Tenascin-Y: A Protein of Novel Domain Structure Is Secreted by Differentiated Fibroblasts of Muscle Connective Tissue

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Abstract. Tenascin-Y was identified in chicken as a novel member of the tenascin (TN) family of ECM proteins. Like TN-C, TN-R, and TN-X, TN-Y is a multidomain protein consisting of heptad repeats, epidermal growth factor-like repeats, fibronectin type III–like (FNIII) domains and a domain homologous to fibrinogen. In contrast to all other known TNs, the series of FNIII domains is interrupted by a novel domain, rich in serines (S) and prolines (P) that occur as repeated S-P-X-motifs, where X stands for any amino acid. Interestingly, the TN-Y-type FNIII domains are 70–100% identical with respect to their DNA sequence. Different TN-Y variants are created by alternative splicing of FNIII domains. Although, based on sequence comparisons TN-Y is most similar to mammalian TN-X, these molecules are not species homologues. TN-Y is predominantly expressed in embryonic and adult chicken heart and skeletal muscle and, to a lower extent, also in several non-muscular tissues. Two major transcripts of ~6.5 and 9.5 kb are differentially expressed during heart and skeletal muscle development and are also present in the adult. Anti-TN-Y antibodies recognize a ~400-kD double band and a ~300-kD form of TN-Y on immunoblots of chicken heart extracts. In situ hybridization and immunofluorescence analysis of aortic smooth muscle, heart, and skeletal muscle revealed that TN-Y is mainly expressed and secreted by cells within muscle-associated connective tissue. Cultured primary muscle fibroblasts released a ~220-kD doublet and a ~170-kD single TN-Y variant only when cultured in 10% horse serum but not in medium containing 10% fetal calf serum. All TN-Y variants isolated bind to heparin under physiologically relevant conditions that may indicate an important function retained in all tenascins.

THE tenascin (TN)[1]-family of extracellular matrix glycoproteins currently counts three members in vertebrates: TN-C, TN-R, and TN-X (for review see Erickson, 1993; Chiquet-Ehrismann et al., 1994; Chiquet-Ehrismann, 1995, and references therein). They exhibit a modular structure, typical for extracellular matrix proteins, that evolved through the shuffling of mobile domains. The mechanism of shuffling modules with defined subfunctions may have proven to be an efficient mechanism to create new, multifunctional proteins (Patthy, 1991; Bork, 1992; Engel et al., 1994; Doolittle, 1995).

All tenascins are built of four types of sequence motifs which can fold independently into structural units: 3–3.5 NH2-terminal heptad repeats, 4.5–18.5 tenascin-type epidermal growth factor (EGF)-like repeats, a series of fibronectin type III–like (FNIII) domains, and a fibrinogen-like domain at their COOH terminus. The latter is the most conserved part among all tenascins (Erickson, 1994). A portion of FNIII domains called the variable domains is subject to alternative splicing that creates various splice forms differing in number and combination of their variable domains. Depending on the species, TN-C has six to eight variable domains, some of which evolved only recently by internal gene duplications (Gulcher et al., 1990). Only one variable domain has been identified so far in chicken and rat TN-R (Nörenberg et al., 1992; Fuss et al., 1993) but the cysteine-rich NH2-terminal region of chicken TN-R undergoes differential splicing as well (Nörenberg et al., 1992). TN-X splice variants are produced by different mouse cell lines (Matsumoto et al., 1994) and were reported to occur in some porcine tissues (Geffrotin et al., 1995).

In the case of TN-C, two subunit trimers are disulfide linked to form a six-armed molecule, a hexabrachion (Erickson and Iglesias, 1984). Although TN-R has all the features to assemble into a six-armed molecule as well, it seems to form trimers, dimers and monomers only (Pesheva et al., 1989; Nörenberg et al., 1992). The microscopic

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1. *Abbreviations used in this paper:* DIG, digoxigenin; DOC, deoxycholate; HS, horse serum; MHC, major histocompatibility complex; SP, serine/proline; TN, tenascin.

structure of TN-X has not been reported yet, but there is evidence that TN-X may also assemble into higher order structures (Bristow et al., 1993; Matsumoto et al., 1994).

The search for functional properties of the tenascin prototype, TN-C, revealed complex effects in modulating cell adhesion and neurite outgrowth. This turned out to be true for TN-R as well, and thus could be a general feature of the tenascins (reviewed in Chiquet-Ehrismann, 1991, 1993; Husmann et al., 1995; Schachner et al., 1994). In vitro studies using recombinant proteins composed of various combinations of TN-C domains helped to assign specific functions such as cell binding activity, anti-adhesive activity, or the ability to bind to other extracellular matrix molecules to different sites within TN-C (Spring et al., 1989; Prieto et al., 1992; Aukhil et al., 1993; Götz et al., 1996). Sometimes, results obtained using bacterial as opposed to eukaryotic expression systems are inconsistent. Aukhil et al. (1993) mapped two heparin-binding sites within TN-C, whereas Fischer et al. (1995) showed that a single region within the fibrinogen globe of TN-C is necessary and sufficient for binding of the intact protein to heparin. Since TN-X has also been shown to bind to heparin (Matsumoto et al., 1994), this ability may be retained in all tenascins, and thus is likely to play an important functional role in vivo. Differential expression of TN-C splice forms is a subtle way to adapt its in vivo activities to tissue specific needs. It was shown that the smallest TN-C splice variants bind more strongly to fibronectin (Chiquet-Ehrismann et al., 1991) and that the presence of alternatively spliced FNIII domains induces the loss of focal adhesions in endothelial cells (Murphy-Ullrich et al., 1991).

The interest in TN-C is based on its dynamic expression in many developing organs, whereas only a few adult tissues express TN-C (reviewed in Chiquet-Ehrismann, 1995). Expression of TN-R is restricted to the central nervous system while TN-X is quite ubiquitously expressed in embryonic and adult tissues but is most abundant in fetal and postnatal muscle tissue and testis (Rathjen et al., 1991; Bristow et al., 1993; Matsumoto et al., 1994; Geffrotin et al., 1995). In a number of tissues, the expression of various tenascins has been observed to overlap. This either indicates that different functional properties of the tenascins are needed or that expression is redundant in a particular tissue. In this context, the lack of an obvious phenotype in mice deprived of TN-C (Saga et al., 1992) deserves further investigation, and yet unidentified tenascin-like genes may come into play that have not been considered so far.

To date, two tenascin genes are known in chicken, namely TN-C and TN-R. The result of a low stringency hybridization of chicken genomic DNA with probes derived from either the human TN-X or chicken TN-C EGF-like region pointed to the existence of further tenascin-like genes in chicken (Baumgartner and Chiquet-Ehrismann, 1993; Chiquet-Ehrismann et al., 1994). This report presents the characterization of a new member of the tenascin family in chicken that we termed TN-Y. We describe the cloning of cDNAs comprising the complete TN-Y coding region and the purification of the encoded protein from chicken heart extracts and primary cell culture conditioned media. Furthermore, we investigated the distribution of TN-Y mRNA and protein in situ and present evidence for muscle fibroblasts as a source of TN-Y.

Materials and Methods

Isolation of TN-Y cDNA Clones from Chicken Heart cDNA Libraries

Clone pcx (see Fig. 1 A) was amplified from chicken genomic DNA using two sets of nested primers. The first primer-set (set 1: 5' GGCACNG-CAGGNGACTCCATG 3' and 5' GGTCTCAGYTTCATYTC 3') corresponds to nt 6013-6033 and nt 6238-6254. Subsequent internal amplifications were performed with the human TN-X derived primer-set 2 (5' GAGGGGCCTGGTGG 3' and 5' TTCATTTCCGTGAAGGG 3'), corresponding to nt 6113-6126 and nt 6229-6245. The cDNA clones depicted in Fig. 1 A are derived from two cDNA libraries (except ptny3) by two different strategies: cy cDNA clones were obtained by screening a λgt11 adult heart cDNA library (Clonetech, Palo Alto, CA), and pcz cDNA clones are PCR products from a λZAP cDNA library of E18 chicken heart, constructed with the λZAP-cDNA synthesis kit (Stratagene, La Jolla, CA) according to the manufacturer's protocol.

One screening procedure included 10⁶ λgt11 phages, plated at a density of 2 × 10⁵ pfu/plate (24 × 24 cm). Duplicate lifts onto Hybond N or N⁺ nylon membranes (Amersham Intl., Amersham, UK) were treated according to standard procedures and UV cross-linked. All membranes were hybridized to random-labeled digoxigenin (DIG)-dUTP TN-Y derived cDNA-probes according to the manufacturer's protocol (Boehringer Mannheim Biochemica, Mannheim, Germany), using probes at a final concentration of 10-15 ng/ml. Detection of positive plaques was carried out following the DIG luminescence detection kit protocol (Boehringer Mannheim Biochemica, Mannheim, Germany). Positive clones were picked and purified by one or two additional rounds of screening. For reprobing, filters were stripped 2 × 20 min in 0.2 M NaOH, 0.1% SDS at 37°C, followed by a short wash in 2× SSC and stored at 4°C. The cDNA fragments were cut out with EcoRI from large scale phage DNA preparations and subcloned into pBluescript KS vectors (Stratagene, La Jolla, CA).

Screening with pcx resulted in the isolation of cy1 and cy3. To select for cDNA clones that provide further 5' sequence information, the same filters were rehybridized to DIG-labeled cy3. From the 11 positive cDNA clones (cy4 to cy14) which were obtained, cy5 and cy6 were completely sequenced, the others were characterized by partial sequencing and restriction mapping. The 5' and the 3' terminal sequences of all clones isolated exclusively encoded for FNIII domains which were either identical or highly homologous to each other even within one cDNA. Further screening of either the λgt11 adult heart or the λZAP embryonic day 18 heart cDNA library with probe cy6 resulted in the isolation of this type of cDNA clones. These findings indicate that identical and highly similar FNIII domains occur several times in different orders within the TN-Y transcript and render cDNA alignment difficult. Furthermore, they implicate a complex pattern of differential splicing of the TN-Y transcripts.

