Murine tenascin-W: a novel mammalian tenascin expressed in kidney and at sites of bone and smooth muscle development

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Summary

We cloned and characterized a novel member of the tenascin family of extracellular matrix proteins – the murine orthologue of zebrafish tenascin-W. Full-length recombinant tenascin-W was expressed and purified from mammalian cell cultures. Rotary shadowing followed by electron microscopy showed that tenascin-W forms hexabrachions. We studied its expression during development and in the adult by immunohistochemistry, in situ hybridization and immunoblotting. Tenascin-W is expressed during palate formation, osteogenesis and smooth muscle development. In the adult, tenascin-W is found in the kidney, cardiac semilunar valves, corneal limbus and periosteum. Tenascin-W and tenascin-C expression overlap in many of these areas. Bone-morphogenic-protein-2 treated C2C12 cells secrete tenascin-W and are able to adhere to and to extend actin-rich processes on a tenascin-W substratum. In vitro, cells bind to tenascin-W in an RGD-dependent manner. This adhesion is increased by transfection of α8 integrin, which localizes with tenascin-W in the periosteum and kidney.

Key words: Tenascin, Hexabrachion, Expression, Development, Adhesion, Extracellular matrix

Introduction

Extracellular matrix (ECM) is important in the maintenance of normal tissue architecture and the regulation of cell behaviour and gene expression. Many of the ECM proteins involved in these functions belong to families of large, multi-modular molecules consisting of serially repeated domains (Engel, 1996). Tenascins are such a family, with each member composed of related subunits built from variable numbers of repeated domains. Each tenascin has one or more epidermal growth factor (EGF)-like repeats, a variable number of fibronectin type III (FNIII) modules and a C-terminal fibrinogen-like globe (Jones and Jones, 2000a). Three members of the tenascin family have been characterized in mammals: tenascin-C, tenascin-X and tenascin-R (Erickson, 1993; Chiquet-Ehrismann, 1995). A fourth member of the family, tenascin-Y, is the avian homologue of tenascin-X (Hagios et al., 1996). The tenascins have distinctive patterns of expression during development. Tenascin-C is found around migrating cells, at sites of epithelium-mesenchyme interactions, at sites of smooth muscle and connective tissue morphogenesis, and throughout the central and peripheral nervous system, and is often induced under pathological conditions (Jones and Jones, 2000b; Chiquet-Ehrismann and Chiquet, 2003). Tenascin-X and tenascin-Y are expressed around developing muscle and blood vessels (Matsumoto et al., 1994; Hagios et al., 1996), and tenascin-R expression is limited to the nervous system (Rathjen et al., 1991).

Each of the tenascins found in the mouse have been knocked out by homologous recombination in order to study their functions during development. Mice lacking a functional tenascin-C gene were first reported to develop normally (Saga et al., 1992), but more recent reports demonstrate several neurological phenotypes (reviewed in Mackie and Tucker, 1999). Tenascin-X knockout mice also develop outwardly normally but eventually display connective tissue disorders resembling Ehlers-Danlos syndrome (Mao et al., 2002). Similarly, tenascin-R knockout mice are viable and fertile, although the molecular composition of their perineuronal nets is altered (Weber et al., 1999). The relatively mild developmental phenotypes described in each of these knockout mice might be explained by functional redundancy within the tenascin family but, as yet, there is no evidence that the expression pattern of one tenascin changes in response to the knockout of another. This leaves open the possibility that a fourth, uncharacterized, tenascin could be present in the mouse embryonic ECM.

A candidate for this fourth mammalian tenascin is tenascin-W, which has to date only been described in zebrafish (Weber et al., 1998). Zebrafish tenascin-W was isolated by screening a zebrafish cDNA library constructed 20-28 hours after fertilization for the EGF-like domains conserved in all tenascins. The expression pattern of tenascin-W was then investigated by in situ hybridization in embryos and juvenile zebrafish. At both stages, tenascin-W expression overlapped extensively with the expression of tenascin-C.

In order to learn more about the possible role for tenascin-W in compensating for tenascin-C in the knockout mice, as well as to learn about the properties of tenascin-W protein, we

have cloned the complete coding sequence of murine tenascin-W, studied its expression by immunohistochemistry, western blotting and in situ hybridization, and cultured cells on recombinant tenascin-W. Our results show considerable overlap between the expression of tenascin-W and tenascin-C in the mouse, but distinctive effects of these proteins on cells in vitro. We have also identified a possible integrin receptor for tenascin-W that does not act as a receptor for intact tenascin-C.

Materials and Methods

Cloning, expression and purification of mouse tenascin-W

Mouse tenascin-W was cloned from a cDNA library of 19 d whole mouse embryos (DupLEX-A DLM-110; OriGene). In a first step, the following PCR primers derived from a 'novel human mRNA from chromosome 1, similar to Tenascin-R' (accession number AL049689), were used for nested PCR reactions with the Expand High Fidelity PCR System (Roche) using the mouse cDNA library as template. The first reaction was performed with the primer set 5′-TAGCAGCCCACAGCATCTACTTGCC-3′ / 5′-ATTGCTGTTCTG-CTGAACCTGACTGCA-3′ and the second reaction with 5′-ATGGATCCAGAAATTGACGGCCCCAAAAACCTAG-3′ / 5′-AT-AAGCTTGTGGAGAGGGTGGTGGATACATTTC-3′. The second primer set included a *Bam*HI and a *Hin*dIII restriction site, respectively, to enable the directional cloning into the bacterial expression vector pQE30 (Qiagen) supplying a C-terminal His-tag for the purification of the recombinant protein. The recombinant protein was expressed and purified by affinity chromatography to a Ni-NTA matrix (Qiagen) according to the manual of the supplier. It was purified under native conditions and was eluted with 250 mM imidazole. The recombinant protein encompassed the three most C-terminal FNIII repeats of tenascin-W (amino acids 794-1057) of the complete amino acid sequence of mouse tenascin-W encoded by nucleotides 2380-3171 of the tenascin-W nucleotide sequence. To complete the 5′ part, the following PCR reactions using the above cDNA library as template were performed: for the first PCR reaction, the primer pair 5′-AGGAGATGGTGGCTGTATTTTCGG-3′ / 5′-AGCCTCTTGCTGAGTGGAGATGCC-3′ was used, followed by a second PCR reaction with the primer set 5′-TAGAATTCGGT-CACCTGATTGGTCACTAGG-3′ / 5′-TTATGATGTGCCAGATTA-TGCC-3′. To complete the 3′ part of the tenascin-W cDNA, the following PCR reactions were performed: in the first reaction, the primer pair 5′-CTCAAATTGATGGCTACATTTTGACC-3′ / 5′-AA-GCCGACAACCTTGATTGGAGAC-3′ was used, followed by the primer pair 5′-TACCAGTTCCCAAATGGCACCG-3′ / 5′-AAACCT-CTGGCGAAGAAGTCC-3′. In each case, the longest products were cloned. These overlapping tenascin-W cDNA clones were assembled into one full-length mouse tenascin-W cDNA and cloned into the expression vector pCEP/Pu (provided by J. Engel, Biozentrum, Basel, Switzerland). At the 3′ end of the tenascin-W cDNA a 6× His-tag was inserted in front of the stop codon to allow the purification of full-length mouse tenascin-W protein expressed in mammalian cell culture.

To check for potential splicing variants within the region of the FNIII repeats, we performed PCR reactions with primers 5′-CACAGTTGTGCCCATCCG-3′ / 5′-GCCATCAATTTG-3′ spanning repeats 2 to 9 using the 19-day whole-embryo library as template. PCR products were analysed after electrophoresis in 1% agarose gels.

