Journal of Cellular Biochemistry 41:71–90 (1989)

Biochemical and Structural Studies of Tenascin/Hexabrachion Proteins

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Tenascin is a large, disulfide-bonded glycoprotein of the extracellular matrix. The predominant form of tenascin observed by electron microscopy is a six-armed oligomer, termed a hexabrachion. We have determined the molecular mass of the native human hexabrachion to be 1.9×10^6 Da by sedimentation equilibrium analysis and by electrophoresis on non-reducing agarose gels. On reducing polyacrylamide gel electrophoresis (SDS-PAGE), human tenascin showed a single prominent band at 320 kDa and minor bands of 220 and 230 kDa. The molecular weight of the native human hexabrachion is thus consistent with a disulfide-bonded hexamer of the 320 kDa subunits.

Upon treatment with neuraminidase, the apparent molecular weights of all human and chicken tenascin subunits on reducing SDS-PAGE were decreased by about 10 kDa. Prolonged incubation with α-mannosidase, however, caused no apparent change in the apparent molecular weight of tenascin subunits. Sedimentation in a cesium chloride gradient gave a higher buoyant density for human tenascin than for fibronectin, suggesting that it has a higher degree of glycosylation. The far-UV circular dichroism spectrum indicates a predominance of β-structure and a lack of collagen-like or α-helical structure.

When human hexabrachions were reduced and acetylated, the resulting fragments were single arms which sedimented at 6 S in glycerol gradients and migrated at 320 kDa on non-reducing gels. Treatment of tenascin with trypsin and α-chymotrypsin also produced large fragments which were fractionated by gradient sedimentation and analyzed by non-reducing SDS-PAGE and electron microscopy. We present a structural model for the assembly of the observed fragments into the elaborate native hexabrachion.

Key words: extracellular matrix, cytotactin, fibronectin, proteolysis, glycosylation, disulfide bonds

Proteins with a distinctive "hexabrachion" structure have been isolated from cell cultures of chicken and human tissues [1–12]. First characterized by several groups searching for new extracellular matrix (ECM) proteins of widely varying function, hexabrachion proteins from different sources have been shown to have very similar

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Received November 17, 1988; accepted June 2, 1989.

multi-armed structures by electron microscopy (EM) and are immunologically cross-reactive. The history of the discovery and characterization of these proteins is reviewed in ref. 13.

Chiquet and Fambrough [1] found the "myotendinous antigen" in embryonic chicken tissue including tendon primordia, premuscle masses, and developing smooth muscle by using the monoclonal antibody M1. Also secreted by cultured chick embryo fibroblasts (CEF), the antigen was described as a collagenase-resistant, disulfide-bonded oligomer of subunits similar in size to fibronectin, but antigenically distinct. The M1 antigen barely entered a non-reducing 3% PAGE gel, indicating a native molecular weight in excess of 10^6 Da [2].

In their search for human glioma marker antigens, Bourdon et al. [4–6] used the monoclonal antibody 81C6 to define the "glioma mesenchymal extracellular matrix" antigen (GMEM) in numerous cultured tumor and fibroblast cell lines. The antigen was also found in glioblastomas, several types of fibrosarcomas, and normal adult kidney, liver, and spleen. Detergent-solubilized GMEM from the surface of cultured cells was shown to be distinct from fibronectin and other ECM proteins.

"Cytotactin," identified by Grumet et al. [7] in embryonic chicken brain, was found in many of the same locations [12] where the M1 antigen had been detected and had a very similar reduced subunit structure as observed on SDS-PAGE [2]. It is now established that cytotactin is identical to the myotendinous antigen or chicken hexabrachion [9,11]. A very similar protein called J1 was previously identified in mouse brain [8].

Chiquet-Ehrismann et al. [12] demonstrated that a polyclonal antibody against chicken myotendinous antigen stained a protein that was expressed in embryonic tissues and tumors in rat. They proposed the more general name "tenascin," which we will use to refer to this class of closely related proteins from different species.