To obtain more cDNA clones without the use of hybridization with the repetitive sequences, PCR amplification on 4-8 × 10⁴ phages of the λZAP-cDNA library per 50-μl reaction was performed with Taq-polymerase (Boehringer Mannheim Biochemica, Mannheim, Germany) using nested gene-specific primers in combination with nested pBluescript SK vector primers. The gene-specific primers were designed for sequences unique for the FNIII domain YD2 as it appeared to be less abundant in the TN-Y transcript than the YE-type domains. Two different cDNA clones pcz1a and pcz1b (Fig. 1 A) were amplified by this approach and provided new 5' sequence information. The two clones cy66 and cy102 could be isolated from the λgt11 adult heart cDNA library in two successive screenings, using a probe that encompassed the 28-amino acid extension and the half FNIII domain lead to the isolation of cy66 which was then used as a probe to isolate cy 102 (see Fig. 1 A).

RNA Isolation, Reverse Transcription/Polymerase Chain Reaction (RT/PCR)

Various tissues of adult and embryonic day 4, 8, 11, and 18 chicken were dissected, quickly frozen, and then powdered in liquid nitrogen. Total RNA was prepared as described (Chomczynski and Sacchi, 1987) and used for poly(A)⁺ RNA preparation with the mRNA purification kit (Pharmacia, Uppsala, Sweden) according to the manufacturer's protocol. First strand cDNA of poly(A)⁺ RNA was synthesized by AMV reverse transcriptase (Boehringer Mannheim) with oligo d(T)$_{12-18}$ primers (United States Biochemical, Cleveland, OH) or random hexamer primers. Gene specific primers P1 and P3 (Fig. 2 A) with an EcoRI and SalI restriction

site attached on either side to allow directional subcloning into pBlue-script KS vectors (Stratagene, La Jolla, CA) were used to amplify the 4.4-kb cDNA ptny3 from E18 chicken heart cDNA with the Expand Long Template PCR System (Boehringer Mannheim).

Construction of Deletion Clones and DNA Sequencing

Nested deletions of cy5, cy6, cy66, cy102, and ptny3 were prepared with the Nested Deletion Kit (Pharmacia, Uppsala, Sweden) as proposed by the manufacturer. Mini DNA-preparations of the nested deletion clones were linearized with appropriate restriction enzymes and sorted on 0.8% agarose gels according to their sizes. This was absolutely essential to obtain a correct order of the tandemly repeated FNIII domains which are highly or completely identical. Sequences were obtained using Sequenase Version 2.0 (United States Biochemical).

Northern Blot Analysis and In Situ Hybridization

Electrophoresis of 10 μg of total RNA (see RNA-isolation) per lane in formaldehyde containing gels was performed as described (Sambrook et al., 1989), followed by overnight transfer onto nylon membranes (positively charged; Boehringer Mannheim). The blot was stained in 0.5 M Na-acetate (pH 5.2) containing 0.04% methylene blue to visualize the 28S and 18S rRNA. Membranes were hybridized using 50 ng/ml anti-sense ribo-probe of cy5 and hybridizing bands were detected using the DIG-luminescent detection kit (Boehringer Mannheim). In situ hybridization was done on freshly prepared cryosections (15 μm) of different chicken embryonic tissues as described by Koch et al. (1995). DIG-labeled anti-sense ribo-probes were in vitro transcribed from the TN-Y-derived cDNA clone cy5, from the TN-C derived cDNA clone cTn8 (see Spring et al., 1989) and from a cDNA clone derived from chicken laminin γ1, which was kindly provided by Dr. Roberto Perris (manuscript in preparation).

Isolation, Purification, and Electron Microscopy of TN-Y Protein Variants

Commercially available frozen hearts from adult chicken were dissected from fat but not from the major vessels, homogenized at 4°C with a Poly-tron homogenizer in 20 mM Tris (pH 7.5) washing buffer, containing protease inhibitors PMSF (1 mM) and NEM (2.5 mM) (Fluka, Buchs, Switzerland), and centrifuged for 30 min at 19,700 g. This washing step was repeated once, followed by homogenization of the pellet in 5–10 times the volume of extraction buffer (pH 8.0) that contained 25 mM Tris, 400 mM NaCl, and 10 mM EDTA and protease inhibitors as described above. Extraction proceeded overnight at 4°C, the suspension was centrifuged at 23,500 g and the supernatant was passed through a filter and concentrated by 45% ammonium sulfate precipitation at 4°C. The residual pellet was treated with 2% deoxycholate (DOC), 2 mM EDTA in washing buffer for 1 h at 4°C, and aliquots of supernatants and pellets from each step were tested by Western blotting using anti-TN-Y antibody (not shown). The major part of TN-Y polypeptides from either heart extracts or conditioned media of cell cultures was detected in the 45% ammonium sulfate precipitate and was dialyzed against 1× TBS. Subsequently, the sample was passed over a gelatine agarose column (Sigma, Buchs, Switzerland) and the flow through was applied immediately to an anti-TN-Y affinity column. The column was washed extensively with TBS/0.01% Tween (wash 1), followed by a wash step with the same buffer including 1% Triton X-100 (wash 2). The TN-Y protein was eluted with 8 M LiCl, pH 11.0, at 4°C. Fractions were immediately neutralized, dialyzed in 1× TBS, 0.01% Tween at 4°C, and shock frozen in liquid nitrogen. Samples for electron microscopy were prepared and analyzed as described in Koch et al. (1992).

SDS-PAGE and Immunoblotting

Affinity-purified TN-Y or samples of conditioned media were separated by 6 or 3–15% gradient SDS-PAGE under reducing or non-reducing conditions. Protein bands were either stained with Coomassie Blue or electroblotted (Milliblot™; Millipore Corp., Bedford, MA) to Immobilon-P membranes (Millipore) which were incubated with affinity-purified anti-TN-Y antibody, diluted 1:2,000, and detected with horseradish peroxidase conjugated secondary antibody (Cappel, Durham, NC), diluted 1:2,000, followed by ECL-detection (Amersham Intl.).

Heparin Binding Assay

Fractions of affinity-purified, dialyzed TN-Y were passed over a heparin agarose (Sigma) column followed by extensive washes with 1× TBS (20 mM Tris, pH 7.4, 150 mM NaCl), 0.01% Tween and eluted with 1 M NaCl in 1 × TBS, 0.01% Tween. Loaded material, flow through, washes and eluted fractions were analyzed by SDS-PAGE and immunoblotting.

Staining of Cryosections

Air-dried and fixed (4% paraformaldehyde) cryosections were blocked in 1× TBS/0.3% BSA, incubated first with anti-TN-Y antibody and then with the appropriate rhodamine-labeled secondary antibody (Cappel, Durham, NC) with intermediate washes. Sections were overlaid with 90% glycerol in PBS and covered with coverslips. In some cases, adjacent sections were stained by hematoxylin and eosin to reveal the tissue architecture.

Tissue Cultures

Hind limb muscles from 10–12-d-old chicken embryos were dissected, trypsinized with a 1:10 dilution of trypsin (0.25% stock, GIBCO BRL) in PBS for 3 × 15 min at 37°C, and collected in medium containing 10% horse serum (HS) or FCS. After centrifugation, the pellet was resuspended in an appropriate volume of serum-free minimum essential medium (MEM; GIBCO BRL) containing 4% chicken embryo extract (prepared from 11-d-old chicken embryos with an equal volume of 0.11% (wt/vol) glucose in PBS). Cells were either plated directly in the presence of 10% HS (GIBCO BRL) or 10% FCS (GIBCO BRL) on tissue culture dishes coated with 0.3 μg/ml gelatine (Bio-Rad) to obtain a myoblast/muscle fibroblast co-culture, or they were preplated on non-coated plastic tissue culture dishes for 1 h at 37°C to separate adhering muscle fibroblasts from non-adhering myoblasts. The myoblast-enriched supernatant was either discarded or plated on gelatine-coated tissue culture dishes. Adhering muscle fibroblasts were slightly trypsinized in a 1:10 dilution of trypsin, pelleted by centrifugation and resuspended in MEM containing 4% chicken embryo extract with either 10% HS or 10% FCS. Cells were plated at a density of about 6 × 10⁴ /ml in 24-well plastic tissue culture dishes and aliquots of conditioned media were collected at different time intervals.