The full-length tenascin-W cDNA was transfected into HEK293 EBNA cells (ATCC number CRL-10852) using the transfection reagent fugene-6 (Roche). Transfected cells were selected for with puromycin and the medium containing the secreted tenascin-W protein was collected. The protein was purified by sequential chromatography over a gelatine-agarose column (Sigma) to remove any contaminating fibronectin in the preparation and by adsorption to a Ni-NTA matrix (Qiagen). The tenascin-W was eluted from the nickel column by 250 mM imidazole and dialysed against PBS.

Electron microscopy was performed by the rotary shadowing technique as described previously (Chiquet-Ehrismann et al., 1988). Briefly, 50 μg ml^{-1} tenascin-W in 0.2 M ammonium hydrogen carbonate was mixed 1:1 (v/v) with glycerol and sprayed onto freshly cleaved mica discs. These were dried in high vacuum, rotary shadowed with platinum at an angle of 5-7°, replicated and photographed.

Heparin binding of tenascin-W was tested by incubation of the purified tenascin-W with heparin-agarose beads (Sigma-Aldrich) in PBS and in PBS diluted 1:2 with water. The beads were washed with the respective incubation buffers and the bound protein eluted by boiling of the beads in sodium-dodecyl-sulphate/polyacrylamide-gel electrophoresis (SDS-PAGE) loading buffer. The protein input, the supernatant, the wash and the elution fractions were analysed by SDS-PAGE and stained by GelCode (Pierce). In PBS, all of the tenascin-W was recovered in the supernatant, whereas, in the diluted PBS, ~90% of the protein was retained by the heparin-agarose.

In situ hybridization

Timed (12.5 days) CD-1 mouse embryos were dissected from the uterine horns and fixed in ice-cold 4% paraformaldehyde in PBS overnight. The embryos were then rinsed in PBS, dehydrated in methanol and stored in 100% methanol at –80°C until processed for in situ hybridization. Antisense and sense tenascin-W RNA probes (corresponding to the sequences coding and complementary to FNIII repeats 6 and 7) were labelled with digoxigenin-UTP (Boehringer Mannheim) using the instructions provided by the manufacturer. In situ hybridization was carried out using methods modified from the protocol published by Wilkinson and Nieto (Wilkinson and Nieto, 1993) and described by us previously (Tucker et al., 2001a), except that the proteinase K treatment was extended to 10 minutes. Hybridization was carried out for 2 days at 68°C. Following hybridization, the embryos were washed three times for 30 minutes each in 5× SSC/50% formamide and three times for 30 minutes each in 2× SSC/50% formamide at 65°C. Embryos were then rinsed in 1× TBS with 1% Tween-20, blocked in TBS with 10% goat serum and then incubated overnight in alkaline-phosphatase-conjugated anti-digoxigenin (Boehringer Mannheim) that had been preabsorbed with mouse embryo powder. NBT/BCIP (Boehringer Mannheim) was used for the alkaline phosphatase colour reaction.

Anti-tenascin-W antibody production and immunohistochemistry

The bacterially expressed recombinant fragment of tenascin-W described above was used to raise polyclonal antisera in rabbits using standard immunization procedures. This antiserum reacted specifically with purified full-length tenascin-W as well as with endogenous tenascin-W in tissue extracts of mouse organs. Timed (10.5 days, 11.5 days, 12.5 days, 13.5 days, 14.5 days, 15.5 days and 16.5 days) CD-1 mouse embryos were fixed as described above for in situ hybridization, rinsed, cryoprotected in 25% sucrose in PBS overnight, and embedded for cryosectioning. Sections were cut at 14 μm in a Leitz cryostat, collected on presubbed slides (Fisher) and allowed to air dry for 6 hours. Cross and sagittal sections from each developmental stage were then rinsed in PBS, blocked in PBS with 0.5% bovine serum albumin (BSA) and incubated overnight at room temperature in diluted anti-tenascin-W serum, diluted preimmune serum or blocking solution alone. Following rinses, the sections were then incubated in secondary antibody (Alexa-488- or Alexa-594-tagged goat anti-rabbit IgG, Molecular Probes) for 2 hours. For double-label immunohistochemistry, sections were incubated in both the anti-tenascin-W serum and rat monoclonal anti-tenascin-C MTn-12 (Sigma) and incubated in two appropriate secondary antibodies

(Molecular Probes) labelled with different fluorophores. Photographs were taken using Ektachrome (Kodak) slide film, including double exposures of the double-label immunohistochemistry.

For immunofluorescence and F-actin staining of C2C12 cells, cultures were transfected with a full-length α8 cDNA in pcDNAI [kindly provided by L. Schnapp (Schnapp et al., 1995a)], with this α8 cDNA and a plasmid encoding enhanced green fluorescent protein (EGFP) under the control of a β-actin promoter (kindly provided by A. Matus, Friedrich Miescher Institute, Basel, Switzerland), or with the EGFP plasmid alone using the transfection reagent fugene (Roche Diagnostics). 36 hours after transfection, the cells were harvested and re-plated in serum-free medium on wells coated with tenascin-W (50 μg ml^{-1}) or untreated wells of 3.5 cm dishes containing four separate wells (Greiner). 1 hour after plating, the cells were fixed with 4% paraformaldehyde in PBS for 30 minutes and permeabilized with 0.1% Triton X-100 in PBS for 5 minutes. Then, they were either incubated with anti-α8 antiserum at a dilution of 1:200 (Schnapp et al., 1995b) followed by Alexa-488-conjugated antibodies (Molecular Probes) at a 1:1000 dilution together with TRITC-phalloidin (Sigma-Aldrich) at a 1:500 dilution. Each incubation was for 1 hour and cells were washed in PBS after each incubation. Finally, the specimens were mounted in Mowiol (Calbiochem) and examined and photographed using a Axiophot microscope (Carl Zeiss MicroImaging) connected to a 3CCD camera (Sony).

Western-blot analysis of tenascin-W expression

Approximately equal volumes of dissected tissue samples were solubilized for 5 minutes at 95°C in SDS-PAGE sample buffer, followed by electrophoresis on 6% polyacrylamide gels. Proteins were electro-transferred onto PVDF membranes (Millipore). After the transfer, the membranes were stained with Amidoblack to control for equal protein loading. After blocking for 1 hour at room temperature in TBS containing 0.05% Tween and 3% skimmed milk powder, membranes were incubated overnight with anti-tenascin-W antibody (1:400) and then for 1 hour with anti-rabbit IgG coupled to horseradish peroxidase (HRP) (1/10000). Specific staining was revealed using ECL enhanced chemiluminescence kits (Amersham).

Supernatant was collected from J_1 ES cells cultured on primary mouse embryonic fibroblasts as a feeder layer in medium complemented with LIF (Gibco).

Mouse C2C12 cells (CRL-1772; American Type Culture Collection) were routinely cultured in Dulbecco's modified Eagle's medium (Life Technologies) containing 10% foetal calf serum (Amimed). During the treatment with bone morphogenic protein-2 (BMP2; Research Diagnostics) cells were cultured in medium containing 5% newborn calf serum (Amimed) with or without the addition of 200 ng ml^{-1} of BMP2. Medium was exchanged every second day up to 6 days. Aliquots of 20 μl of the conditioned media were analysed by SDS-PAGE and immunoblots for the presence of tenascin-W. After 6 days of treatment, these cells were used for adhesion assays as described below.

