAVSYRGAWYRNCHYANLNGLYGSTVDHQGVSWYHWKGFESVFPFTEMKLRPRNFQVPTRGT
3564 SCAVSYRGAWYRNCHYANLNGLYGSTVDHQGVSWYHWKGFESVFPFTEMKLRPRNFQVPTRGT

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Figure 3. Sequence alignment of the carboxy-terminal region of mouse (upper sequence) and human TN-X (lower sequence). Amino acid sequences were aligned using the GCG program bestfit. Vertical bars represent identical residues and dots indicate conservative substitutions. The numbering of mouse TN-X is from Fig. 1 and the sequence and numbering of human TN-X is taken from (3), except for the inclusion of a glutamic acid at position 3359, which was present in a cDNA clone of TN-X previously reported by the same authors (35). Dashes in human TN-X indicate insertions to maximize homology.

mRNA. This confirms previous results on TN-C expression (41).

In contrast to the expression in tissues, which showed a single mRNA band around 13 kb, there existed two distinct transcripts of TN-X in some tumor cells (Fig. 6 A), namely, a ~13- and an 11-kb transcript. The large form was especially prominent in mouse glioma 203 and renal carcinoma Ren-Ca cells, whereas the small form was found in MethA sarcoma and RSV-M glioma. For comparison the expression pattern of TN-C is shown in Fig. 6 B, confirming our previous data (41). No correlation between the expression levels or splicing patterns of TN-X and TN-C, respectively, can be observed in the cell lines analyzed.

Identification and Purification of the TN-X Protein

To identify the presence of TN-X protein in mice, Western blot analysis of crude homogenates of mouse adult heart was carried out using the polyclonal antibody pAb40 prepared against a GST-TN-X fusion protein. On immunoblots, the antibodies recognized a band of ~500 kD in the pellet, but

not in the supernatant following CAPS extraction (Fig. 7 A). This molecular size correlates well with the predicted protein subunit size based on the length of the TN-X mRNA. This result indicated that TN-X is present in the heart, but not extractable without denaturing and reducing agents. We thus attempted to use cultured murine cells to purify TN-X protein.

Based on the presence of TN-X mRNA on Northern blots (Fig. 6 A), renal carcinoma (Ren-Ca) cells were considered as a source for the purification. The release of TN-X by these cells into the conditioned medium was explored by immunoprecipitation analysis with affinity-purified TN-X antibody pAb800. This antibody precipitated from medium of metabolically labeled Ren-Ca cells a single 500-kD molecule (Fig. 7 A). To isolate TN-X from conditioned medium of unlabeled cells, we prepared an affinity column coupled with anti-TN-X antibody pAb800. This column allowed us to purify a single molecule migrating at ~500 kD (Fig. 7 B), consistent with the immunoblotting and immunoprecipitation results. When the purified TN-X was run under nonreducing conditions it did not enter the gel (not shown), suggesting

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cCyp21ps 2892 GGAGTGAAGCTGGTACCACTGGAGGGGTACCACTGCAAGCTTCGAGTCTCTGTGCCGTTTCACGCAAAATGAAGCTTCCGACCGGCAAAAATTCACAGGT
TN-X 2482 GGAGTGAAGCTGGTACCACTGGAGGG .....GCTTCGAGTCTCTGTGCCGTTTCACGCAAAATGAAGCT. .GGAGCC. CAG. AAAGTTCACAGGT
cCyp21 3038 GGAGTGAAGCTGGTACCACTGGAGGG .....GCTTCGAGTCTCTGTGCCGTTTCACGCAAAATGAAGCT. .CGGTCC. CAG. AAAGTTCACAGG

2792 .TCCACAGGGGACCTGAGCCTGCTGCCACCTCACTCACACCCCGGTAGTCTGCCGAGCACTGAGGGGTGTGCCAGAGAAGAGCCAGCAATGCCAC
2565 CCCCACAGGGGACCTGAGCCTGCTGCCACCTCACTCACACCCCGGTAGTCTGCCGAGCACTGAGGGGTGTGCCAGAGAAGAGCCAGCAATGCCAC
2955 CCCCACAGGGG. CACTGAGCCTGCTGCCACCTCACTCACACCCCGGTAGTCTGCCGAGCACTGAGGGGTGTGCCAGAGAAGAGCCAGCAATGCCAC