Results

Structural Similarities and Differences in the Primary Structure of TN-Y Compared to the Other Tenascins

Since the fibrinogen globe is the most conserved part of the known tenascins, we designed nested primers specific for the human TN-X fibrinogen part to perform PCR reactions on chicken genomic DNA. A genomic fragment of 460 bp (pcx, Fig. 1 *A*) was amplified that contains 130-bp coding sequence interrupted by an intron of 330 bp. Subsequent screening strategies (as explained in Materials and Methods) led to the identification of the cDNA clones depicted in Fig. 1 *A*. The complete sequence, coding for one splice variant of TN-Y was deduced from cy102, cy66, cy1, and ptny3. Clone ptny3 represents a subcloned RT-PCR-amplification product. It was the smaller of two major PCR fragments of 7.5 and 4.4 kb which were amplified, using the primers P1 and P3 (Fig. 1 *B*). Two major bands of the appropriate relative sizes 8.0 and 4.9 kb were also obtained with the primer set P4 and P3 (Fig. 1 *B*). This implies the existence of at least two major splice variants of TN-Y. We identified and sequenced two sets of cDNA-clones that arose by alternative splicing at least at two different sites of the TN-Y transcript (Fig. 1 *A*). Sequencing of sequential nested deletion clones of cy102, cy66, ptny3, and cy1 revealed an open reading frame of 5,827 bp encoding the smallest TN-Y-variant. The cDNA clones cy102 and cy1 comprise 5′ and 3′ untranslated sequences, respec-

tively, and thus delimit the coding region of the TN-Y mRNA. The predicted primary sequence of 1,914 amino acids for this variant and a structural model is presented in Fig. 2. The putative signal peptide cleavage site was determined according to the −1, −3 rule given by von Heijne (1986). The NH$_2$-terminal sequences, that contribute to the central globe of TN-C (Spring et al., 1989) and which are also present in TN-R (Nörenberg et al., 1992; Fuss et al., 1993) and TN-X (Bristow et al., 1993), are mostly absent from TN-Y. The NH$_2$ terminus of TN-Y is defined by a small proline-rich peptide (residue 20 to 36) which is followed by three and a half heptad repeats (residues 37 to 61). Heptad repeats form α-helical coiled-coil structures (Parry, 1982) which are thought to be responsible for the trimerization of TN-C (Spring et al., 1989) and TN-R (Nörenberg et al., 1992; Pesheva et al., 1989). In all tenascin molecules, this region is surrounded by a different number of cysteine residues that could contribute to the stabilization of multimeric structures. In TN-Y, four cysteines are found in this region compared to 7–10 cysteines in the other tenascins. Thus TN-Y monomers could also associate into oligomeric structures which may be of lower order than those described for TN-C (Erickson and Iglesias, 1984) and TN-R (Pesheva et al., 1989; Nörenberg et al., 1992).

Residues 90-134 represent one and a half EGF-like repeats where the intact repeat complies with the conserved cysteine spacing pattern $X_4CX_3CX_5CX_4CX_1CX_8C$, typical for the tenascins (Spring et al., 1989). The final 1/2 EGF-like repeat (residue 135-148) appears to be degenerated and only the cysteines 1 and 3 are retained.

The EGF-like region is followed by one FNIII domain (YA; residues 151-239), which is separated from the other FNIII domains by a novel sequence. This section contains a serine/proline (SP)-rich motif with the consensus sequence SPX (whereby X can be any amino acid) which is repeated 23 times within 98 amino acids (residues 260-352), followed by 99 amino acids that show no particular homology to any known sequence motif. This "SPX-containing domain" is unique to TN-Y and has not been reported to appear in other tenascins. Interestingly, it is joined to the second half of a FNIII domain (residues 452–512). To check the exon-intron organization of the TN-Y gene in the novel 5' region, we analyzed genomic DNA and genomic clones (not shown). Exon primers were used to sequence the exon-intron boundaries which are pointed out in Figs. 1 and 2. Our results confirmed the correct splicing of the described cDNA clones covering the 5' region of TN-Y. Interestingly, the SPX-domain and the half FNIII domain are encoded within a single exon which is
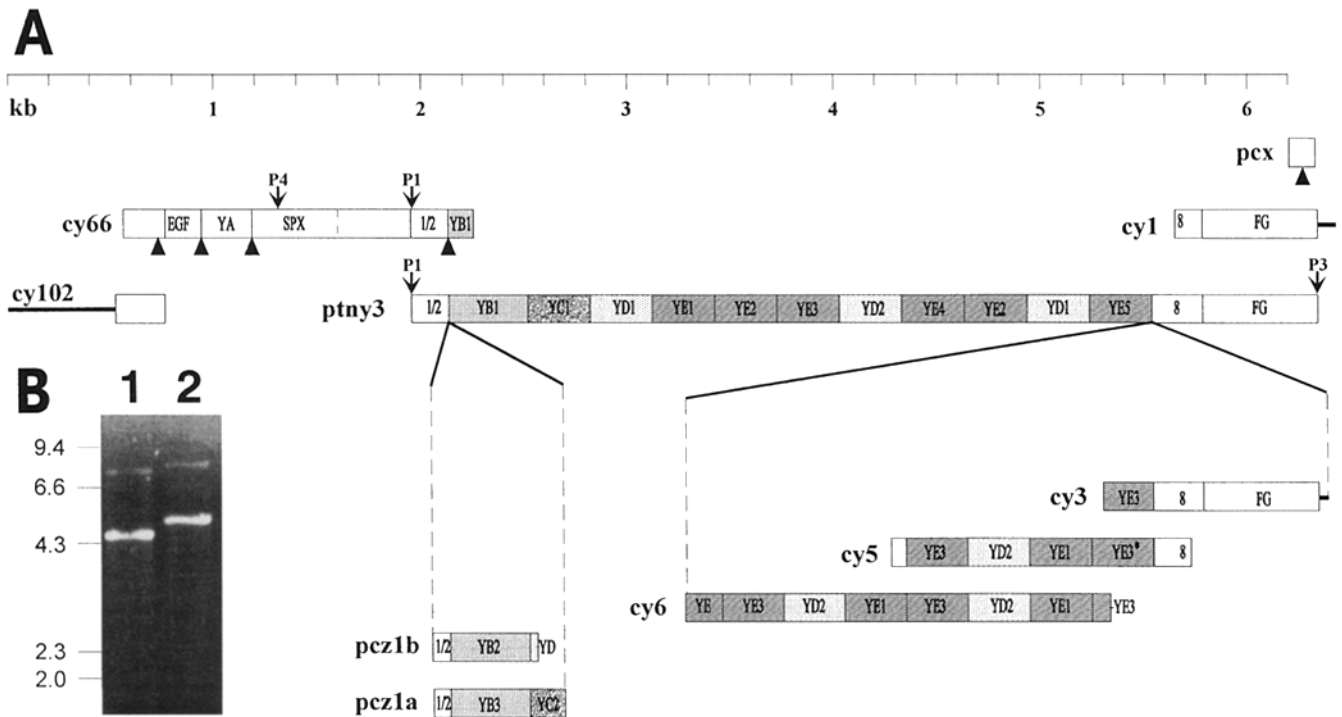

Figure 1. Alignment and PCR-amplification of TN-Y cDNA clones. (A) cy-clones were isolated by screening an adult heart cDNA library; p-clones were PCR amplified from either chicken genomic DNA (pcx), from E18 chicken heart cDNA library (pcz1a and pcz1b) or by RT-PCR from E18 chicken heart mRNA (ptny3). The scale bar at the top indicates the length of the cDNA clones in kilobases (kb). Clones cy102, cy66, ptny3 and cy1 comprise the full-length sequence of the smallest TN-Y variant. Two sets of clones deriving from alternative splicing at two sites are indicated (pcz1b/pcz1a and cy3/cy5/cy6). The different repetitive Y-type FNIII domains are defined by a distinct shading; other domains are shown as open boxes. Bars mark the 5' and 3' untranslated regions. EGF, EGF-like repeats; SPX, SPX-containing domain; 1/2, half FNIII domain; 8, FNIII domain 8; FG, fibrinogen globe; YA-YE, TN-Y-type FNIII domains (subtypes numbered). YE3* lacks the first codon but is otherwise identical to YE3. Filled arrowheads mark intron positions identified in genomic clones and arrows indicate the binding sites of primers P1, 3, 4 used for RT-PCR amplification from E18 chicken heart mRNA (B): lane 1, cDNA fragments amplified by primer-set P1/P3. The 4.4-kb fragment corresponds to ptny3; lane 2, cDNA fragments amplified by primer-set P4/P3.