Cell adhesion assays

Cell adhesion assays were performed with either C2C12 cells described above or with human T98G (CRL-1690; American Type Culture Collection) cells. Microtitre plates (60 wells; Nunc) were coated for 1 hour at room temperature with 5 μl per well PBS containing 0.01% Tween and the indicated concentration of ECM protein. All wells were then blocked for 30 minutes with PBS containing 1% BSA. Before plating, cells were detached using trypsin-EDTA. Trypsin was blocked with 100 μg ml^{-1} soybean trypsin inhibitor in PBS, and cells were resuspended in serum-free medium and counted. 1500 cells per well were plated for 60 minutes. The set of integrin-function-blocking antibodies was obtained from Chemicon International (ECM430). Cells were incubated for 30 minutes in suspension with the indicated antibody at 10 μg ml^{-1} before plating. To investigate the effect of RGD peptides (GRGDS and SDGRG, both from Sigma) 0.1 M or 0.5 M peptide was added to the cells before plating. Cells were fixed by the addition of 4% formaldehyde in PBS for 30 minutes at room temperature and stained with 0.1% crystal violet in H_2O for 30 minutes. Cells were photographed with a Nikon microscope (Nikon Diaphot) equipped with a Nikon camera and counted.

Adhesion assays with α8 or mock-transfected human K562 (kind gift of U. Müller, Friedrich Miescher Institute, Basel, Switzerland) were performed as described (Denda et al., 1998).

Human SW480 carcinoma cells transfected with α9 integrin as well as the corresponding control cells were a kind gift of D. Sheppard (Yokosaki et al., 1994).

Results

Structure of mouse tenascin-W

We used a 19-day whole-mouse-embryo cDNA library to clone a full-length mouse tenascin-W cDNA and deduced its primary structure (Fig. 1; accession number AJ580920). A putative signal peptide of 20 amino acids (underlined), with a predicted cleavage site after the 26th amino acid, is followed by three-and-a-half heptad repeats flanked by cysteines (amino acids 99-132). They are followed by three-and-a-half EGF repeats (amino acids 155-259) and nine fibronectin type III (FNIII; amino acids 260-1056) modules. The C-terminal part of the protein is formed by a fibrinogen globe (amino acids 1057-1296). All these modular elements are consistent with tenascin-W belonging to the tenascin family. When we compared the protein sequences of the fibrinogen globes of all known tenascins, we found that this mouse tenascin-W sequence is 91% identical to a protein encoded by the GenBank entry termed 'human tenascin-R-like cDNA' (accession number AL049689.1) and 64% identical to the zebrafish tenascin-W, whereas its identity with other mouse tenascins is around 50%. Therefore, we conclude that the so-called human tenascin-R-like transcript and the mouse tenascin-W presented here are the species homologues of zebrafish tenascin-W.

A striking feature of the tenascin-W sequence is its high degree of homology between FNIII repeats within one molecule. A comparison between all FNIII repeats of mouse tenascin-W reveals that repeats 1, 2 and 9 are clearly different from the central group of FNIII repeats 3-8 (Fig. 1B). Comparing the sequences between mouse and zebrafish tenascin-W, it is possible to assign homologous repeats between species (Fig. 1C). Thus, zebrafish has only two of the central highly conserved repeats and the fifth FNIII corresponds to the ninth of the mouse tenascin-W. This indicates that either the zebrafish tenascin-W sequence reflects a shorter variant produced by alternative splicing or the mouse has acquired more of the repeats during evolution by exon duplications. The human tenascin-W sequence that can be found in the DNA database (accession number AL049689.1) corresponds exactly to the mouse sequence presented here and also contains nine FNIII repeats.

In order to analyse the structure and function of tenascin-W, we expressed FNIII repeats 7-9 in bacteria and raised an antiserum against this recombinant fragment of tenascin-W. Furthermore, we purified full-length tenascin-W from transfected HEK293 cells. The purified full-length tenascin-W was analysed by gel electrophoresis and the specificity of the

anti-tenascin-W antiserum was tested on an immunoblot of purified full-length tenascin-W and tenascin-C (Fig. 2A). The anti-tenascin-W antiserum reacted specifically with tenascin-W and showed no cross-reactivity with tenascin-C. The migration of tenascin-W was analysed under reducing and non-reducing conditions, and compared with migration of tenascin-C. Under reducing conditions, tenascin-W and tenascin-C migrated as monomers, whereas, under non-reducing conditions, tenascin-C migrated as a disulfide-linked hexamer. Because non-reduced tenascin-W exhibited a similar molecular weight to tenascin-C, we wanted to know whether tenascin-W can form hexamers as well. To address this point, purified mouse recombinant tenascin-W was visualized by electron microscopy after rotary shadowing. Indeed, tenascin-W molecules formed six-armed structures, called hexabrachions, similar to those previously reported for tenascin-C (Fig. 2B) (Erickson and Inglesias, 1984).

Because tenascin-W contains four potential heparin-binding sites (BBxB; underlined in Fig. 1) we tested whether tenascin-W can be retained on a heparin-agarose column. This binding was found to be weaker than that of tenascin-C (Fischer et al., 1995), tenascin-X (Matsumoto et al., 1994) or tenascin-Y (Hagios et al., 1996) and could only be detected below physiological salt concentration (not shown).

Expression of tenascin-W during embryonic development and in the adult

The expression of tenascin-W was investigated by immunohistochemistry in sections from timed CD-1 mouse embryos in parallel with tenascin-C. Tenascin-W expression first appeared at embryonic day 11.5 (E11.5) in the maxillary process (Fig. 3A). Tenascin-C was expressed in the maxillary process as well, and the two proteins were partially co-localized (Fig. 3B,C). The expression of tenascin-W in the maxillary process was confirmed by in situ analysis at E12.5 showing expression of tenascin-W mRNA only in the maxillary process (‘moustache-like’ structures; Fig. 3E,F). Tenascin-W was still expressed in this region at E14.5 (Fig. 3D) and at E16.5 in the newly formed mandible (Fig. 4M). After E11.5, tenascin-W expression extended to other regions but with a very specific pattern. At day E14.5, tenascin-W