2693 ACCAAGGAGCCCTCTCTGCCACAATCTCACAGCACCACTTTTACAGGGGGGGAGGGGAGGGGAAATGGAGCAATAAAGGAGAAACTTGAGGCACCT 2598
2665 ACCAAGGAGCCCTCTCTGCCACAATCTCACAGCACCACTTTTACAGGGGGGGAGGGGAGGGGAAATGGAGCAATAAAGGAGAAACTTGAGGCACCT 2759
2886 ACCGAGGAAACCTCTCTGCCACAATCTCACAGCACCACTTTTAC. .GGGGGGGGGGGGGAACT. . .CAAATAAGGAGAAAC. TTTA. .AAG 2800

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Figure 4. Comparison of the 3' region of the mouse TN-X sequence with complementary sequences of the 3' untranslated region of mouse genomic Cyp21ps (cCyp21ps) and Cyp21 (cCyp21). The complementary sequences of Cyp21ps at nucleotide position 2598–2892 (available from EMBL/GenBank/DBJ under accession number M15008) and of

Cyp21 at 2800 to 3038 (accession number M15009) are shown and compared with the mouse TN-X sequence from nucleotide position 2482–2759. Vertical lines between nucleotide sequences indicate identity between sequences. Dots in cCyp21 and cCyp21 are inserted to maximize homology to TN-X. The termination codon at 2581–2583 and polyadenylation signal at 2737–2742 of TN-X is shown with bold letters or underlined, respectively.

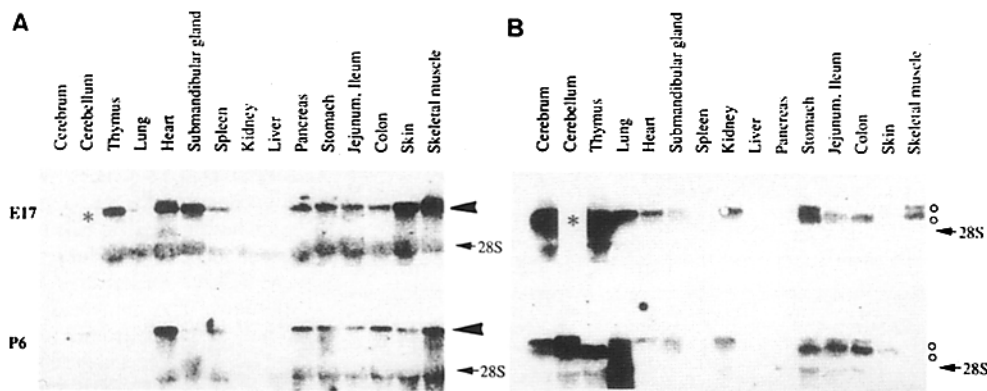


Figure 5. Tissue distribution of TN-X transcripts in comparison to TN-C. Total RNA was prepared from various tissues of E17 and P6 mice. Samples were subjected to RNA blotting. (A) The filter was probed with the TN-X pG40. A ~13-kb band indicated with an arrowhead is observed in most tissues. The position of the 28S rRNA is indicated. (B) After removal of the TN-X probe the same membrane was probed for TN-C mRNA. The 7- and 5.5-kb TN-C tran-

scripts are indicated with open circles. Cross-hybridization of the 550-bp TN-X probe with rRNAs was reproducibly higher than that of TN-C. This may relate to our previous finding that the G+C% level of TN-X is higher than that of TN-C (28). The star in the cerebellum lane of E17 indicates that no RNA was loaded.

that the protein is a disulfide-linked oligomer like all other known members of the tenascin family. Taking these data together, we conclude that the 500-kD protein is indeed the subunit of TN-X and that it is secreted into the conditioned medium of Ren-Ca cells.

TN-X Is a Heparin-binding Protein

It is known that TN-C interacts with other extracellular matrix components, most notably with TN-R (40), fibronectin (13, 14), heparin (7, 31) and proteoglycans (5, 27). We therefore tested whether TN-X also interacts with other components of the extracellular matrix. We examined the binding of TN-X present in conditioned medium of Ren-Ca cells in solid phase binding assays to TN-C, fibronectin, laminin, and collagens type I and IV, but could not detect any significant binding to any of these proteins (not shown). Next we investigated whether TN-X binds to heparin. In the first experiment, conditioned medium of Ren-Ca cells was passed over a heparin-agarose column. The flow through, the wash, and the high salt eluate fractions were collected and analyzed

by ELISA for the presence of TN-X, revealing that most of the TN-X present in the loaded conditioned medium was retained by the column and could be eluted by 1 M NaCl (Fig. 8). Since this binding could have been indirect, we also tested whether the purified TN-X binds to heparin-agarose. The same experiment was repeated by loading purified TN-X in PBS onto the heparin-agarose column. Again most of the TN-X was retained by the column and could be eluted by high salt (Fig. 8). Therefore, the heparin-binding activity appears to be shared by both TN-C and TN-X.