The tenascins or hexabrachion proteins share several characteristic structural features. The most commonly seen oligomer has six extended arms ranging in length from an average of about 87 nm for the human protein to an average of 67 nm for hexabrachions from most chicken tissues. The inner one-third of each arm appears to be relatively thin in rotary shadowed specimens, while the outer two-thirds is thickened and ends in a 5 nm distal knob. Three arms are joined together in an apparent "T"-junction on either side of the hexabrachion's central knob (about 6 nm in diameter). Separate three-armed structures are also seen, especially in affinity-purified chicken tenascin, as well as nonamers and rare dodecamers in preparations of the human protein [13].

Based on a presumed homology among these proteins, we have studied detailed structural questions concerning both chicken and human hexabrachions. Our primary interest has been the relationship between the subunit structure of the native, reduced, and enzymatically cleaved hexabrachions. In particular, does each arm of the hexabrachion correspond to one reduced subunit as seen by reducing SDS-PAGE; and how are these subunits assembled into the native molecule? We have studied the secondary structure of human tenascin with circular dichroism to determine which types of secondary structure chiefly account for the hexabrachion arms. Exoglycosidase treatments were used to characterize the terminal carbohydrate residues of chicken and human tenascins. We also report other biochemical properties of the purified hexabra-

METHODS

Cells and Cell Cultures

Primary cultures of chicken embryonic fibroblasts were prepared from 11-day chicken embryo skin. Stock cells were frozen at the third passage and then used for experimentation up to passage 11. Cells were grown in a 5% CO_2 atmosphere in Dulbecco's modified Eagle's medium (DMEM) with high glucose supplemented with penicillin (100 IU/ml) and streptomycin (100 μg/ml) and 10% v/v heat-inactivated fetal bovine serum until they reached confluence. At confluence, medium was changed to DMEM-H supplemented with pen-strep, 5% non-heat-inactived fetal bovine serum. The human glioma cell line U251MG, clone 3, from Dr. Darell D. Bigner, Pathology Dept., Duke University, was grown in DMEM-H supplemented with pen-strep, 10% heat-inactivated fetal bovine serum and amphotericin-B (50 μg/ml) until the cells reached confluence. At confluence, these cells were cultured in DMEM-H, pen-strep with amphotericin-B, and 1% non-heat-inactivated fetal bovine serum.

Materials

Sodium dodecyl sulfate (SDS), bisacrylamide, N,N,N′,N′-tetramethylethylenediamine (TEMED), 4-chloro-1-naphthol, and agarose were purchased from BioRad. Sigma Chemical Co. was the source for cyanogen bromide-activated Sepharose 4B, neuraminidase from *Clostridium perfringens,* jack bean α-mannosidase, bovine pancreatic trypsin and α-chymotrypsin, soybean trypsin inhibitor, 2-nitro-4-carboxyphenyl-N,N-di -phenyl carbamate (NCDC), and iodoacetamide used in these experiments. von Willebrand protein was purified as in [14]. All tissue culture media and supplements except amphotericin-B (Gibco Laboratories) were prepared by the Lineberger Cancer Research Center at the University of North Carolina School of Medicine, Chapel Hill, NC. Other proteins used include bovine serum albumin (BSA) from Boehringer Mannheim Biochemicals and human plasma fibronectin from the New York Blood Bank.

Immunoreagents

The monoclonal antibody "M1" to the chicken myotendinous antigen and a rabbit polyclonal to the same protein, as described in [1], were a gift of Dr. Douglas Fambrough. The monoclonal antibody 81C6 to the "glioma mesenchymal extracellular matrix" protein [4] was provided by Dr. D.D. Bigner. Affinity-purified horseradish peroxidase (HRP)-conjugated anti-mouse and anti-rabbit antibodies were obtained from BioRad.

Purification of Tenascin

Cell supernatant was removed from cultured chicken embryo fibroblasts or human glioma U-251MG cells every 2 or 3 days and used either for affinity adsorption (M1 coupled to Sepharose-4B for chicken cell media or 81C6-Sepharose-4B for human) or $(NH_4)_2SO_4$ precipitation followed by glycerol gradient sedimentation. (See ref. 9 for a detailed description of both methods.) Gradient-fractionated material often achieved peak protein concentrations in excess of 500 μg/ml and contained at least 80% tenascin as estimated from visual inspection of silver-stained gels. These gradient fractions (to be referred to as "gradient-purified tenascin") were especially useful for studies involving