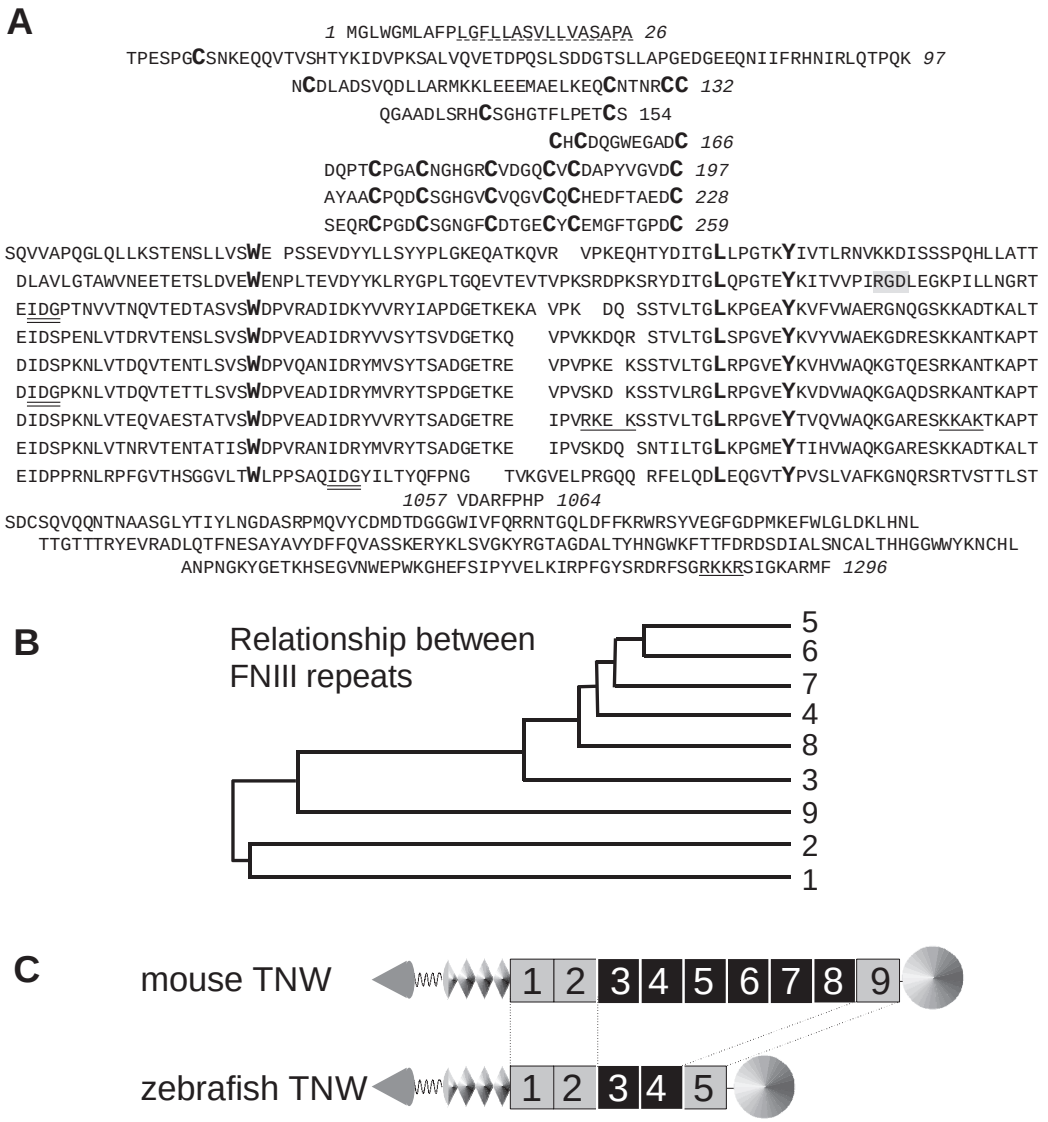


Fig. 1. Primary structure of the mouse tenascin-W protein. (A) Mouse tenascin-W amino acid sequence translated from the DNA database entry (accession number AJ580920). The signal peptide is underlined (dashed). Amino acids 99-132 represent the heptad repeats flanked by cysteines, amino acids 155-259 the EGF repeats, amino acids 260-1056 the FNIII repeats and amino acids 1057-1296 the fibrinogen globe. The RGD sequence in the second FNIII repeat is boxed (grey), the IDG motifs are double underlined and BBXB sites are underlined.
(B) Relationship between FNIII repeats created by the program pileup of the GCG software package.
(C) Schematic representation of the domain structure of mouse and zebrafish tenascin-W depicting an N-terminal domain (wedge), heptad repeats (wavy line), EGF-like repeats (diamonds), FNIII repeats (squares; central highly similar repeats shaded in black) and fibrinogen globe (circle). Homologous FNIII repeats between mouse and zebrafish tenascin-W are interconnected by dashed lines.

was found in the smooth muscle cells in the stomach (Fig. 4A) and the intestine (Fig. 4E). In the same tissue sections, tenascin-C was expressed in the stomach, mostly localized with tenascin-W (Fig. 4B), but not in the intestine (Fig. 4F). A similar observation was made at E15.5 (Fig. 4C,D,G,H). At the same stage, tenascin-W appeared in the periosteum of the ribs (Fig. 4K), again overlapping the expression of tenascin-C (Fig. 4L). Tenascin-W expression was not seen associated with developing bones before E15.5, as shown, for example, in vertebral arches (Fig. 4I), whereas tenascin-C was already found in this structure at this embryonic stage (Fig. 4J). Later, at E16.5, tenascin-W expression was restricted to specific regions, localizing with tenascin-C: mandible (Fig. 4M), palate (Fig. 4N) and teeth (Fig. 4O). In masseter, however, tenascin-W was expressed but tenascin-C was not (Fig. 4P). During all embryogenesis, tenascin-W was never found to be present in lung, cartilage, liver, brain or peripheral nerves (data not shown), whereas tenascin-C is known to be expressed in all these structures during development.

In the adult, the pattern of tenascin-W expression was restricted to kidney (Fig. 5B), aortic valve (Fig. 5C), corneal limbus (Fig. 5D) and periosteum around the ribs (Fig. 5E). Tenascin-W overlapped expression of tenascin-C in all these regions. Tenascin-W was absent from cerebellum (Fig. 5A), stomach (Fig. 5F) and intestine (Fig. 5G), where tenascin-C was still expressed.

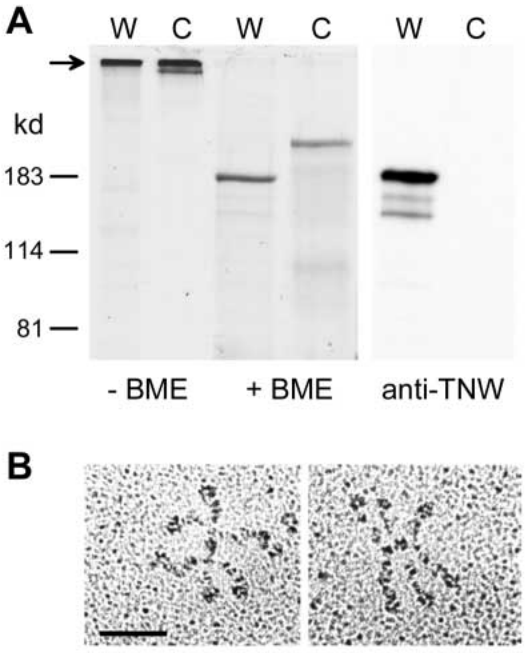


Fig. 2. Purification of tenascin-W. (A) Protein gels of purified tenascin-W (W) in comparison to tenascin-C (C) reveals that both proteins form high molecular weight disulphide-linked oligomers (arrow), as can be seen by their slow migration in the absence of reducing agent (–BME). Migration under reducing condition (+BME) reveals for tenascin-W a M_r of 180 kDa. An immunoblot with anti-tenascin-W antibody (anti-TNW) reveals that this antiserum specifically reacts with tenascin-W, but not with tenascin-C. (B) Electron microscopy after rotary shadowing of recombinant mouse tenascin-W reveals that it forms hexameric oligomers. Scale bar, 50 nm.

Analysis of endogenous tenascin-W in tissues and cell cultures

The expression of tenascin-W was investigated in the embryo and adult mouse by immunoblot analysis (Fig. 6). In extracts of whole embryos from E10.5 and E12.5, a single 180 kDa band was detected with the anti-tenascin-W antibody on immunoblots (Fig. 6A). In adult mouse, the same 180 kDa tenascin-W was detected in extracts from the periosteum of the ribs and in kidney, as well as in J1 embryonic stem cells in culture (Fig. 6A). To confirm that there are no splice variants of tenascin-W, we performed reverse-transcription polymerase chain reactions (PCR) on mRNA from E16.5 mouse whole embryos (Fig. 6B). Only one band could be amplified by PCR with primers encompassing the second to the ninth FNIII repeats. The size of the amplified band corresponded to the expected size, with six FNIII repeats between repeats 2 and 9.