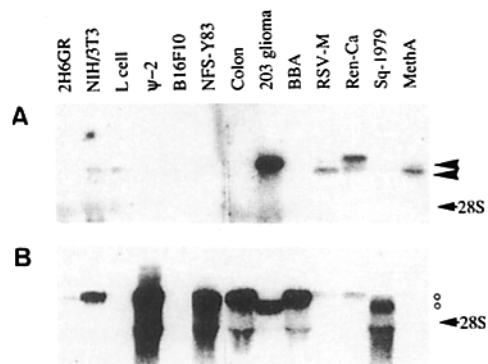


Figure 6. Detection of TN-X transcripts in mouse cell lines. (A) Total RNAs from various cells were subjected to RNA blotting as described. The blot was hybridized with pG40. The TN-X transcripts of 13 and 11 kb are indicated with arrowheads. (B) Expression of mouse TN-C was investigated using the same membrane after removal of the TN-X probe. The 7- and 5.5-kb TN-C transcripts are indicated with open circles. Position of 28S rRNA is indicated.

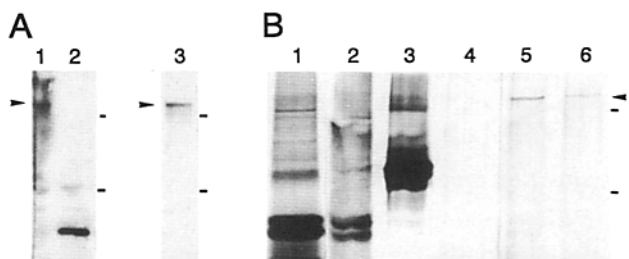


Figure 7. Identification and purification of the mouse TN-X protein. (A) Immunological detection of TN-X by Western blot analysis (lanes 1 and 2) and by immunoprecipitation (lane 3). Adult heart homogenates were used for the immunoblots using the TN-X-specific antibody pAb40. After CAPS (pH 11.0) extraction, the pellet (lane 1) and supernatant (lane 2) were resolved by SDS-PAGE, blotted onto a membrane, and immunostained with pAb40. 20 μ g of protein was loaded in each lane. A ~500-kD band, indicated with an arrowhead can be detected in the pellet fraction. Immunoprecipitation of TN-X from conditioned medium of metabolically labeled renal carcinoma (Ren-Ca) cells using TN-X specific antibody pAb800 revealed a 500-kD band indicated by an arrowhead (lane 3). (B) A silver stained gel shows the affinity purification of TN-X using immobilized pAb800. Lane 1 represents the crude conditioned medium loaded sequentially onto a gelatin agarose and pAb800 column; lane 2 represents the flow through; lane 3 is the eluate from the gelatin-agarose column (mostly fibronectin); lanes 4-6 are three eluate fractions of the pAb800 column and represent the 500-kD subunit of the purified TN-X protein indicated by an arrowhead. Molecular size markers for 450 (non-reduced fibronectin dimer) and 200 (myosin) kD are indicated with bars.