several steps of manipulation, where a high starting protein concentration ensured a significant yield. In these studies (proteolysis and reduction) the minor contaminants did not affect the results or interpretation. Affinity-purified tenascin seldom eluted at concentrations over 50 μg/ml and was concentrated to greater than 200 μg/ml in an Amicon Centricon C-30 concentrator for use in several experiments. (We frequently experienced heavy loss of protein during the concentration process.) Chicken gizzard tenascin was extracted from tissue homogenized in 200 mM sodium CAPS (2-(cyclohexylamino)-1-propane-sulfonic acid), 0.15 M NaCl, pH 11, neutralized, treated with DNAse and hyaluronidase, filtered and affinity purified on M1 sepharose as in ref. 9.

An alternative purification has recently been developed using a Mono-Q (Pharmacia) anion exchange column and glycerol gradient sedimentation [15,16] (Aukhil et al., in preparation). This procedure gave tenascin at 500 μg/ml, of purity identical to affinity-purified tenascin, based on SDS-PAGE and all other assays. This "Mono-Q tenascin" was used to confirm three studies already done with affinity-purified protein: ultraviolet spectroscopy and determination of extinction coefficient, circular dichroism, and sedimentation equilibrium.

Electrophoretic Analyses and Immunoblots

Samples were boiled for 30 to 60 s in buffer containing 2% SDS with or without 2-mercaptoethanol and loaded on 5% (for reduced samples) or 3–15% gradient (for nonreduced samples) Laemmli-type polyacrylamide slab gels [17] with a 3% stacker. Gel molecular weight standards included human plasma fibronectin (reduced = 255 kDa, nonreduced = 510 kDa), myofibrils (C protein = 140 kDa, myosin = 212 kDa), thyroglobulin (330 kDa), and laminin (reduced = 225 kDa and 400 kDa, nonreduced = 820 kDa). All slab gels not intended for immunoblotting were silver stained by the method of Merril et al. [18]. For blotting, proteins were transferred to nitrocellulose at 0.8 mA/cm^2 for approximately 1 h using an LKB semi-dry apparatus with graphite electrodes [19]. Nitrocellulose membranes were blocked with 1% bovine serum albumin in phosphate-buffered saline, incubated with primary antibody for 2 h and with horseradish peroxidase conjugated second antibody for 1 h, and then washed and visualized with 4-chloro-1-napthol and hydrogen peroxide. Membranes were agitated continuously during the incubations and were washed between steps with phosphate-buffered saline.

For analysis of intact (non-reduced) tenascin, 0.75% agarose gels were prepared with 0.1 M Tris borate, pH 8.6, containing 0.1% SDS, and run using the same buffer system. Proteins were transferred to nitrocellulose using an LKB semi-dry apparatus at 0.8 mA/cm^2 for 30–45 min and stained with India ink by the method of Hancock and Tsang [20]. Plasma fibronectin, laminin, and von Willebrand protein (which runs as oligomers of 500 kDa to 4 $\times$ 10^6 Da in this system [14]) were used as gel standards.

Electron Microscopy

Samples in buffer containing volatile salt (0.2M ammonium formate in the case of reductions and proteolytic digestions, 0.2M ammonium bicarbonate, pH 9.5, in all other cases) and 30% glycerol were sprayed onto freshly cleaved mica chips, dried in vacuum, and rotary shadowed with platinum [21]. Contour length measurements of fragments and individual native hexabrachion arms (from the "T"-junction to the tip of the distal

knob) were made with a Numonics planimeter/digitizer on micrographs printed at 200,000× magnification.

Protein Concentration and Determination of Extinction Coefficient

As a primary assay of protein concentration we used the heated Biuret method [22], which has been reported to give the same color reaction for proteins. As standards we used three proteins with known extinction coefficients: bovine serum albumin, $\epsilon = 0.667$; tubulin, $\epsilon = 1.18$; and fibronectin, $\epsilon = 1.28$ ($\epsilon = A_{277}$ for 1 mg/ml, 1 cm path). In several tests of the heated Biuret assay the three proteins gave identical color reactions, within ±3%. When assaying tenascin in different buffers, identical buffer components were added to the reference proteins. This was especially important for samples containing glycerol, which significantly increased color development in the heated Biuret assay.