A

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                              MTPGLPLLLLALALRRAQG 19
                              NAGASPGPPPVPPELSC 36
                                        GAEV 40
                                    LEAVLGR
                                    LRALEGE
                                    VRALQGQ 61
                      CGDSLGQAGTGSTAARRLCDTPGAGGC 89
                                CGCRLGESGDGLPC 103
                  PAPRCPLDCSDQGRCRAGRCHCFEG..FTGPFC 134
                  ATPVCPPGRGGPH.CTL 150
```

YA   EIPSVTLRLAARNQTSFRVTWPRPAKPVDGYEVAVIPMDEPAALTTHELPGSAVTFEVTGLTPGQAFEIFIQAQREQHLGAPGTLRVRT 239
LLAQSLPNHGGPRGTPTFLA 259

SPVAPASPSARQSPASPWSPVSLGSLGSPASLRSPVSPESSAAPASLESPWSPASPRSPESPASPVSPRSPTSPWSPASPQSPLSPASPISPV 352
LPNVPSLHELGVKLSSYNGSLLQRLESHLRATNFPLRGNQTVPAVARAILSYLLRRSPASLRYQFLRHLQQNPHPKPQVLPGAAGEALVDLDGLRGHAE 451

Y1/2   TVVIRYRLLEEPEGDEGEVRVPGDTTVARVPGLVPGATYRVEVHGVV.RGRVSKSYTSLVTA 512

YB1 { GLGDTSEPPPEWENLYDMEVTEPQGAMA
KAAPSEEEPPQRPRLGMLTVSHVTPSSIQLEWSVLEGTFDSFTVQYRDAQGQPQALAVDGGSRTVTVPGLSPSRHYKFNLYGVWGRKRIGFISTDAVT 638

YC1 DSAKPKEELPSQPRLGELTASHVSPDSVQLEWSVPEGSFDSFTVQYKDAQGQPQVVPVDGGLRTVTVPGLSPSRRYKFNLYGVWGRKRLGPMSTDAVT 736

YD1 AAEETEEEPPSQPRLGELTASHVSPNSVQLEWSIPEGSFDSFTVQYIDVQGQPQELHLDSGSRTVTVSGLLPSHPYKFNLYGVWGQRRLGPISTDTIT 834

YE1 AAAPAQKEPPSQPRLGELTASHVGPDSVQLEWSVPEGSFDSFTVQYKDAQGQPQVVPVDGGLRTVTVPGLSPSRRYKFNLYGVWGRKRLGPMSTDAVT 932

YE2 ..APAQKEPPSQPLLGELTASHVGPDSVQLEWSVPEGSFDSFTVQYKDAQGQPQVVPVDGGLRTVTVPGLSPSRRYKFNLYGVWGRKRLGPMSTDAVT 1028

YE3 AAAPAQEEPPSPPRLGELTASHVGPDSVQLEWSVPEGSFDSFTVQYKDAQGQPQVVPVDGGLRTVTVPGLSPSRRYKFNLYGVWGRKRLGPMSTDAVT 1126

YD2 AAEETEEEPPSQPRLGELTASHVSPNSVQLEWSIPEGSFDSFTVQYIDVQGQPQELHLDSGSRTVTVSGLLPSHPYKFNLYGVWGQTRLGPISTDTIT 1224

YE4 AAAPAQEEPPSPPRLGELTASHVGPDSVQLEWSVPEGSFDSFTVQYKDAQGQPQVVPVDGGLRTVTVPGLSLSRRYKFNLYGVWGRKRLGPMSTDAVT 1322

YE2 ..APAQKEPPSQPLLGELTASHVGPDSVQLEWSVPEGSFDSFTVQYKDAQGQPQVVPVDGGLRTVTVPGLSPSRRYKFNLYGVWGRKRLGPMSTDAVT 1418

YD1 AAEETEEEPPSQPRLGELTASHVSPNSVQLEWSIPEGSFDSFTVQYIDVQGQPQELHLDSGSRTVTVSGLLPSHPYKFNLYGVWGQRRLGPISTDTIT 1516

YE5 AAAPAQKEPPSQPRLGVLTASHVGPDSVQLEWSVPEGSFDSFTVQYKDARGQPQVVPVDGGLRTVTVPGLSPSRRYKFNLYGVWGRKRLGPMSTDAVT 1614

8   GAPGTLWVGTLWPRSAHLHWAPPHVPPEGYNLIYGPPGGPVKTLQLPPEATSKELWGLEPSGRYRVQL...WGRG.LEPLETTFDT 1696
PPLPHPHP 1704

RDCAEEQLNGPGPSREVLIFLGGDRQRPLHVFCDMESNGGGWLVFQRRMDGGTDFWRGWEEYVHGFGNVS 1774
GEFWLGNAALHTLTASGPTELRVDLRTPSDSAFARYRDFAVSGPEDNFRLHLGAYSGTAGDALSYHAGSP 1844
FSTRDHDPRGRPRPCAVAYTGAWWYRNCHYANLNGRYGVPYDHQGINWYPWKGFEYSIPFTEMKLRPQRD 1914

B

H₂N–ΜΜΜ–◆◆–| YA |–SPX–⟨½⟩ YB1 YC1 YD1 YE1 YE2 YE3 YD2 YE4 YE2 YD1 YE5 8 ●–COOH
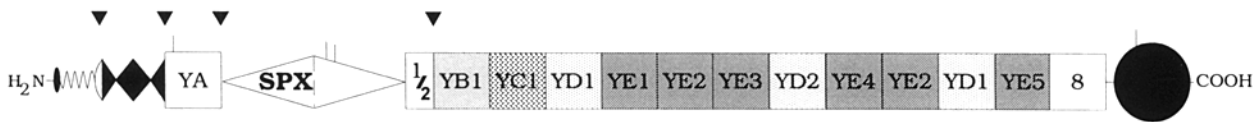
Figure 2. Model of a TN-Y arm. (*A*) 1,914 amino acids that define the small TN-Y variant are depicted according to their domain structure. The numbering of the residues is given on the right, FNIII domains are designated on the left. Intron-insertion sites (*arrowheads*) and putative N-glycosylation sites (*underlined*) are indicated. The signal peptide (1-19) is followed by a short proline-rich peptide (20-36) and 3 1/2 heptad repeats (37-61). A short cysteine-rich sequence (62-89) leads to the EGF-like region (90-150). The FNIII domain YA (151-239) precedes the SPX-containing domain (240-451) that contains 23 perfect S-P-X motifs (*shaded boxes*) and is together with the half FNIII domain contained in one exon. The following 11 FNIII domains YB1-YE5 (513-1614) are defined as repetitive Y-type FNIII domains, referring to their 70–100% sequence identity. FNIII domain 8 (1615-1696) corresponds to the equivalent FNIII domain in TN-C, TN-R, and TN-X; the four residues highlighted in bold (*W, L, Y, T*) are conserved in every FNIII domain. A short proline-rich peptide (1697-1704) leads to the COOH-terminal fibrinogen globe (1705-1914). (*B*) The TN-Y polypeptide is arranged as a structural model using different symbols for the various domains as proposed by Spring et al. (1989). A small ellipse indicates the NH₂ terminus, the wavy line stands for the heptad repeats, and the half circle to follow indicates a cysteine-rich region that precedes the complete and two half-EGF-like repeats which are shown as black diamonds. FNIII domains are drawn as boxes; distinct shading patterns for the repetitive FNIII domains were used to label the different types YB, YC, YD, YE. The SPX-containing domain, unique to TN-Y, that interrupts the FNIII domain series is shown as a rhomb, bisected by a dotted line that demarcates the part with repeated S-P-X-motifs from the second part which is joined to the half FNIII domain. A short dash connects the COOH-terminal fibrinogen globe, drawn as a black circle. The potential N-glycosylation sites are shown as thin vertical lines and intron insertion sites at the 5′ end are indicated as black arrowheads above the model. The sequence data are available from GenBank/EMBL/DDBJ under accession number X99062.

separated from the flanking FNIII domains by two introns. Further introns were found to separate the FNIII domain YA from the EGF-like repeats and the latter from the NH₂-terminal part including the heptad repeats. All splice junctions examined in the 5′ region revealed phase 1 introns that split the codon after the first position (Patthy, 1991).

The following 11 FNIII domains are 70–100% identical in terms of their nucleotide sequence and cannot be aligned in a collinear fashion with either TN-X, -C or -R (nor can FNIII domain YA). Therefore, we named them with a capital letter designating the type of domain followed by numbers for the varying subtypes. The preceding Y identifies them as FNIII domains of TN-Y to prevent

confusion with the TN-C nomenclature. They are referred to as repetitive FNIII domains and can be classified into four types of FNIII domains, YB, YC, YD, and YE. This classification was based on NH_2-terminal differences within the otherwise highly similar FNIII domains (see Fig. 2 A). The YB domains differ from all other FNIII domains by an NH_2-terminal 28 amino acid extension. Five YE subtypes (YE1-YE5) and two YD subtypes (YD1, YD2) could be identified within the ptny3 cDNA (Figs. 1 A and 2 B). YE1-YE5 occur singly, in a doublet or in a triplet, and are separated by either a YD2 or YD1 domain. Only single YB- and YC-type domains are represented in ptny3, indicating that they may occur less frequently than YE and YD domains. The same types of domains such as YB2 and YB3 or YE3 and YE4 are 88 to 99% identical on the amino acid and on the nucleotide level. Interestingly, the subtypes YD1 and YE2 occur twice as identical domains within clone ptny3. Only a single subtype of each, YB (YB1) and YC (YC1) were contained in this clone. Two more YB and one more YC subtypes were identified in the cDNA clones pcz1a and pcz1b, which were generated by an alternative splicing event 3' of the half FNIII domain. A second set of cDNA clones, cy3, cy5, and cy6 were generated by differential splicing after domain YE5 (Fig. 1 A). They contain the subtypes YD2, YE1, and YE3 that come as tandemly repeated triplet. FNIII domain YE3* lacks the first three nucleotides but is otherwise identical to YE3. If this difference genuinely reflects the organization of the TN-Y gene, cy6 might lie further 5' of the position shown in Fig. 1 A. These findings make clear, that identical and highly similar FNIII domains occur several times in different orders within the TN-Y transcript. Furthermore, they implicate a complex pattern of differential splicing of the TN-Y gene-products. The FNIII domain that follows the series of repetitive Y-type FNIII domains in ptny3 is most homologous to the corresponding one in all other tenascins and therefore is referred to as FNIII domain 8, according to the tenascin nomenclature proposed in several publications (Aukhil et al., 1993; Bristow et al., 1993; Chiquet-Ehrismann, 1993). Domains YD2, YE1, and YE3 of ptny3 differ in one single nucleotide each from their coun-