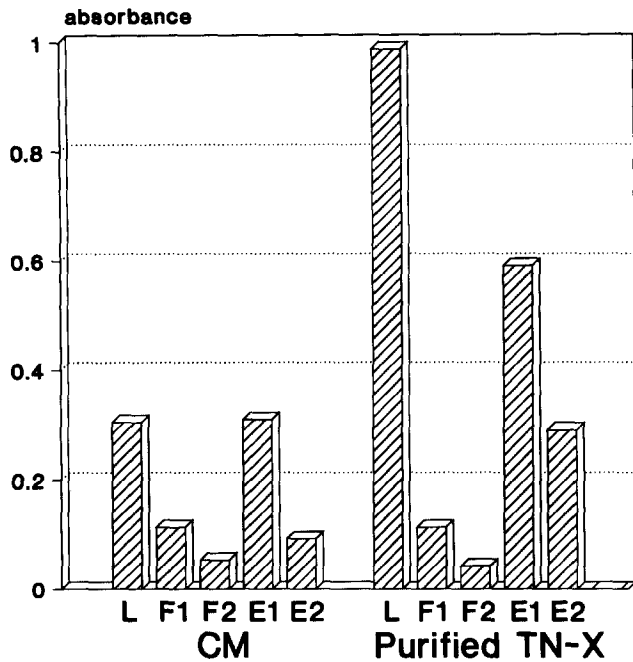


Figure 8. TN-X binds to heparin. Conditioned medium (CM) of ReCa cells was passed over a heparin-agarose column and the TN-X present in the loaded medium (L), the flow through and wash fractions (F1, F2), and the eluate fractions (E1, E2) was determined by ELISA, showing that most of the TN-X loaded was retained by the column and was recovered in the eluate using 1 M NaCl. The same type of experiment was performed using purified TN-X and the amount of TN-X loaded (L), present in the flow through fractions (F1, F2) and the eluate fractions (E1, E2) determined by ELISA. Again most of the TN-X was quantitatively retained by the heparin column and could be recovered in the 1 M NaCl eluate.

Tissue Distribution of TN-X Protein

The distribution of TN-X protein was examined in various tissues and compared with other well-characterized extracellular matrix proteins. In brain, lung, and mammary gland we could not find any significant staining. Serial cryosections of mouse heart and skeletal muscle of E15.5, P0, and adult mice were stained by indirect immunofluorescence with preimmune IgG, anti-TN-X (pAb800), anti-TN-C, anti-fibronectin, or anti-laminin antibodies. Preimmune IgG did not show any staining (data not shown). TN-X was present at all stages examined along skeletal muscle fibers (Fig. 9, *d* and *f*) and cardiac muscle cells (Fig. 9, *b* and *h*), typical for extracellular matrix staining. Fibronectin and laminin were abundant in a fine fibrillar distribution, in contrast to TN-C which could not be detected in the heart and skeletal muscle at any developmental stage examined (data not shown).

Interestingly, in skin and digestive tract, especially esophagus, gut, and stomach, the distributions of TN-X and TN-C were shown to be distinct and nearly reciprocal (Fig. 10). For instance, in E15.5 esophagus TN-X was abundant within the lamina propria (Fig. 10 *g*), whereas TN-C was prominent in the surrounding smooth muscle (Fig. 10 *m*). In gut and stomach, TN-X was most prominent in the mesenchyme surrounding smooth muscle (Fig. 10, *i*, *j*, and *k*), while TN-C was mainly expressed within the smooth muscle and in the dense mesenchyme adjacent to the epithelium (Fig. 10, *p* and *q*). In skin, TN-X was abundant throughout the dermis and the staining is fading towards the epidermis (Fig. 10 *l*), whereas TN-C was most intensely stained adjacent to the epidermis and the staining is fading out into the dermis (Fig. 10 *r*). It is interesting to note that the TN-C staining in E15.5 esophagus is disappearing until P0 and the TN-C expression in the gut is induced in the same time