C2C12 cells are known to differentiate into osteoblastic cells after treatment with BMP2. The osteoblast markers alkaline phosphatase and osteocalcin were first detected in conditioned medium 3 days after adding BMP2 and a large increase was observed after 6 days in culture (Katagiri et al., 1994). Because the immunostaining of mouse embryos revealed the expression of tenascin-W in the periosteum, we tested whether BMP2 induces tenascin-W in C2C12 cells concomitant with osteogenic differentiation. Indeed, BMP2 treatment of

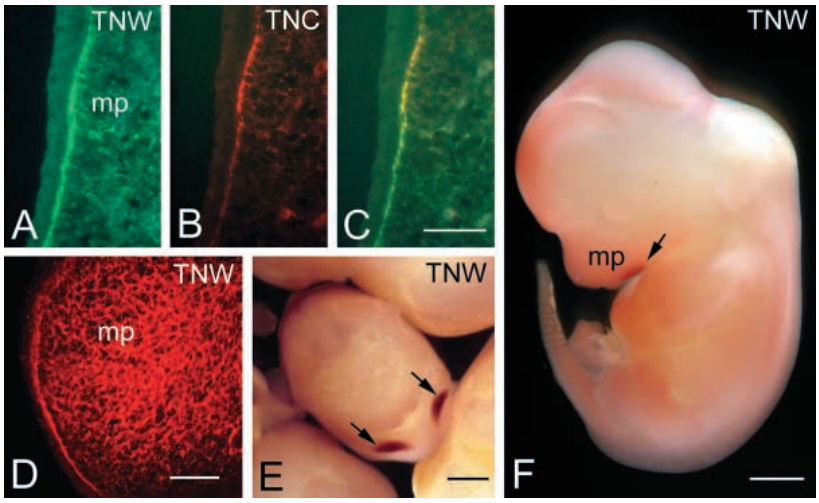


Fig. 3. Tenascin-W is first expressed in maxillary processes of mouse embryos. Immunohistochemistry of tenascin-W (A), tenascin-C (B) and a merged picture (C) in a section through an E11.5 mouse embryo reveals co-expression in the maxillary process (mp). Tenascin-W is still expressed in the mp at E14.5 (D). In situ hybridization analysis of E12.5 mouse embryos with probes against tenascin-W reveals expression in 'moustache-like' structures corresponding to the mp when viewed from below (arrows, E) and in whole embryos (arrow, F). Embryos incubated with sense control probes were unlabeled (not shown). Scale bars, 100 μm (A-D), 1 mm (E,F).

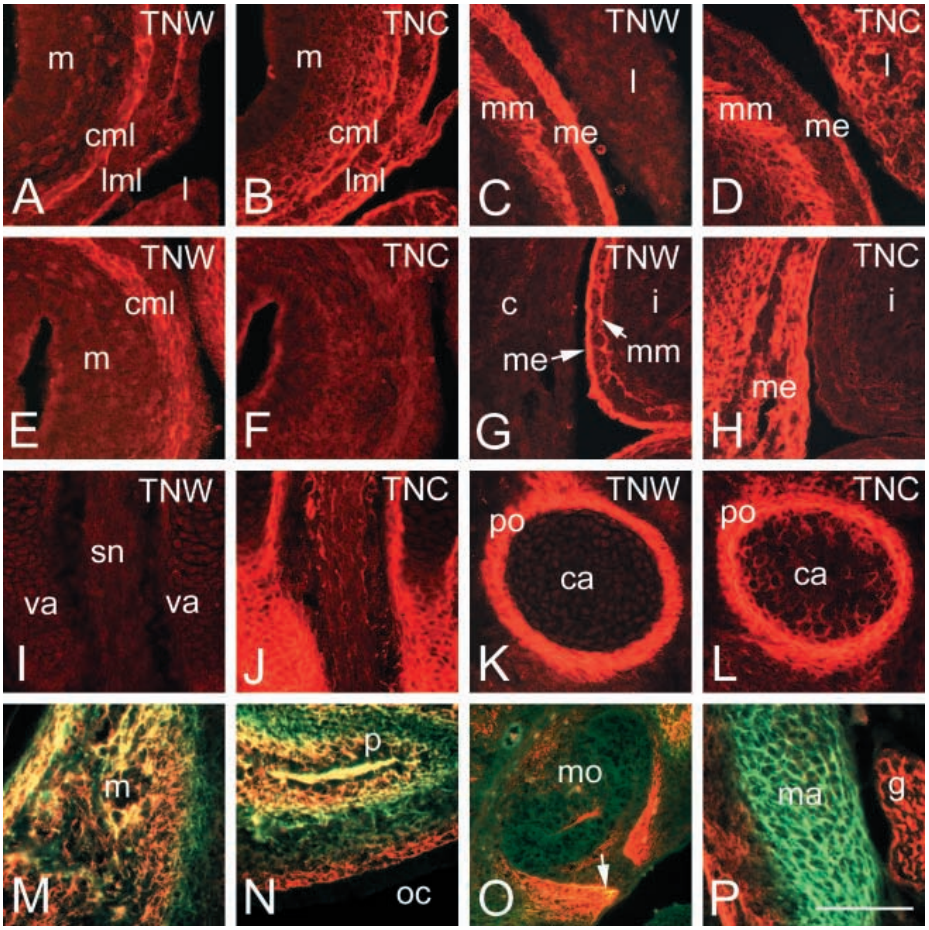
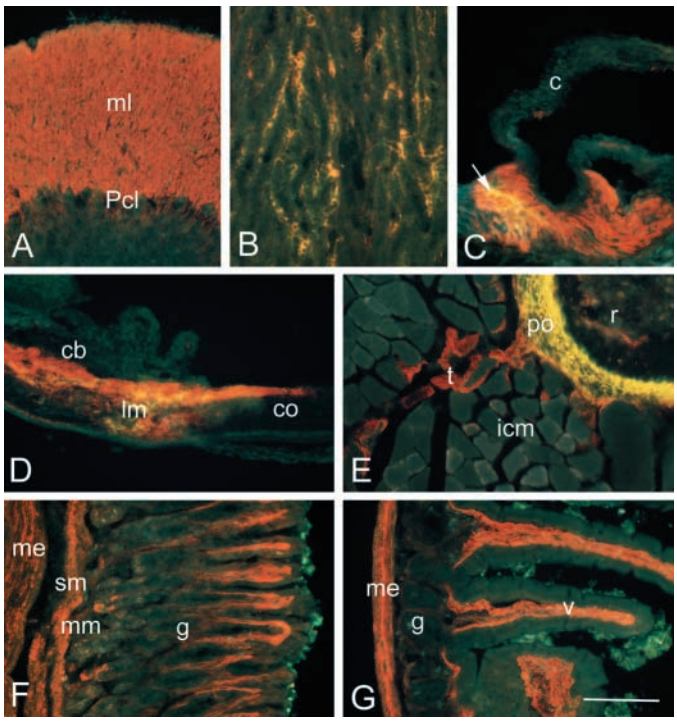


Fig. 4. Similar but distinct expression patterns of tenascin-W and tenascin-C in mouse embryos. Immunohistochemistry of tenascin-W and tenascin-C (as indicated) at E14.5 (A,B,E,F,I,J), E15.5 (C,D,G,H,K,L) and E16.5 (M-P). At E16.5 (M-P), double labelling is shown with tenascin-W in green, tenascin-C in red and overlap in yellow. Tenascin-W is expressed in the circular muscular layer (cml) of the stomach at E14.5 (A) and in the muscularis mucosa (mm) and muscularis externa (me) at E15.5 (C). At E14.5, tenascin-C is expressed in both the cml and longitudinal muscular layer (lml) of the stomach (B). At E15.5, tenascin-C is found in the mm, but staining in the me (i.e. combined cml and lml) is faint. Tenascin-C is expressed in the developing liver (l) at both E14.5 (B) and E15.5 (D), but tenascin-W is not. Tenascin-W is expressed in the cml of the ileum at E14.5 (E) and in both the me and mm at E15.5 (G). It is not seen in the colon (c). Tenascin-C is not detected in the ileum at either E14.5 (F) or E15.5 (H) but, unlike tenascin-W, it is expressed in the developing musculature of the colon at E15.5 (H). At E14.5, tenascin-W is not present in the cartilaginous vertebral arches (va, I), but expression appears at E15.5 in the periosteum (po) around the ribs (K). Tenascin-C was expressed at both embryonic stages in the cartilaginous vertebral arches (J) and rib cartilage (ca, L). At E16.5, tenascin-W was expressed in mandible (m, M), palate (p, N) and molars (mo, O), mostly as a subset of tenascin-C expression (e.g. arrow, O). At the same stage, only tenascin-W was expressed in the matrix of the masseter (ma, P). sn, spinal nerve; oc, oral cavity; g, salivary gland. Scale bar, 200 μm.