terparts in cy6 and cy5. We cannot exclude that such minimal sequence variations are due to PCR errors. FNIII domains with only one amino acid difference such as YE3 and YE4 show a number of additional differences within their DNA-sequence as well and are therefore considered as divergent domains.

Comparison of the COOH-terminal fibrinogen part and the FNIII domain 8 of TN-Y to the same region in the other tenascins revealed only 59% identity to human and mouse TN-X compared to over 80% identity between chicken, human, and mouse TN-C in this region (Fig. 3). This together with the different domain organization indicates that we did not isolate the chicken TN-X, but a new member of the tenascin family that we termed TN-Y (Chiquet-Ehrismann et al., 1994, 1995).

Expression of TN-Y in Developing and Adult Chicken

The expression of TN-Y transcripts was investigated by Northern blot analysis (Fig. 4) using cy5 anti-sense riboprobes (see Fig. 1 A). Two major TN-Y mRNA bands, roughly estimated to be 6.5 and 9.5 kb long, were detected on Northern blots of total RNA from tissues of different embryonic stages and adult chicken (Fig. 4). This result is in agreement with the amplification of two major TN-Y cDNA fragments in the RT-PCR shown in Fig. 1 B. A lower molecular mass band of ~5 kb appears after long blot exposure (Fig. 4, left panel) and is also visible in adult ovaries and lung. We cannot exclude that this band represents yet another splice variant of TN-Y which is differentially expressed during development and in the adult. However, we tend to consider the 5-kb band as a background signal since it is not reproducibly detected.

The 6.5- and the 9.5-kb TN-Y transcripts could not yet be detected in 4-d-old chicken embryos but were found to be most abundant in skeletal muscle and heart from later embryonic stages as presented in the developmental profile, as well as in adult tissues (Fig. 4). The 4-d-old embryos were dissected into trunk and head and the 8-d-old embryos into trunk, heart and head. RNA from the trunk was included in the developmental Northern blot. The RNAs
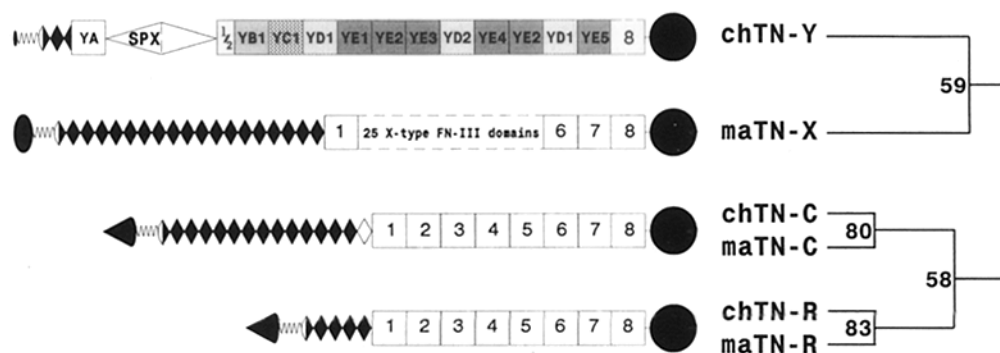

Figure 3. A comparison of the tenascins in chicken and mammals, and their structural relationship. The dendrogram was created by the multiple alignment program "pileup" based on the amino acid sequence of the fibrinogen globe and the adjacent FNIII domain 8 (according to Chiquet-Ehrismann et al., 1994). Numbers in the dendrogram indicate sequence identities in %. TN-Y and TN-X are as similar (59%) as are TN-C and TN-R (58%). On the left, only the smallest variant of each tenascin is shown. Symbols used for TN-Y are explained in Fig. 2. Differences at the NH_2 termini of the tenascins are indicated by black ellipses and pie segments; wavy lines represent heptads which are followed by EGF-like repeats shown as black diamonds (chicken and mammalian TN-C differ in one EGF-like repeat as indicated by a white diamond). Constant FNIII domains 1-8 in TN-C and TN-R and the equivalent domains in TN-X (6-8), and in TN-Y (8) are shown as white boxes. The 25 X-type FNIII domains are indicated by a large dotted box (not to scale). Black circles represent the NH_2-terminal fibrinogen globes in all tenascins.
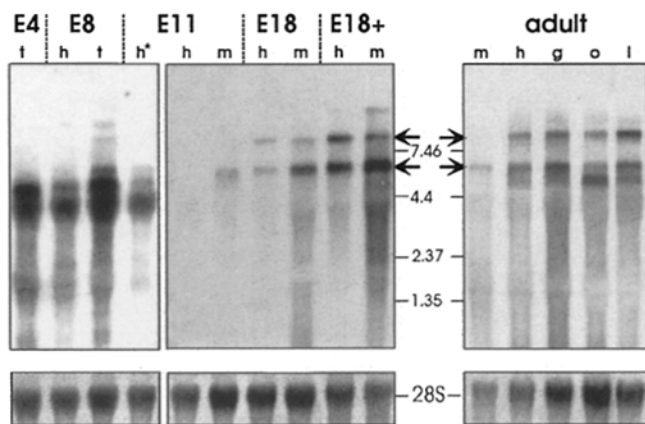
Figure 4. TN-Y mRNA expression in chicken heart, skeletal muscle and adult tissues. Northern blots of equal amounts (10 μg) of total RNA or poly(A)$^+$ RNA from various tissues of embryonic day 4, 8, 11, 18 and adult chicken were hybridized with digoxigenin-labeled anti-sense cy5 riboprobe. Two major TN-Y transcripts of ~6.5 and 9.5 kb (*arrows*) are detected in various samples, a potential third transcript of 5 kb can be detected in some tissues (E4 t; E8 h/t; E11 h*; adult o/l). *t*, trunk; *h*, heart (h* and h of E11 means same sample, different exposure times); *m*, skeletal muscle; *g*, gizzard; *o*, ovaries; *l*, lung. (*Left panel*) Exposed for 1 h; (*middle and right panels*) exposed for 10 min. RNA marker sizes are given in kb. At the bottom, ribosomal 28S RNA from each sample shows that comparable amounts of RNA were loaded.

from the heads were tested separately and turned out not to contain TN-Y mRNA. Weak TN-Y mRNA expression could be detected in gizzard and skin of 11-d- and in brain of 18-d-old embryos, respectively, but not in liver from these stages (not shown). In the adult, TN-Y continued to be expressed in heart and muscle and was also found in gizzard, ovaries and lung (Fig. 4, *right*).

The 6.5- and 9.5-kb TN-Y mRNA variants are differentially expressed during development. Both transcripts can first be detected in the trunk (but not in the heart) of 8-d-old and in heart of 11-d-old chicken embryos after long exposure of the blot (Fig. 4, *left*) and gradually increase with the age of the embryo. Both variants are equally abundant in embryonic and adult heart whereas the 6.5-kb transcript dominates in skeletal muscle. During embryogenesis, TN-Y mRNA expression is higher in skeletal muscle than in heart but the inverse pattern is found in the adult, where expression in skeletal muscle is reduced (Fig. 4, *right*).

Purification and Electron Microscopy of TN-Y Protein

To get information about the structure and the distribution of the TN-Y protein, we prepared a polyclonal antiserum directed against glutathione S-transferase- and β-galactosidase-fusion proteins with cy6 and cy3 (see Fig. 1 *A*) which were expressed in bacteria. Columns of affinity-purified anti-TN-Y antibody were used to isolate TN-Y protein from adult chicken heart and from conditioned media of embryonic muscle primary cultures grown in medium containing 10% horse serum and 4% chicken embryo extract (for details see below). TN-Y from adult heart was predominantly found in the supernatant after

high salt/EDTA extraction, but not in the DOC-treated fraction when analyzed by SDS-PAGE. It is also released into the culture medium by muscle fibroblasts under the above mentioned culture conditions. Thus TN-Y is a secreted molecule.

Immunoaffinity-purified chicken TN-Y separated by SDS-PAGE under reducing conditions appears as three major bands, with a high molecular mass doublet and a lower molecular weight band (Fig. 5). The apparent molecular mass of the heart-derived TN-Y is ~400 kD for the double band, and 300 kD for the lower band, respectively (Fig. 5, *A* and *B*). TN-Y isolated from cell culture conditioned medium is of markedly lower size, namely 220 kD (for the double band) and 170 kD (Fig. 5 *C*). All six variants were recognized by the polyclonal anti TN-Y antibody on immunoblots (Fig. 5, *B* and *C*).

Immunoaffinity-purified TN-Y from heart was examined by rotary shadowing electron microscopy. Electron micrographs revealed monomeric TN-Y molecules, appearing as rod-like structures with two terminal knobs (Fig. 6). The length of the TN-Y arms ranged from 70 to 125 nm. An accumulation of short molecules (70–75 nm) and intermediate sized molecules (95–100 nm) was observed but larger TN-Y arms of ~120 nm were found as well. The micrographs suggest that TN-Y from the heart does not form disulfide-linked multimers, although it cannot be excluded that oligomers were dissociated during extraction and purification. Except for the difference in length, the molecules resemble monomeric TN-C molecules as they were isolated from chicken gizzard (Chiquet et al., 1991).