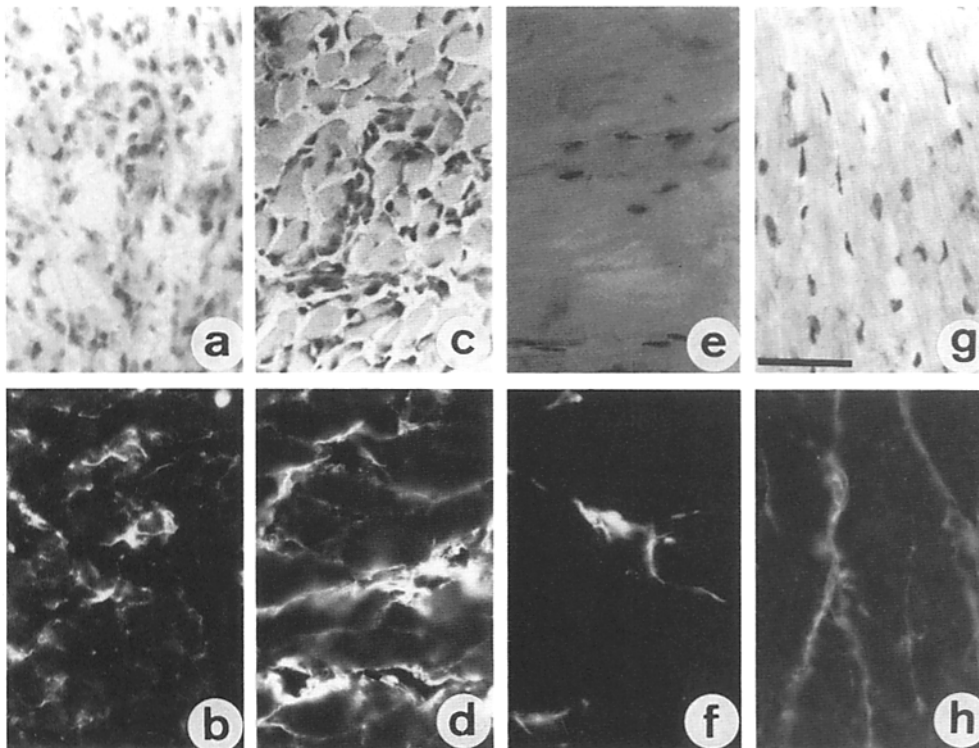


Figure 9. Distribution of TN-X in murine heart and skeletal muscle. Sections of adult heart (*g* and *h*), adult skeletal muscle (*e* and *f*), E15.5 fetal heart (*a* and *b*), and P0 skeletal muscle (*c* and *d*) were stained with hematoxylin and eosin (*a*, *c*, *e*, and *g*), TN-X specific antibody pAb800 (*b*, *d*, *f*, and *h*). Bar, 50 μ m.