Tenascin purified by antibody affinity chromatography generally was low in concentration (20–50 μg/ml), and a significant absorbance in the region of 320 nm indicated some turbidity, which complicated the ultraviolet spectrum. A much cleaner spectrum was obtained by further purifying and concentrating affinity-purified tenascin by anion exchange chromatography [15], or from material purified directly by anion exchange and gradient sedimentation [15,16] (Aukhil et al., in preparation). The ultraviolet spectra of tenascin purified by these two methods were identical and also similar to that of fibronectin. Using the heated Biuret to determine the absolute protein concentration, the extinction coefficient for human tenascin was determined to be $\epsilon = 0.97$.

As a secondary assay of protein concentration, we used the Bradford assay [23], adding 0.9 ml of 1/5 diluted BioRad reagent to 0.1 ml of sample. It is known that proteins vary considerably in their color yield in this assay. Fibronectin produced the lowest A_{595} of the four proteins tested, for a given mg/ml concentration. Relative to fibronectin, the A_{595} was 1.1 to 1.2 times higher for tenascin; 1.6 times higher for tubulin; and 2.0 to 2.5 times higher for bovine serum albumin. We generally used fibronectin as a reference protein for tenascin in the Bradford assay. Note that BSA is a very poor choice of standard in this assay: if the color of tenascin or fibronectin is referred to a BSA protein standard without correction, their concentrations would be underestimated by a factor of 2.0 to 2.5.

Circular Dichroism Spectroscopy

Protein samples were dialyzed against PBS (0.15M NaCl, 0.01M sodium phosphate, pH 7.4). Protein concentration was determined from the ultraviolet spectrum and extinction coefficient. For the experiment shown, tenascin (purified by gradient sedimentation and Mono-Q chromatography) and bovine fibronectin (purified by gelatin agarose affinity chromatography followed by Mono-Q) were diluted in PBS to final concentrations of 0.208 and 0.157 mg/ml, respectively. The ultraviolet CD spectra of these samples were obtained at 20.0°C in a 0.10 cm path length cell by using a Jobin Yvon Auto Dichrograph Mark V interfaced to an Apple IIe computer. Data were collected in 0.20 nm steps, and the average of five scans is reported. The mean residue ellipticity Θ (in deg cm^2 $dmol^{-1}$) was calculated assuming a mean residue weight of 110 for both tenascin and bovine fibronectin. The maximum absorbance at 200 nm was kept below 1.3 to minimize stray light artifacts. The instrument was calibrated with d-10-camphorsulfonic acid dissolved in water [24]. The ratio of $\Theta_{192.5}/\Theta_{290.5}$ was determined to

be −1.91. This differs from the value of −2.0 reported by Yang et al. [24] and likely reflects the age of our xenon lamp.

Centrifugation Procedures: Glycerol Gradient Zone Sedimentation, Cesium Chloride Density Gradients, and Sedimentation Equilibrium

For preparative purification and estimation of sedimentation coefficients, samples were sedimented on 15–40% glycerol gradients in 0.2M ammonium bicarbonate on a 12 ml gradient and centrifuged at 41,000 rpm for 18 h at 20°C in a Beckman SW-41 Ti rotor. Sedimentation coefficients were estimated relative to standard proteins in a separate tube: α_2 macroglobulin, 18.3 S; catalase, 11.3 S; and bovine serum albumin, 4.6 S.

The buoyant densities of human tenascin and fibronectin, and mouse laminin from EHS sarcoma (a generous gift of Dr. H. Kleinman, NIH), were determined by equilibrium sedimentation in CsCl as described by Davis et al. [25]. Samples were first analyzed under denaturing conditions (4M guanidine HCl) to disrupt possible association with proteoglycans; 2.10 g guanidine HCl and 1.70 g CsCl were dissolved in 3.0 ml H_2O, and 0.6 ml protein solution was added. (Total volume in nitrocellulose tube was 5.6 ml.) Samples were centrifuged in a Beckman 50.1 swinging bucket rotor for 67 h at 45,000 rpm, 20°C. Fractions of 200 μl were collected after piercing the bottom of the tube. The density of each fraction was determined by weighing a 100 μl sample in a calibrated glass micropipet. Protein peaks were identified by SDS-PAGE. The two to three peak fractions were dialyzed against PBS and recentrifuged under native conditions: 2.05 g CsCl added to 5.0 ml total H_2O plus protein.