C2C12 cells led to the accumulation of tenascin-W in the conditioned medium after 4 days in culture, with a further increase after 6 days (Fig. 7A).

Cell adhesion to tenascin-W

BMP2-treated and untreated C2C12 cells were tested for adhesion to tenascin-W in comparison to fibronectin (Fig.

Fig. 5. Expression of tenascin-W and tenascin-C in the adult mouse. Each figure shows double staining of tenascin-W (green) and tenascin-C (red), with the overlap being yellow. Tenascin-C immunoreactivity is found in the molecular layer (ml) of the cerebellum (A). Tenascin-W was not detected here or elsewhere in the adult central nervous system. Tenascin-W and tenascin-C co-localize in the matrix of kidney tubules (B). Tenascin-C is found in the matrix of major blood vessels but tenascin-W is expressed only at the base of the cusps (c) of the aortic (arrow, C) and pulmonary valves. Tenascin-W is also expressed as a subset of the tenascin-C expression pattern in the corneal limbus (lm, D) and the periosteum (po) of the rib (r) (E). Tenascin-C is seen in the matrix of adult stomach (F) and intestine (G), but tenascin-W is not. c, valve cusp; cb, ciliary body; co, cornea; g, glands; icm, intercostal muscle; me, muscularis externa; ml, molecular layer; mm, muscularis mucosa; Pcl, Purkinje cell layer; sm, submucosa; t, tendon; v, villus. Scale bar, 200 μm.

7B,C). Both batches of cells adhered well to tenascin-W, reaching maximal adhesion at 10 μg ml^{-1} coating concentration. Whereas equal numbers of both batches of cells adhered to fibronectin, adhesion to tenascin-W was increased in the BMP-treated cells. The cellular morphology of the cells differed between the fibronectin and the tenascin-W substrata (Fig. 7D-F). The cells on fibronectin were flat with regular shapes and extensive lamellipodia, whereas the cells on tenascin-W displayed many long, narrow processes and rounded cell bodies. The morphology of the BMP2-treated cells was the same as that of the untreated cells.

To determine the cellular receptor(s) responsible for cell adhesion to tenascin-W, we tested the effect of integrin function-blocking antibodies. Because these antibodies recognize human proteins only, we used the human glioblastoma cell line T98G for these studies. These cells adhere well to tenascin-W and show a similar morphology to the C2C12 cells, with actin-rich processes (see below). Antibodies to α1, α2, α3, α4, α5, α6 and αV were unable to inhibit adhesion of T98G cells to tenascin-W (Fig. 8A). Nevertheless, this adhesion depended on β1 integrin, because 10 μg ml^{-1} of the anti-β1-integrin-blocking antibody P4C10 completely inhibited adhesion to tenascin-W. IDG tripeptide motifs have been reported to be the recognition sequence for α9β1 integrin (Yokosaki et al., 1998). Because mouse tenascin-W contains three IDG motifs (Fig. 1A), we investigated whether α9 integrin could be a receptor for tenascin-W. We obtained SW480 colon carcinoma cells transfected with a vector containing the cDNA for α9 integrin and mock-transfected cells (Yokosaki et al., 1994), and plated them on tenascin-W-coated wells. However, the α9- and mock-transfected SW480 cells failed to adhere to tenascin-W, whereas they adhered well to fibronectin and collagen (Fig. 8B).

Integrin α8 is expressed in developing rib, kidney and in smooth muscle from the gastro-intestinal tract (Schnapp et al., 1995b; Denda et al., 1998). Because this expression pattern coincides with the presence of tenascin-W, it seemed that integrin α8 was also a good candidate receptor for tenascin-W. We tested this hypothesis using the leukaemia cell line K562 transfected with α8 integrin (Muller et al., 1995). α8-Integrin-transfected K562 cells could indeed adhere to tenascin-W but the mock-transfected control cells did not (Fig. 8C). Therefore, we propose that α8β1 integrin is a receptor for tenascin-W. Because α8β1 is known to be an RGD-dependent receptor and mouse tenascin-W contains an RGD sequence in its second FNIII repeat, we tested whether cell adhesion to tenascin-W could be inhibited by RGD peptides. Inclusion of the GRGDS peptide inhibited adhesion of T98G cells to fibronectin as well as to tenascin-W, whereas the control SDGRG peptide had no effect (Fig. 8D). The adhesion of C2C12 cells to tenascin-W was also inhibited by RGD peptides (not shown).

We next analysed the morphology and the actin cytoskeleton of C2C12 cells plated on tenascin-W in comparison to cells plated on fibronectin. The shape of the cells on tenascin-W was very different from the cells on fibronectin. This was particularly obvious after F-actin staining with phalloidin (Fig. 9A,B). The cells on fibronectin were well spread and contained stress fibres, whereas the cells on tenascin-W had many actin-rich processes but no stress fibres and the cell bodies remained relatively round. Finally, we tested whether overexpression of α8 integrin in C2C12 cells would further increase cell adhesion of these cells to tenascin-W. We transfected either an α8 expression plasmid, an EGFP plasmid or the two plasmids together into C2C12 cells. 2 days after transfection cells were

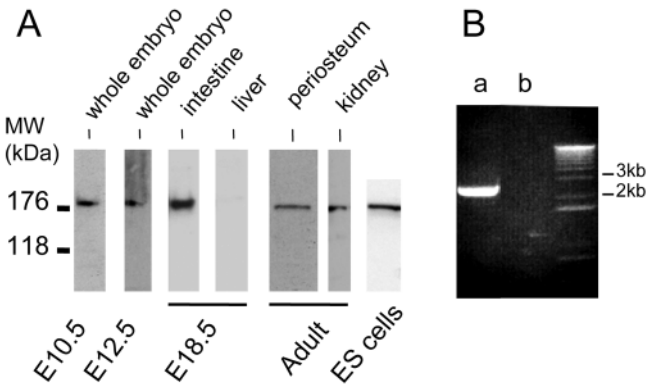


Fig. 6. Identification of endogenous tenascin-W. (A) Western blot analysis with an anti-tenascin-W antibody of extracts from E10.5 and E12.5 whole embryos, intestine and liver of E18.5 embryos, periosteum and kidney of adult mice and of embryonic stem (ES) cells conditioned medium. All of the samples reveal a single tenascin-W band of 180 kDa except for liver, which is negative for tenascin-W. (B) Agarose gel of nested-PCR products amplified from an E18.5 whole embryo mouse library using primers covering the second to the ninth fibronectin type III repeats of mouse TN-W, with (a) and without (b) template.