Tissue Distribution of the TN-Y Protein and mRNA

We investigated the distribution of TN-Y in breast muscle and heart of 18-d-old chicken embryos and adult chicken. Consecutive sections were stained with anti-TN-Y and anti-TN-C antibody, respectively (Fig. 7, *A* and *B*). The TN-Y protein was found to be distributed in a fibrillar manner mainly between bundles of muscle fibers (in the perimysium) in embryonic and adult breast muscle (Fig. 7 *A*); in the latter it was additionally found between single muscle fibers, in the endomysium (Fig. 8 *D*). To address the question of which cells express TN-Y, in situ hybridization was performed on adjacent sections of 18-d embryonic breast muscle and compared to TN-C (Fig. 7, *C* and *D*) and laminin (Fig. 7 *F*) expression. The in situ hybridization revealed muscle fibroblasts which populate the perimysium to be the source of TN-Y mRNA (Fig. 7, *C* and *E*). In contrast, fibroblasts within the tendon expressed TN-C mRNA (Fig. 7 *D*) while no TN-Y mRNA could be detected in these cells (Fig. 7 *C*). However, diffusion of the TN-Y protein into the tendon is observed (Fig. 7 *A*). Coexpression of TN-Y and TN-C was found in the connective tissue surrounding blood vessels, but note that smooth muscle cells around arteries express TN-C, while TN-Y protein and mRNA are absent (Fig. 7). Laminin and TN-Y expression in embryonic breast muscle is compared at a higher magnification, clearly showing TN-Y mRNA restricted to the cells within the perimysium (Fig. 7 *E*), while laminin mRNA is expressed by both muscle fibroblasts and myofibers (Fig. 7 *F*).
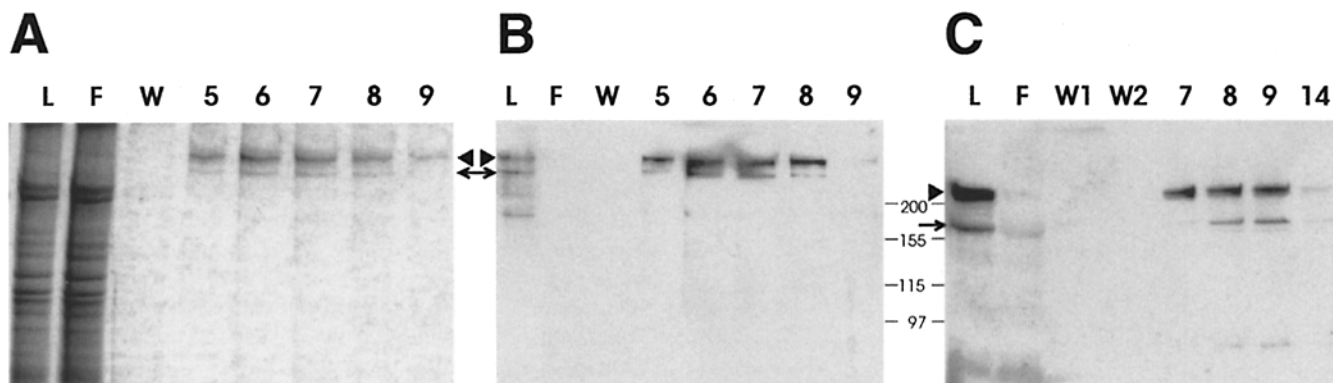
Figure 5. Analysis of immunoaffinity-purified TN-Y from adult chicken heart and from cell culture conditioned media. Heart extracts (*A* and *B*) or conditioned media from cocultures of primary muscle fibroblasts and myoblasts (*C*) were loaded (*L*) on an anti-TN-Y antibody column. Flow through (*F*) and washes (W1, W2) were collected and TN-Y fractions were eluted with 8 M LiCl, pH 11. Samples were analyzed by SDS-PAGE, performed under reducing conditions. (*A*) Coomassie blue-stained 6% SDS–polyacrylamide gel of TN-Y purified from heart, and (*B*) immunoblot of the same samples incubated with anti-TN-Y antibody. A ~400-kD doublet (*arrowheads*) and a ~300-kD variant (*arrow*) of TN-Y from heart were specifically eluted from the column (fractions 5-9) and detected by the anti-TN-Y antibody, but were not present in the flow through or wash fractions. (*C*) Immunoblot of TN-Y purified from primary muscle cell conditioned medium incubated with anti-TN-Y antibody. A ~220-kD doublet (*arrowhead*) and ~170-kD variant (*arrow*) were detected in eluted fractions (7-14). Protein marker sizes are given in kD.

TN-Y is also absent from the aortic smooth muscle wall in embryonic heart but is abundant in the tunica adventitia, a layer of connective tissue that surrounds the vessel wall and anchors it to the surrounding tissue (Fig. 8 *B*). The epicardium and the pericardium, two continuous connective tissue layers that delimit the myocardium and the pericardial cavity, respectively, stained positive for TN-Y in embryonic and adult heart. Sporadic TN-Y staining within the myocardial connective tissue was observed as well (Fig. 8, *B* and *C*). In situ hybridization of 18-d-old chicken heart revealed weak, sporadic TN-Y expression within the muscle tissue and the epicardium (not shown), which parallels the distribution of the TN-Y protein in adult heart (Fig. 8 *C*).

TN-Y Has Heparin-binding Activity

TN-C and TN-X have been shown to bind heparin under physiological salt conditions (Chiquet et al., 1991; Matsumoto et al., 1994). For TN-C, this ability was confined to a single region within the fibrinogen-like domain (Fischer et al., 1995). To test whether TN-Y may also interact with heparin, we carried out binding experiments with purified TN-Y on heparin-agarose columns. Both TN-Y from heart (Fig. 9 *A*) and from conditioned medium (Fig. 9 *B*) were retained on the heparin column under physiological salt conditions and could be eluted with 1 M NaCl. This indicates that heparin-binding ability may be a common feature of tenascin-like proteins.

TN-Y Production by Primary Muscle Fibroblasts

Chicken embryo skin fibroblasts (CEFs) cultured in medium containing 10% fetal calf serum release TN-C into the culture media (Chiquet and Fambrough, 1984*b*). Based on the TN-Y expression pattern in breast muscle connective tissue (see Fig. 7), we considered muscle fibroblasts to be a putative source for TN-Y purification. Cocultures of

myoblasts and muscle fibroblasts released the 220- and 170-kD variant of TN-Y into the medium if they were grown in medium containing 10% HS and 4% chicken embryo extract, but not if they were kept in 10% FCS and 4% chicken embryo extract as is demonstrated by immunoblots using the polyclonal anti-TN-Y antibody (Fig. 10 *B*). Under non-reducing conditions the 220-kD polypeptide was absent and instead oligomeric TN-Y barely entered the gel (Fig. 10 *C*). It appears therefore that TN-Y in conditioned medium, in contrast to the protein extracted from adult heart tissue, forms multimeric structures. The 170-kD band persisted under non-reducing conditions, indicating that it is probably not included in oligomers of TN-Y or that it represents a degradation product. To ascribe the TN-Y production to either myoblasts or muscle fibroblasts, the two cell types were separated and plated in 10% horse serum and 4% chicken embryo extract. TN-Y protein was detected in Western blots from conditioned media of both cell types with a markedly higher production in the muscle fibroblast culture (not shown). Therefore fibroblasts contaminating the myoblast/myotube-enriched culture may be the source of TN-Y protein synthesis, but we cannot exclude the possibility that undifferentiated myoblasts also express TN-Y. These results point to the existence of a TN-Y inducing factor in horse serum that acts on fibroblasts. Such a factor may be considered as a differentiation-inducing activity, which regulates the production of a cell type–specific gene product of muscle fibroblasts.

Discussion

A Novel Tenascin Family Member with Unique Features

This is the first report presenting cDNA and protein data on TN-Y, the third tenascin-like molecule identified in
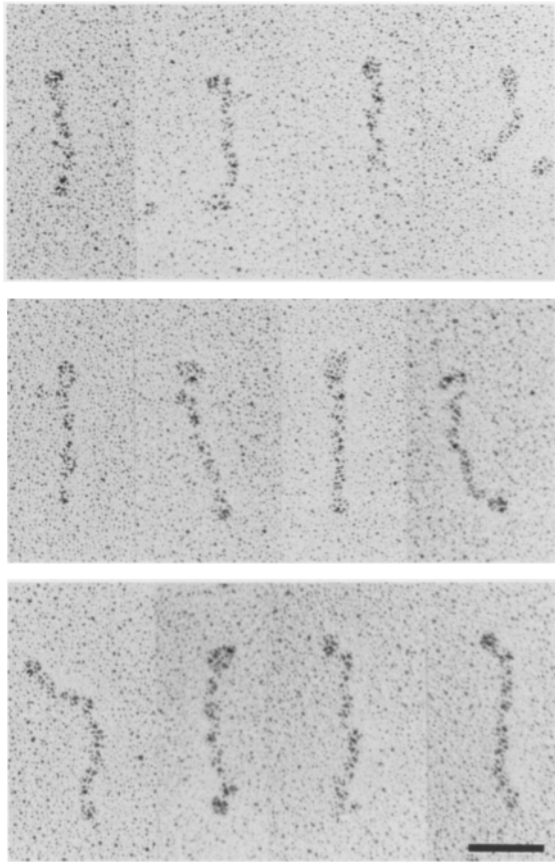
Figure 6. Electron-micrographs of purified TN-Y from chicken heart. Monomeric TN-Y of three major sizes were identified in rotary-shadowed samples of TN-Y purified from anti-TN-Y Sepharose columns: (*top*) small sized TN-Y ($\sim$70 nm); (*middle*) intermediate-sized TN-Y ($\sim$100 nm); (*bottom*) large sized TN-Y ($\sim$120 nm), standard deviation, 20 nm. The molecules appear as rod-like structures with two terminal knobs. Bar, 50 nm.

chicken. TN-Y is a typical tenascin in that it contains all the domains present in other known family members in the same arrangement, namely heptad repeats, followed by EGF-like repeats, FNIII domains and a domain homologous to fibrinogen. However, TN-Y is unique with respect to its NH_2 terminus, to the insertion of a new domain which is rich in serines and prolines and to its series of almost identical FNIII domains.
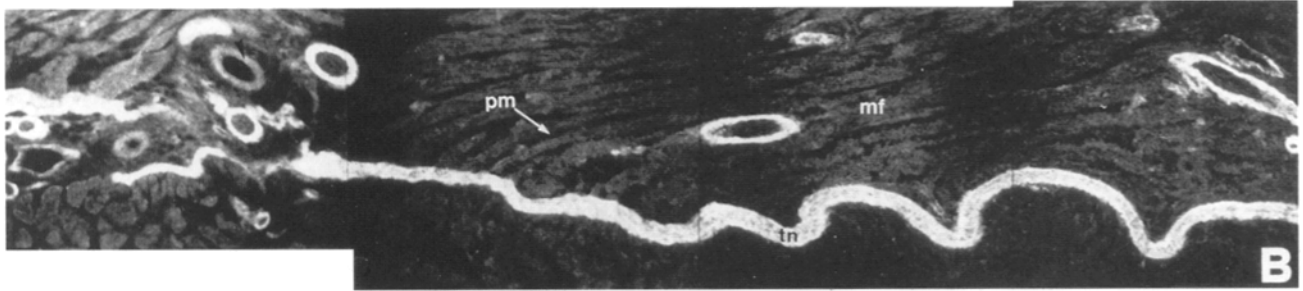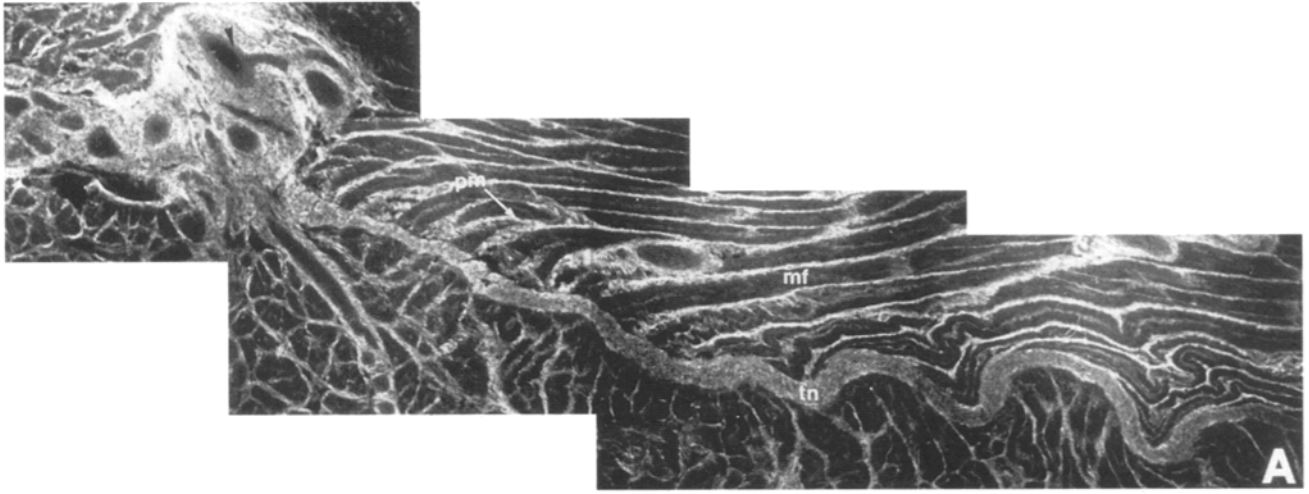