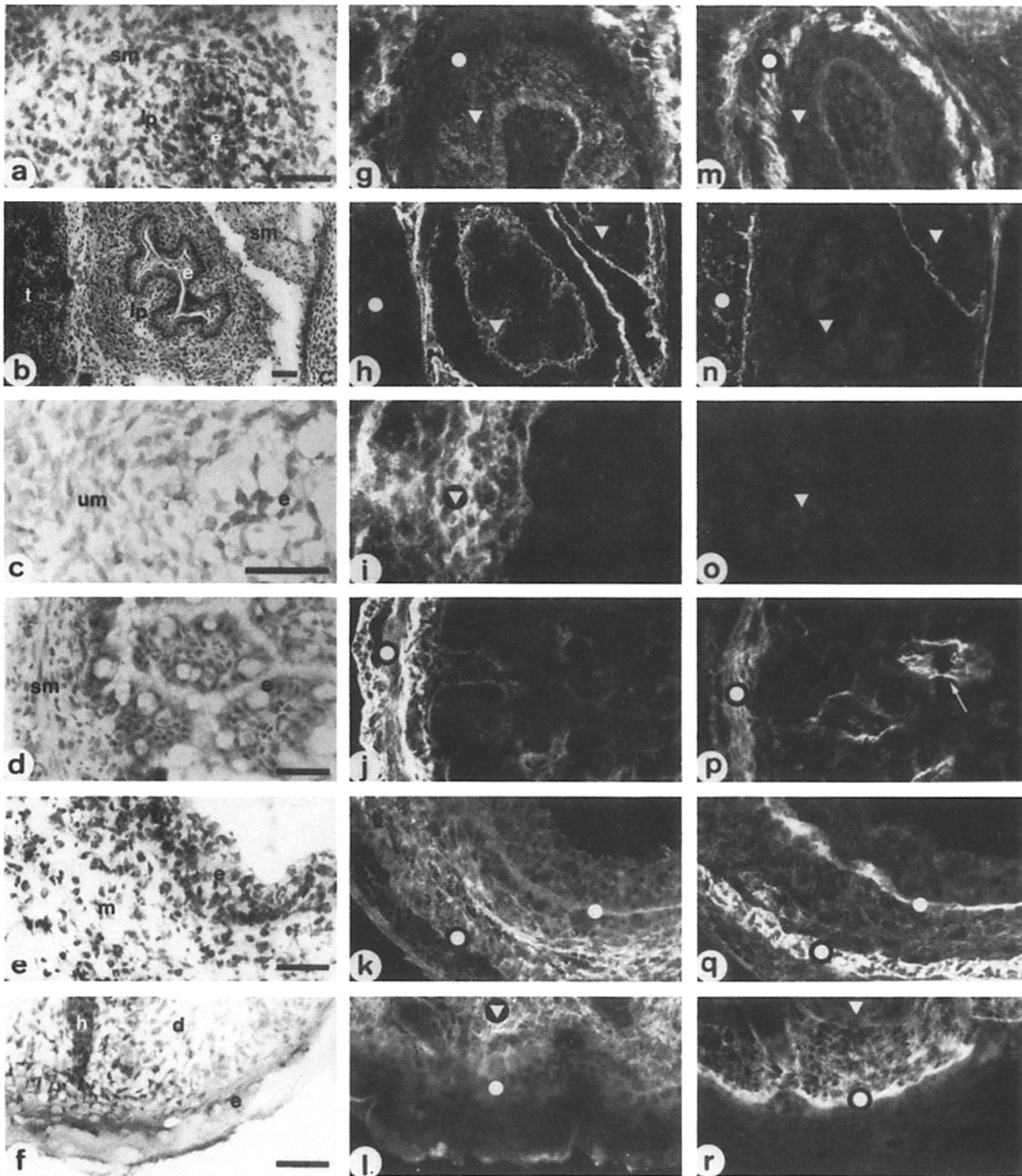


Figure 10. Reciprocal staining patterns for TN-X and TN-C in the digestive tract and skin. Sequential sections of E15.5 (*a*, *g*, and *m*) and P0 (*b*, *h*, and *n*) esophagus; E15.5 (*c*, *i*, and *o*) and P0 (*d*, *j*, and *p*) gut; E15.5 stomach (*e*, *k*, and *q*) and skin (*f*, *l*, and *r*) were stained with hematoxylin and eosin (*a-f*), TN-X antibody pAb800 (*g-l*) or anti-TN-C antibody (*m-r*). Triangles indicate areas where TN-X is prominently expressed but not TN-C, whereas circles mark areas of strong TN-C but not TN-X expression. An arrow in *p* shows primitive villi that consist of the mesenchymal core (lamina propria), which is covered by epithelial cells. In contrast to TN-X, TN-C is predominantly found in the lamina propria, as previously shown by *l*. *c*, cartilage; *e*, epithelium; *d*, dermis; *h*, hair follicle; *lp*, lamina propria; *m*, mesenchyme; *sm* in *b*, skeletal muscle; *sm* in *d*, smooth muscle; *t*, thymus; *um*, undifferentiated mesenchyme. Bar, 50 μ m.

period, whereas TN-X in the same organs remains constant during this period of development.

To determine whether TN-X is produced in lymphoid cells such as T and B lymphocytes, immunofluorescence of Peyer's patches of the ileum and of spleen was performed. Peyer's patches are composed of densely packed lymphocytes (mainly B lymphocytes) that differentiate into plasma cells upon appropriate antigenic stimulation. TN-X was exclusively colocalized with capillaries in the nodules and thus is unlikely to be produced by lymphocytes (Fig. 11). This staining pattern was similar to the other extracellular matrix proteins fibronectin and laminin (not shown). In the spleen as well, TN-X was only prominent around the central artery and other arterioles and veins, but not within the tissue containing T- and B-lymphocytes (not shown). These results indicate that TN-X is not produced in lymphoid cells.

Also in other organs such as the liver and the kidney TN-X was found associated with blood vessels such as arteries, arterioles, veins, and capillaries (Fig. 11). In the liver, the wall of the central vein, consisting of endothelial cells supported by a sparse population of collagen fibers, was intensely stained by anti-TN-X antibody (Fig. 11 *b*). On the contrary, fibronectin and laminin were found in all liver plate regions and very little staining with anti-TN-C antibody was observed in the adult liver (not shown). In the kidney, TN-X was detected in arteries (not shown). The association of TN-X with blood vessels in general may explain the nearly ubiquitous distribution observed for the TN-X mRNA.