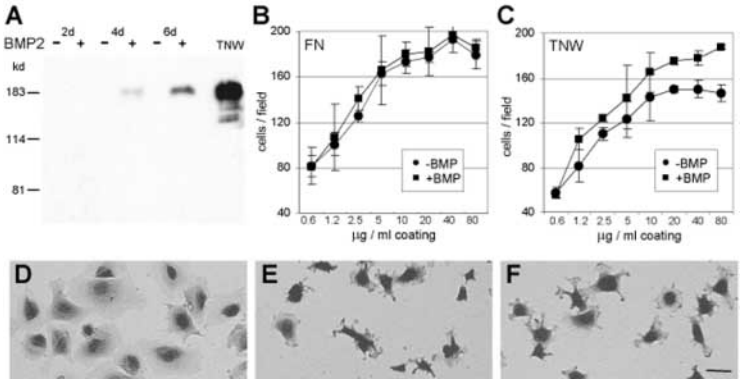


Fig. 7. C2C12 osteoblasts express tenascin-W and adhere to a tenascin-W coated substratum. (A) Tenascin-W accumulates in the conditioned medium of BMP2-treated C2C12 cells (+) but not in untreated cultures (–) collected after 2, 4 and 6 days of treatment as revealed on the immunoblot with anti-tenascin-W antibody. The C2C12-produced tenascin-W migrates with the purified recombinant tenascin-W loaded as control (TNW). (B) Adhesion assay of C2C12 cells after culturing for 6 days with or without BMP2 on fibronectin. (C) Adhesion assay of C2C12 cells after culturing for 6 days with or without BMP2 on tenascin-W. Treated and untreated cells adhered equally well to fibronectin but BMP2 treatment increased the adhesivity of the cells to tenascin-W. (D,E) Photographs of the C2C12 cells of the adhesion assay shown in (B,C). Cells without BMP treatment on fibronectin (D), on tenascin-W (E) and BMP2-treated cells on tenascin-W (F). The cell morphology of treated and untreated cells remains the same. Scale bar, 20 μm (D-F).

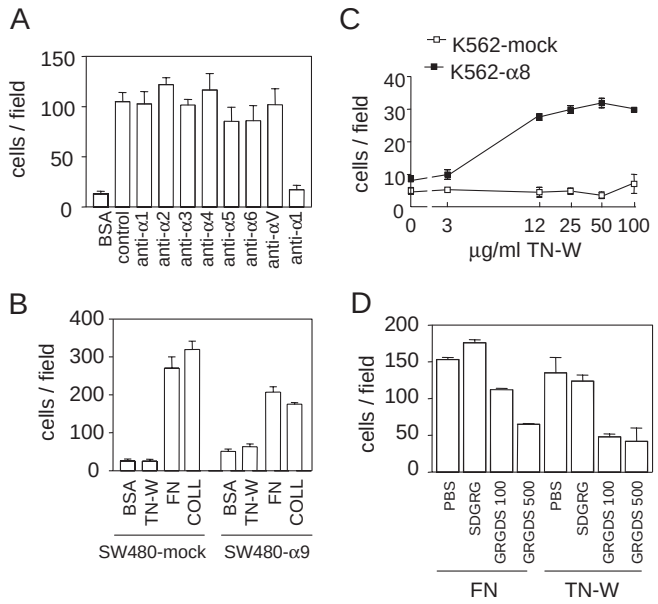


Fig. 8. Adhesion to tenascin-W is α8β1-integrin dependent. (A) Adhesion assay of T98G glioblastoma cells on tenascin-W coated at 20 μg ml^{-1}. Function-blocking antibodies against specific integrin subunits were included at 10 μg ml^{-1} as indicated. None of the α-specific antibodies tested inhibited adhesion, but the anti-β1 antibody reduced the adhesion to the level of the BSA coating. (B) Adhesion assay of SW480 colon carcinoma cells transfected with α9 integrin (SW480-α9) or with the empty plasmid (SW480-mock) on tenascin-W (TN-W), fibronectin (FN) or collagen (COLL). (C) Adhesion assays with α8-integrin- or mock-transfected K562 leukaemia cells on increasing concentrations of tenascin-W reveals that α8-transfected cells acquire adhesiveness to tenascin-W. (D) Adhesion of T98G cells to tenascin-W (TN-W, coated at 20 μg ml^{-1}) and fibronectin (FN; coated at 20 μg ml^{-1}) in the presence or absence of PBS, 100 μg ml^{-1} and 500 μg ml^{-1} of GRGDS or 500 μg ml^{-1} SDGRG peptides as indicated.

harvested and re-plated onto tenascin-W-coated wells for 1 hour in serum-free medium. After fixation, the α8-transfected cells were stained with anti-α8 antiserum as well as with phalloidin to reveal the F-actin (Fig. 9C,D). We found that the cells with prominent α8 staining were more spread and had prominent stress fibres. Looking at lower magnification at the EGFP fluorescence of the double-transfected cells revealed that more fluorescent cells had attached to tenascin-W than to plain plastic and many of the cells were spreading and exhibited more extensive processes than the EGFP-only transfected cells (Fig. 9E-J). In order to confirm a potential interaction between α8 integrin and tenascin-W in vivo, we performed staining of both proteins in the periosteum around the vertebra of an E16.5 mouse embryo (Fig. 10A,B) and in adult kidney (Fig. 10C,D) and found the two proteins to be co-localized in these organs.

Discussion

The aim of this study was to characterize a mammalian homologue of a new member of the tenascin family previously described in zebrafish and called tenascin-W (Weber et al., 1998). Mouse tenascin-W was cloned from an E19 whole-mouse-embryo library, sequenced, expressed in HEK-293 and purified as described. Structurally, it is mostly comparable to zebrafish tenascin-W, with the major exception that the mouse protein contains nine FNIII repeats instead of five in the zebrafish orthologue. The sequence available in the database corresponding to human tenascin-W (accession number AL049689) shows that the human form contains nine FNIII repeats as well. The structural organization of mammalian tenascin-W, described in Fig. 1, shows that tenascin-W is a true member of the tenascin family. Tenascin-W is the fourth and last member of the tenascin family in mouse and man. Judging from sequences in the genomic databases no further tenascin gene seems to exist. It is known that tenascin-C molecules can assemble in a highly symmetrical hexameric structure called a hexabrachion (Erickson and Inglesias, 1984). Tenascin-C molecules interact by the heptad repeats to form trimers that are further stabilized by inter-chain disulphide bonds (Kammerer et al., 1998). Two such trimers can then dimerize and form a disulphide-linked hexamer. So far, tenascin-C was the only member of the tenascin family known to form hexabrachions (tenascin-R can form trimers) (Norenberg et al., 1992). In this study, we demonstrated that recombinant mouse tenascin-W can form hexabrachions, which can be visualized either by biochemical analysis or directly by rotary shadowing followed by electron microscopy.

Alternative splicing is known to occur in all tenascins at the level of the FNIII repeats. In tenascin-C, nine different FNIII repeats can be included or excluded by RNA splicing (Chiquet-

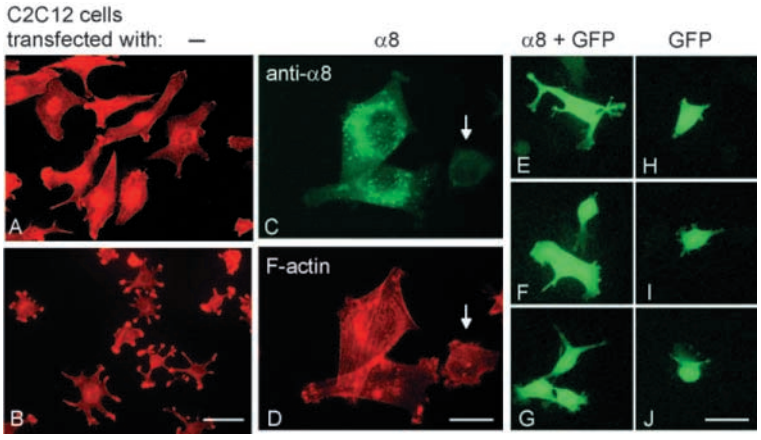


Fig. 9. Overexpression of α8 integrin increases adhesion of C2C12 cells to tenascin-W. Phalloidin staining of C2C12 cells after 1 hour of adhesion on fibronectin (A) reveals stress fibre formation. Phalloidin staining of C2C12 cells reveals the extension of actin-rich processes in cells adherent to tenascin-W (B). C2C12 cells were transfected with an α8 expression plasmid alone (C,D) together with an EGFP plasmid (E-G) or with the EGFP plasmid alone (H-J) and, 2 days later, replated for 1 hour on tenascin-W-coated wells. After fixation, cells shown in (C,D) were stained with anti-α8 antiserum (C) and phalloidin (D). Cells overexpressing α8 are more spread and contain stress fibres, whereas the untransfected cell to the right (arrow) starts to make actin-rich processes. Cells transfected with α8 are more spread and have longer processes (E-G) than EGFP-only transfected cells (H-J). Scale bar, 25 μm (A,B,E-J), 10 μm (C,D).