The 3.5 heptad repeats of TN-Y provide the basis to assemble at least three TN-Y monomers analogous to TN-C and TN-R trimers (Pesheva et al., 1989; Spring et al., 1989; Nörenberg et al., 1992). However, from heart muscle extracts, only monomeric TN-Y molecules could be purified over the anti-TN-Y antibody column, while in muscle fibroblast conditioned culture medium TN-Y seems to be present in an oligomeric form. A similar situation has been reported for TN-C, which typically is a hexamer when isolated from cell culture, but can be extracted only in its monomeric form from adult chicken gizzard (Chiquet et al., 1991). The NH_2 terminus of TN-Y is short and comprises only 4 cysteines compared to 8 and 7 in TN-C and TN-X, respectively. Disulfide bridges between the most NH_2-ter-

minal cysteines of two mature TN-C trimers are thought to connect two trimers to assemble the final six-armed structure of TN-C (Spring et al., 1989; Chiquet et al., 1991). The corresponding cysteine residue is present neither in TN-X nor in TN-Y. Thus, only dimers and trimers have been predicted for TN-X (Bristow et al., 1993) and may also be the prevalent form of TN-Y.

The EGF-like region of TN-Y is encoded by a single exon as in its TN-C (Gulcher et al., 1991) and TN-X (Bristow et al., 1993) counterparts but it is the shortest such region in all tenascins, consisting of one complete EGF-like repeat flanked by two half-EGF-like repeats. Characteristic for a EGF-like repeat are six conserved cysteines where cysteine residues 1-3, 2-4, and 5-6 form disulfide bridges (Cooke et al., 1987; Chiquet-Ehrismann, 1990). The cysteines 1 and 3 are still present in the second half EGF-like repeat of TN-Y while cysteine 2 is lost, possibly due to the lack of its partner cysteine 4.

The ability to bind heparin may be common to all tenascins and the necessary active site in TN-C has been shown to reside within the second half of the fibrinogen globe (Fischer et al., 1995). In fibronectin six discontinuous basic residues within the FNIII domain 13 are brought together on one side of the protein to form a heparin-binding site (Busby et al., 1995). The fibrinogen domain of all known tenascins contains a high number of conserved basic residues which might provide an active surface for heparin binding.

The most intriguing feature about the TN-Y structure is the occurrence of a new domain rich in serines and prolines, which are repeated in the consensus pattern S-P-X over a stretch of $\sim$100 residues. This SPX-rich domain interrupts the series of FNIII domains after FNIII domain YA and is, together with a half FNIII domain, encoded by a single exon. Database searches revealed no homology of the SPX-domain to any known protein. However, a number of structurally and functionally unrelated proteins such as neurofilaments (Zopf et al., 1990), ankyrin (Otto et al., 1991), RNA-polymerases (Bird and Riddle, 1989), and bacterial proteins (Jerlström et al., 1991) were found to have other well conserved serine/proline spacing patterns within repetitive motifs. The function of these motifs within these proteins has not been elucidated in most cases, but it seems likely that serine residues of intracellular proteins can be subject to phosphorylation as proposed for the neurofilaments (for review see Steinert and Roop, 1988). Serine residues in extracellular proteins can have carbohydrates attached to the OH-group as in mucins or proteoglycans. The role of repeating proline residues may be to closely pack regional modifications or structural units by inducing regular β-turn conformations. In collagens, proline and hydroxyproline help to form and stabilize the triple-stranded helix. Whether the SPX-domain of TN-Y is posttranslationally modified and represents a structural unit remains to be elucidated. It could be required to form a complete domain with the adjacent half FNIII domain that may otherwise not be able to fold properly. To our knowledge, this is the first report of the occurrence of a half FNIII domain within a protein. A more tempting explanation would be, that the SPX-domain confers novel structural and functional features to TN-Y which are not shared by other tenascins.
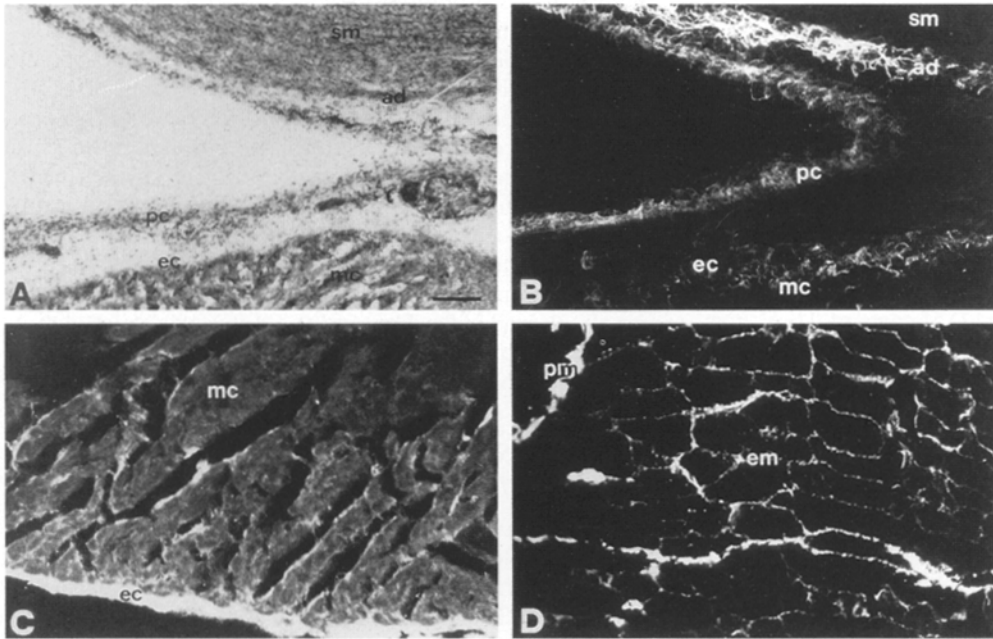
Y-Type FNIII Domains: Is TN-Y a Young Protein?

In a comparative alignment of the most conserved region in all tenascins, the FNIII domain eight and the adjacent fibrinogen globe, we found TN-Y to be more closely related to TN-X than to any other tenascin (Chiquet-Ehrismann et al., 1994). This region in TN-Y is as similar to the one of TN-X as are the corresponding regions in TN-C and TN-R, indicating that TN-Y and TN-X are paralogous (derived from a gene duplication before species divergence) rather than orthologous proteins (the same protein in different species with sequence differences only reflecting species divergence) (see Wilson et al., 1977). The close relationship between TN-Y and TN-X also holds true for the TN-Y FNIII domains YB, YC, YD, and YE which are ~40–60% identical to the 23 TN-X-type FNIII domains (see Bristow et al., 1993; Erickson, 1993), but exhibit only ~30% identity to FNIII domains in TN-C or TN-R. However, there is no clear colinearity in resemblance of single FNIII domains in TN-Y and TN-X as it is the case for domains 1-8 in TN-C or TN-R orthologues. None of the Y-type domains correspond to FNIII domains six or seven which are conserved among all other tenascins.

The FNIII domains YD1 and YE2 occur twice as identical domains within the smallest TN-Y variant, and domains YE1, YE3 and YD2 are tandemly repeated several times en bloc within the cy6 splice variant. The presence of repeated domains which retained 100% nucleotide-sequence identity has been reported only once before, for human apolipoprotein(a) (McLean et al., 1987). This plasma lipoprotein contains 37 copies of the plasminogen kringle 4-like unit, 24 being identical in nucleotide sequence.

It is noteworthy that the series of X-type and Y-type domains (including the half FNIII domain of TN-Y) are most similar to alternatively spliced variable domains of TN-C. This suggests that the X- and Y-type FNIII domains and maybe all variable domains of the tenascins evolved by duplications of a common precursor domain. Assuming that FNIII domains evolved at a constant rate of 1% amino acid exchange every 7–9 million years (Erickson, 1994), duplication of the Y-type domains which are 99–100% identical with respect to their amino acid as well as to their DNA sequence would have occurred only within the last 9 million years. This suggests that TN-Y evolved only recently in evolution. Evidence for recent duplications of the

Figure 7. Immunofluorescence staining and in situ hybridization of breast muscle from 18-d-old chicken embryos. Consecutive sections were stained with anti-TN-Y (A) and anti-TN-C (B) antibodies or were hybridized to digoxigenin-labeled anti-sense riboprobes of TN-Y (C and E), TN-C (D) or laminin (F). TN-Y protein (A) and mRNA (C and E) are prominent in the perimysium (pm) and around blood vessels but are clearly absent from myofibers (mf). Diffuse distribution of the TN-Y protein into the tendon (tn) is observed (A). TN-C protein (B) and mRNA (D) are restricted to the tendon (tn) and are prominent around blood vessels. Note that TN-Y and TN-C are co-expressed around most blood vessels, but that TN-Y is clearly absent from smooth muscle surrounding arterial vessels where TN-C is expressed (arrowhead in all four panels). Higher magnification shows TN-Y expression by muscle fibroblasts of the perimysium (E) in comparison to laminin (F), which is expressed by both muscle fibers and to a lower extent also by muscle fibroblasts. Bar, 100 μm.
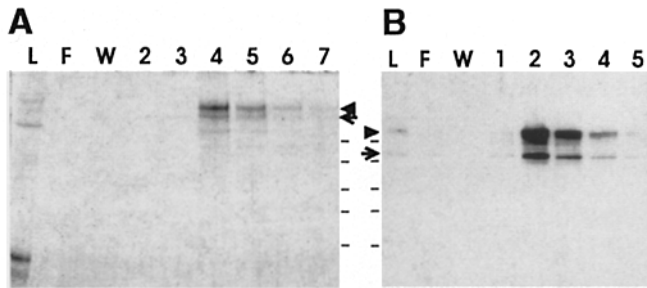