Discussion

This paper represents the first study of the TN-X protein. The purified mouse TN-X subunit has an apparent molecular size of ~500 kD. Assuming that mouse TN-X contains a homologous NH₂-terminal domain and the same number of EGF-like repeats as human TN-X, we can predict, after subtraction of the size of these domains and of the fibrinogen globe, a remainder of ~400 kD representing the fibronectin type III repeats. Assuming an average of 10–11 kD per fibronectin type III repeat we speculate that TN-X may con-

tain up to 36 or 40 fibronectin type III repeats. This is consistent with the predicted protein size calculated from a 13 kb or larger mRNA. In the case of the human TN-X gene, 29 fibronectin type III repeats have already been identified, but it could not be excluded that more of them might be hidden in the gene stretches that have not been sequenced yet (3). Because of the presence of heptad repeats and cysteines in the NH₂-terminal region of the human TN-X gene (3) and due to the fact that our purified TN-X protein did not enter a gel without reduction, we assume that it is forming disulfide-linked oligomers as do all other tenascin family members. Whether TN-X is forming trimers like TN-R, hexamers like TN-C, or any other type of oligomers, remains to be determined.

The cDNA of mouse TN-X that we have isolated encodes the 3' non-coding region of the RNA as well as the fibrinogen globe and the seven most COOH-terminal fibronectin type III repeats. This sequence shows an 87.1% similarity on the amino acid level to its human counterpart (3). Since the most COOH-terminal three fibronectin type III repeats of TN-X, TN-C, and TN-R are arranged in a collinear fashion in front of the fibrinogen globe, we compared these analogous regions among the tenascin family members. Mouse TN-X is in this region 89% identical to human TN-X. When mouse TN-X is compared to mouse TN-C (49) or rat TN-R (21) a sequence identity on the amino acid level of 44 and 41% can be calculated, respectively. TN-C and TN-R are more closely related to each other, showing an identity of 49% within this same region. It is thus concluded that TN-X diverged from a common ancestor tenascin before TN-C and TN-R evolved.

Bristow et al. (3) suggested that the mouse strain aw 18, an intra-H-2 recombinant which is known to have a deletion of ~80 kb encompassing C4 and Cyp21 (45), could be a mouse mutant lacking the TN-X gene. Mice homozygous for aw 18 die within <15 d after birth, have no Cyp21 activity, and show histopathological abnormalities in the adrenal gland (24). Gotoh et al. (25) reported that aw 18 homozygous mutants can be rescued by the administration of synthetic steroids and salts. Furthermore, aw 18 transgenic mice

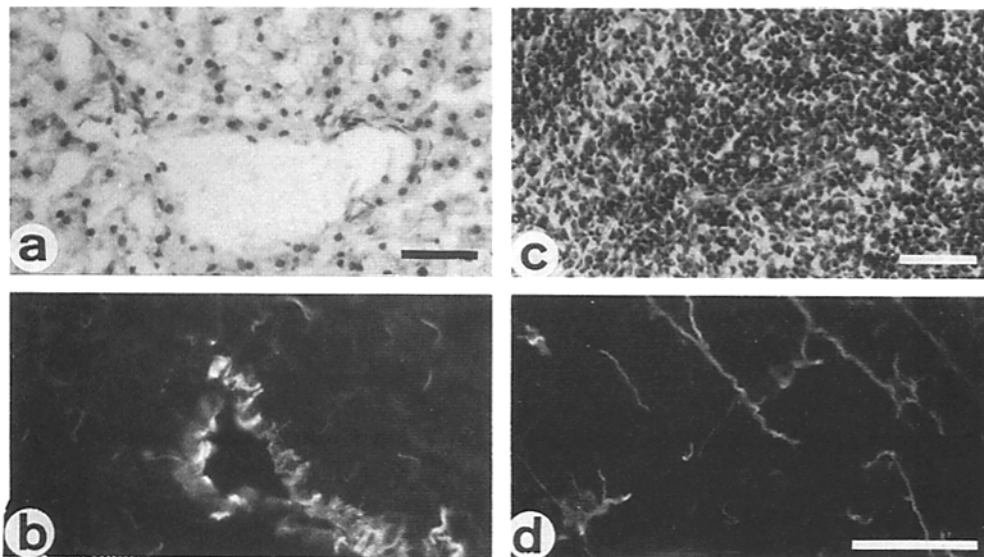


Figure 11. TN-X staining of blood vessels. Sections of liver (*a* and *b*) and Peyer's patches of the ileum (*c* and *d*) were stained with hematoxylin (*a* and *c*) or TN-X antibody pAb800 (*b* and *d*). A central vein surrounded by the liver plates linking the space occupied by the sinusoids is seen in *a* and *b*. TN-X is found associated with blood vessels in both tissues. Bars, 50 μ m.