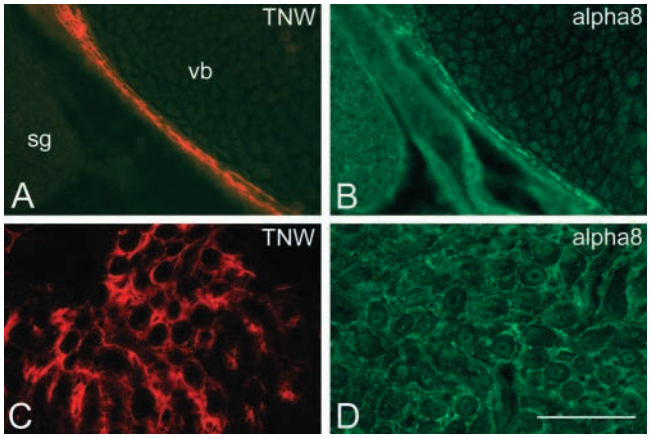


Fig. 10. Co-localization of α8 integrin with tenascin-W. Immunostaining of tenascin-W (red, A,C) and α8 integrin (green, B,D) in sections of an E16.5 mouse vertebra (A,B) and of an adult mouse kidney (C,D). Scale bar, 200 μm.

Ehrismann, 1995). The alternative tenascin-C splicing variants show different expression patterns and can have different functions (Meiners and Geller, 1997; Fischer et al., 1997). Alternative splicing also occurs in tenascin-R (Norenberg et al., 1992) and tenascin-X (Ikuta et al., 1998). Surprisingly, we could only detect a single protein band in whole embryo extracts, which was the same size as the tenascin-W from adult tissues and the recombinantly expressed protein of our full-length cDNA. Furthermore, PCR reactions from cDNA of whole embryos revealed only a single band spanning FNIII repeats 2-9. This indicates that either there are no splice variants or that alternative FNIII repeats could be inserted in exchange of the ones described by us without changing the total number of repeats present in the full molecule. Alternatively, the sensitivity of our detection methods was not high enough to detect other more minor species of tenascin-W. So far, no evidence for alternative splicing of tenascin-W can be found in any entries of mouse, human or zebra fish expressed sequence tag databases.

The expression of tenascin-W during development and in the adult mouse was investigated. Tenascin-W expression is highly regulated during development and in the adult. This characteristic is shared with the other members of the tenascin family. Indeed, tenascin-R is expressed only in the nervous system, tenascin-X is prominently expressed in muscle and skin, whereas tenascin-C is present in more developing tissues, including the nervous system (Chiquet-Ehrismann, 2002). The pattern of tenascin-W expression during development suggests that it is likely to be involved in specific events during embryogenesis, like palate formation, osteogenesis and smooth muscle development. Tenascin-W was often co-expressed in a subset of tenascin-C positive tissues, with two major exceptions – intestine and masseter – in which tenascin-W was present without tenascin-C. This indicates that tenascin-W can function independently of tenascin-C. Tenascin-W was also found in the adult associated with stem cells of the aortic valve, the limbus around the cornea and the rib's periosteum. The strong expression in kidney suggests a role in renal function. Some of our results confirm observations made in zebrafish, including the absence of tenascin-W in the central nervous system and the presumptive role in the formation of the skeleton (Weber et al., 1998). Further studies will be needed to confirm a role of tenascin-W in these developmental mechanisms and to investigate the molecular factors regulating the expression of tenascin-W. Using C2C12 cells, we have shown that tenascin-W expression is induced following osteogenic differentiation initiated by BMP2.

Tenascins are considered as proteins that modulate adhesion of cells to the extracellular matrix. For example, tenascin-C binds to the 13th FNIII repeat of fibronectin and can thereby impair adhesion on this substratum of all cell lines tested so far (Huang et al., 2001). Tenascin-Y, the avian homologue of tenascin-X, can inhibit cell attachment and spreading (Tucker et al., 2001b) and also tenascin-X was described as a poor cell adhesion protein (Elefteriou et al., 1999). By contrast, tenascin-W promoted cell adhesion of C2C12 cells and did not inhibit adhesion of these cells to fibronectin, laminin or collagen (data not shown). Not only could these cells adhere to tenascin-W but also they partially spread and extended actin-rich processes. The morphology of the cells resembled the ones described on a tenascin-C substratum (Fischer et al., 1997). Tenascin-W can thus be considered as an adhesive molecule that promotes partial cell spreading, although without extensive stress fibre formation as on fibronectin. No anti-adhesive property could be detected for tenascin-W when it was mixed with fibronectin (not shown) and, in this respect, it differs from tenascin-C.

Given that tenascin-W provides an adhesive substratum, it was important to characterize the receptors responsible for this adhesion. We first used T98G human glioblastoma cells. These cells were able to adhere to tenascin-W. The adhesion was clearly β1-integrin dependent. Integrins are a large family of heterodimeric cell surface receptors, composed of an α and β subunit. The β1 subunit can associate with many α subunits: α1-α11 and αV (Hynes, 2002). None of the function-blocking antibodies directed against the α integrins that we tested (α1-α6 and αV) were able to inhibit adhesion to tenascin-W. α9 integrin was considered because tenascin-W contains three IDG motifs in its sequence, which are known to be recognition motifs for this α subunit (Yokosaki et al., 1998). Using α9-transfected cells, we could exclude this integrin as a receptor for tenascin-W. Finally, α8 integrin was tested because its expression pattern in the kidney, developing rib and smooth muscle from the gastrointestinal tract was reminiscent of the expression pattern of tenascin-W (Schnapp et al., 1995; Denda et al., 1998). Indeed, α8β1 integrin can serve as a receptor for tenascin-W, because α8-transfected K562 leukaemia cells adhered to tenascin-W. Furthermore, overexpression of α8 integrin in C2C12 increased cell adhesion of this cell type to tenascin-W as well. The adhesion to tenascin-W is RGD dependent. This is in line with previous studies using α8 transfected cells, which were shown to adhere to other RGD-containing ligands such as fibronectin and vitronectin (Schnapp et al., 1995a) and the more recently discovered ligand LAP-TGFβ1 (Lu et al., 2002). The possible in vivo relevance of α8-integrin/tenascin-W interaction was confirmed by the fact that the proteins were localized together in periosteum and kidney. The kidney phenotype of α8 knockout mice emphasized the important role of α8 integrin in kidney development (Muller et al., 1997). However, most of the known ligands of α8 integrin (fibronectin, vitronectin and osteopontin)

are either dispensable for or not involved in α8β1 signalling during kidney development (Miner, 2001). Recently, a new α8β1 integrin ligand called nephronectin was proposed as the relevant ligand mediating α8β1 function in the kidney (Brandenberger et al., 2001). Because the evidence is based solely on its expression pattern, we suggest that tenascin-W be considered as an additional functional ligand for α8β1 during kidney morphogenesis. Future studies will show whether there are other receptors for tenascin-W and whether α8β1 actually functions as a receptor for tenascin-W in vivo. Finally, it will be of interest to investigate whether tenascin-W can substitute at least in some tissues for tenascin-C in tenascin-C-deficient mice.

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