Figure 9. TN-Y shows heparin-binding activity. Purified TN-Y from chicken heart (*A*) or from conditioned medium of primary muscle fibroblasts/myoblasts cocultures (*B*) was passed over a heparin-agarose column. Samples of loaded material (*L*), flow through (*F*), wash (*W*) and fractions eluted with 1 M NaCl (numbered) were analyzed by immunoblotting using the anti-TN-Y antibody. The ~400-kD and ~300-kD variants of TN-Y from heart and the ~220- and ~170-kD variants of TN-Y from conditioned medium were recovered in the high salt fractions (*arrowheads* point to high-molecular mass doublets, *arrows* indicate low-molecular mass variants). Protein marker sizes are indicated by dashes (in kD) from top to bottom: 200, 155, 115, 97, and 66 kD.

variable FNIII domain A within the human TN-C gene (Gulcher et al., 1990) points to a high mobility and probability to duplicate as a characteristic feature of the variable domains.

Database searches with the FNIII domain YA did not reveal significant homology to other FNIII domains of tenascins. Interestingly, with 27% identity, the FNIII domain 14 of fibronectin was the most homologous domain
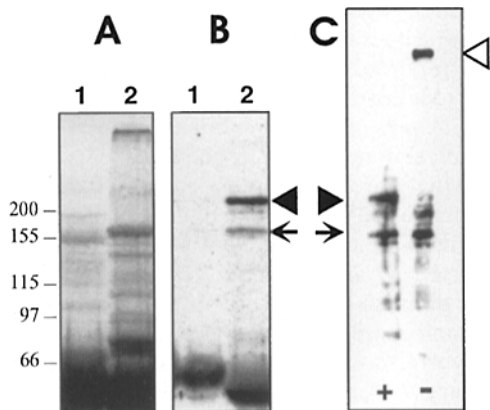

Figure 10. Secretion of TN-Y by cocultured muscle fibroblasts and myoblasts in medium containing HS. (*A* and *B*) Conditioned media from cells grown with 10% FCS (lanes *1*) or with 10% HS (lanes *2*) were analyzed on Western blots which were stained with amido black (*A*) and incubated with anti-TN-Y antibody (*B*). The ~220-kD (*black arrowhead*) and the ~170 kD (*arrow*) variants of TN-Y are only released into the culture medium by cells grown in medium containing 10% HS (*B*, lane *2*). (*C*) Immunoblot of purified TN-Y from conditioned medium separated on a 3–15% gradient SDS–polyacrylamide gel under reducing (+) and non-reducing (−) conditions. A high-molecular mass variant barely enters the gel under non-reducing conditions (*open arrowhead*), while the ~220-kD variant (*black arrowhead*) disappears. The ~170-kD variant remains at the same position under both conditions. Protein marker sizes are given in kilodalton.

found. Since domain YA is only ~20% identical to FNIII domain 1 in TN-C or TN-X, respectively, it is possible that the FNIII domains of TN-Y were assembled from different sources. A mechanism by which this could have occurred is exon shuffling (Gilbert, 1978). This mechanism is thought to be promoted by class 1 introns (that split the codon after the first position) flanking the exons (Patthy, 1991), the type of junction indeed found in TN-Y.

Like in TN-C and TN-R, alternative splicing of FNIII domains creates different variants of TN-Y. The TN-Y isoforms produced by muscle fibroblasts in cell culture are markedly smaller than those isolated from chicken heart extracts. Similarly, size differences between transcripts produced by cells in culture and those isolated from tissue were also found for TN-X (Matsumoto et al., 1994). Distinct environmental influences could affect mRNA splicing of cultured cells. This and the different sets of cDNA clones point to a complex pattern of alternative splicing of the Y-type FNIII domains.

TN-X and TN-Y: Structural Paralogues but Functional Homologues?

As pointed out earlier, TN-Y and TN-X cannot be considered as orthologues from the structural point of view, since they are clearly more distinct from each other than for example chicken and mammalian TN-C, but nevertheless, they share many common features. They are the largest proteins of the tenascin family, and their FNIII domains are of longer average size than those in TN-C and TN-R and lack any glycosylation sites (see Bristow et al., 1993). However, the most striking common feature of TN-X and TN-Y is their predominant expression in skeletal muscle and heart tissue, where the proteins and transcripts of both molecules are found in corresponding regions of muscle-associated connective tissue in mouse (Matsumoto et al., 1994; Burch et al., 1995) and chicken, respectively. From our in situ data and from observations in cell cultures we conclude that TN-Y is expressed by fibroblasts of the muscle connective tissue but not by muscle cells, although we cannot exclude that muscle fiber-associated satellite cells are an additional source of TN-Y. Non-myocyte cell types were also found to be the major source of TN-X in rat muscle (Burch et al., 1995). Fibroblasts between muscle fibers do not normally express TN-C, but are positive for TN-Y in chicken (and for TN-X in rodents). Conversely, tendon fibroblasts and fibroblasts near the myotendinous junction produce TN-C (Chiquet and Fambrough 1984a). This suggests that the different, specialized connective tissue compartments associated with muscle are marked by the presence of at least two different types of fibroblasts.

In skeletal muscle, TN-Y expression is associated with the "late" period of myoblast development, i.e., with the accumulation of secondary and adult myotubes (Stockdale, 1992) which is associated with dramatic changes in muscle-associated connective tissue (Fernandez et al., 1991). TN-Y expression in heart coincides with terminal steps in compartmentalization at a time where the heart function is already well established (around embryonic day 8), and persists during the period of enlargement and thickening of heart muscular structures (see Romanoff, 1960). Another common feature of TN-Y and TN-X is

their absence from the middle portion of the arterial smooth muscle wall (tunica media), where TN-C is strongly expressed (Burch et al., 1995). Here, TN-Y in chicken and TN-X in rodents are present in the tunica adventitia, that surrounds the smooth muscle as a loose connective tissue layer and anchors the vessel to the adjacent tissue (Morse, 1979).

These data suggest a role for TN-Y in the establishment of connective tissue layers in and around skeletal, heart and smooth muscle in the chicken embryo, and the maintenance of these patterns throughout adult life. A similar suggestion has been made for the function of TN-X (Burch et al., 1995). The ubiquitous distribution of TN-Y and TN-X within these tissues make them potential candidates to be involved in anchoring muscle fibers to the surrounding matrix. Since both TN-Y and TN-X have been shown to bind to heparin, this link could at least partially be mediated through interaction with heparan-sulfate proteoglycans present in the endomysium (Fernandez et al., 1991).

TN-X has been mapped to the major histocompatibility complex (MHC) class III region in human and mouse (Matsumoto et al., 1992, 1994) which is part of the MHC locus that comprises MHC I, MHC II, and MHC III in mammals. However, there is no evidence for the existence of a class III region within the avian MHC locus, which seems to have been stripped down to a "minimal essential MHC" with respect to immune function (Kaufman et al., 1995). Up to now, none of the genes present within the mammalian MHC III region have been identified in chicken. In fact, the whole avian genome has been reduced during evolution, comprising only roughly a third of the size of a typical mammalian genome, and is split into few macrochromosomes and a number of minichromosomes (reviewed in Burt et al., 1995). The possibility exists that the class III region was lost from the avian genome entirely. In this case chickens would lack a TN-X orthologue, and TN-Y could have evolved in replacement of TN-X in mammals. Thus it is possible that, although TN-Y and TN-X are not orthologous proteins, they serve equivalent functions in birds and mammals. So far, there is no evidence for a TN-X gene in birds or a TN-Y gene in mammals, respectively. Further studies are needed to clarify the evolutionary and functional relationship between the two proteins.

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