which carry the Cyp21 gene linked to the MMTV-LTR promoter can be rescued as well. These findings suggest that the aw 18 mice are clearly suffering from a deficiency of Cyp21. Therefore, if TN-X was indeed essential for life, as suggested by Gitelman et al. (23), it could not be deleted in the aw 18 mice. This argues that the TN-X gene in the mouse overlaps with Cyp21ps rather than with Cyp21, which would mean that TN-X overlaps the Cyp21 gene located in the same chromosomal position as in man, namely the gene located closer to the centromere. Unfortunately our data cannot clearly distinguish between these two possibilities, because the coding region of TN-X is almost 100% complementary to Cyp21, and the non-coding part to Cyp21ps. This result points towards the possibility that in the Balb/c mouse a third Cyp21 gene may exist, and/or that gene conversion has occurred. Either of these phenomena is well documented for humans (34).

Using our antibodies against TN-X, we found the most prominent expression of TN-X in skeletal and heart muscle, tissues where neither TN-C nor TN-R are found. The high expression of TN-X in muscle was already previously noted on the level of TN-X mRNA expression (3, 23). Thus we tend to think of TN-X as of the muscle tenascin. In our analysis of the tissues of the digestive tract and of skin, we frequently noted intense staining for TN-C adjacent to the epithelium, whereas anti-TN-X more intensely stained regions further away from the epithelium in the loose mesenchyme. Regulation of TN-X and TN-C expression, respectively, seems to be completely independent within the organs analyzed, and during the stages of major changes of TN-C expression in oesophagus and gut, TN-X remains constant. Upon rough inspection TN-X and TN-C show a reciprocal tissue distribution, but there is also some coexpression as, for example, in some of the blood vessels. The blood vessel staining of TN-X in all organs analyzed is the likely reason for the ubiquitous expression of TN-X mRNA. Considering the very distinct tissue distribution of TN-X and TN-C it appears unlikely that TN-X could compensate for the loss of TN-C in the mice lacking TN-C, but still we cannot exclude that TN-X in certain locations might replace TN-C.

There seem to be certain common functional features of the tenascins. None of them is a very good cell adhesion promoting protein. On the contrary, TN-C is often considered as an anti-adhesive or adhesion-modulating protein (10, 11, 43). Also, in the case of TN-X, we could not detect any adhesion-promoting activity for the Ren-Ca cells (not shown). Concerning binding to other extracellular matrix proteins, we could not find any interaction of TN-X with fibronectin, although TN-C is known to bind. However, it should be kept in mind that only the small form of TN-C binds well to fibronectin (14). Thus it may be premature to conclude that there is no interaction possible between TN-X and fibronectin, since the form of TN-X produced by the ReCa cells may be a non-binding variant. Different splicing variants of TN-X may exist that could have different functions, since at least on the mRNA level we could detect two sizes of transcripts produced by the various cell lines analyzed. Another known ligand of TN-C is heparin (2, 7), and also proteoglycans have been observed to interact with tenascin (5, 27). Furthermore, mesenchymal syndecan was shown to bind TN-C via its heparan-sulfate side chains (44). Since indeed also TN-X is binding to heparin, the possibility exists

that this is an important functional activity that has been conserved among different tenascins. The heparin-binding region of TN-C has been localized to the COOH-terminal part including the last three fibronectin type III repeats and the fibrinogen domain (7). Using recombinant tenascin fragments produced in *E. coli* Aukhil et al. (2) found two heparin-binding sites, one of them in the fourth and fifth fibronectin type III repeat the other one in the fibrinogen domain (2). Since we have no evidence for a second heparin-binding region in fragments of authentic TN-C, we consider the heparin-binding activity detected in the fibronectin type III repeats as a potential cryptic site revealed in the recombinant fragment. We therefore believe that the heparin-binding region of tenascin-C is located in the fibrinogen globe, which is the most conserved region among all members of the tenascin family. It is therefore possible that also in TN-X the fibrinogen globe is containing the heparin binding site.

Further studies will be necessary to determine the entire primary structure of TN-X, and biochemical and biophysical investigations should aid in revealing its oligomer structure. Also a more detailed description of its tissue distribution, including earlier developmental stages will be of interest. At present we do not know anything about the function of TN-X in vivo, but there is circumstantial evidence that in humans TN-X may be an essential gene for life (35), thus leading to the most challenging task of finding the function(s) of TN-X.

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