The buoyant densities of tenascin and fibronectin were 1.29 and 1.26 g/cc under denaturing conditions and 1.34 and 1.31 g/cc when recentrifuged in the absence of guanidine HCl. Thus, under both conditions tenascin was about 2.3% more dense than fibronectin. Laminin banded at the same density as tenascin under both denaturing and non-denaturing conditions. The buoyant density of a protein in CsCl depends on hydration effects and interaction with CsCl and does not give directly the partial specific volume in the buffer used for sedimentation equilibrium. This parameter was estimated as follows. The partial specific volume of fibronectin has been calculated from amino acid and carbohydrate composition to be 0.72 cm^3/g [26], and this value gave the correct mass of dimeric fibronectin when used in the Svedberg equation [27]. (The molecular mass reported in ref. 27 was 506 kDa, but this involved an arithmetic error, previously unreported. The correct calculation gives 516 kDa (HPE unpublished), very close to the value expected for the peptide plus carbohydrate). If we assume similar solvation parameters for fibronectin and tenascin, we estimate the partial specific volume of tenascin to be 2.3% less, or 0.70 cm^3/g.

Sedimentation equilibrium and velocity experiments were performed with a Beckman Model E Analytical Ultracentrifuge equipped with a photoelectric scanner. For the equilibrium measurements, a sample of affinity-purified human tenascin with total absorbance at 280 nm of 0.3 was examined at rotor speeds of 3,600, 4,000 and 6,800 rpm, at 15.8 to 17°C. The protein gradient along the cell was monitored as absorbance at 280 nm. The solvent density (1.024 gm/cm^3) was measured as the mass of a known volume of solvent, and $\bar{v} = 0.70$ ml/g was estimated from buoyant density measurements as described above.

Exoglycosidase Treatments

Aliquots of clean, gradient-purified tenascin were dialysed into 0.1 M sodium acetate buffer, pH 5.0, and treated with 1 unit/ml of neuraminidase at room tempera-

ture for up to 3 h. Samples to be treated with α-mannosidase were dialysed into 50 mM sodium citrate buffer, pH 4.5. Enzyme was added to 2 units/ml and samples were incubated for up to 24 h at 37°C. For gel analyses, glycosidase reactions were stopped by adding an equal volume of electrophoresis buffer containing 2% SDS and 5% 2-mercaptoethanol, followed by boiling for 1 min. Samples to be rotary shadowed were dialysed into 0.2M ammonium bicarbonate buffer, pH 9.5, containing 30% glycerol.

Proteolysis of Tenascin

Aliquots of affinity-purified CEF tenascin in 0.1M Tris-HCl, pH 8, or gradient-purified human tenascin (~80% pure) in 0.2M ammonium formate, 10 mM sodium PIPES (piperazine-N, N'-bis-2-ethane-sulfonic acid) buffer, pH 8.0, were incubated at room temperature for 30 min with 1) no added enzyme, 2) 10 μg/ml α-chymotrypsin, or 3) 10 μg/ml trypsin. At the end of the incubation period, inhibitors were added as appropriate (control: no inhibitor; α-chymotrypsin: 4 μg/ml NCDC; trypsin: 10 μg/ml soybean trypsin inhibitor).

For size fractionation, 500 μl samples of digests were sedimented on a glycerol gradient in the formate digestion buffer. Gradient fractions were analyzed by non-reducing PAGE on 3–15% gradient gels and by electron microscopy.

Reduction of Human Tenascin

Gradient-purified tenascin was dialysed into 0.2M ammonium formate buffer as described for proteolysis. Urea was added to 4M in treated and control samples. Aliquots to be reduced were treated with 2 mM dithiothreitol for 30 min, followed by 4 mM iodoacetamide for 30 min, all at room temperature. Comparable volumes of ammonium formate buffer were added to control samples incubated for 1 h at room temperature. All samples were dialysed overnight at 4°C against ammonium formate buffer to remove urea and other reagents and then sedimented as before on 15–40% glycerol gradients. Fractions from the gradient were analyzed by non-reducing 3–15% PAGE and by electron microscopy.

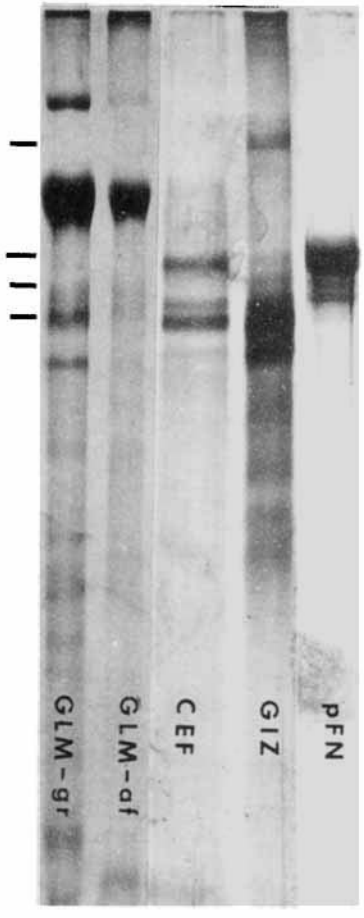
Overall yields of recovered protein for reductions and proteolytic digestions were low (as little as 20%) due to losses on dialysis membranes and in glycerol gradients. As there was no simple and reliable way to assay the small amounts of protein in each step and fraction, we have not attempted to quantitate the yields in these procedures.

RESULTS

Electron Microscopy of Native Hexabrachions

A comparison of hexabrachions from several sources by reduced SDS-PAGE analysis appears as Figure 1A. Affinity-purified human glioma tenascin (GLM-af) consists of a major band at 320 kDa, a sharp band at about 640 kDa, which varies in relative intensity between preparations, and a very weak doublet at about 220–230 kDa. All of these bands are stained by monoclonal antibody and polyclonal antibodies against chicken and human tenascin in immunoblots [9]. In addition, two moderate intensity bands at about 200 and 230 kDa can be seen in the gradient-purified human protein (GLM-gr), but these have not been identified as tenascin.

The arms of human hexabrachions, of which a representative small field and distribution of arm lengths are shown (Fig. 1B,C), averaged 87 nm in length (±1% standard error of the mean) in specimens prepared from pH 9.5, 0.2 M ammonium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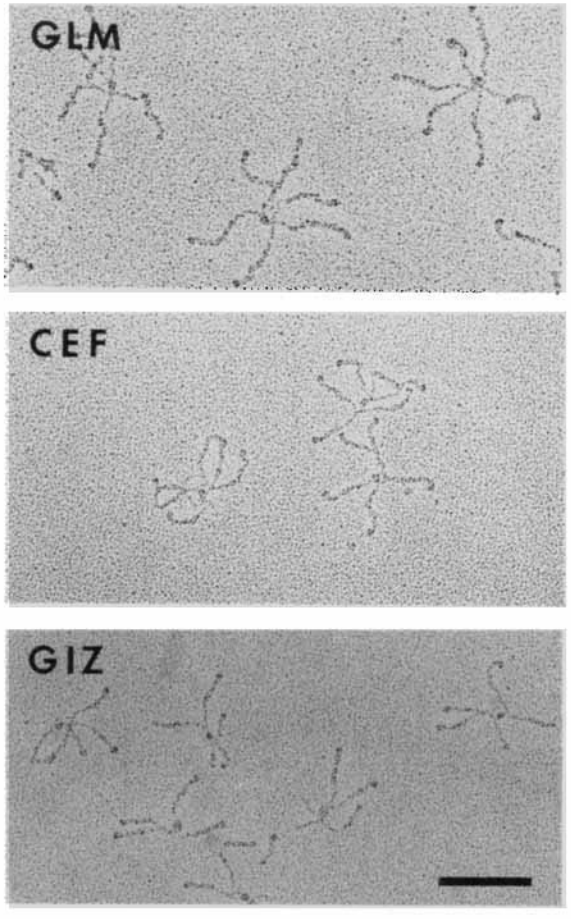
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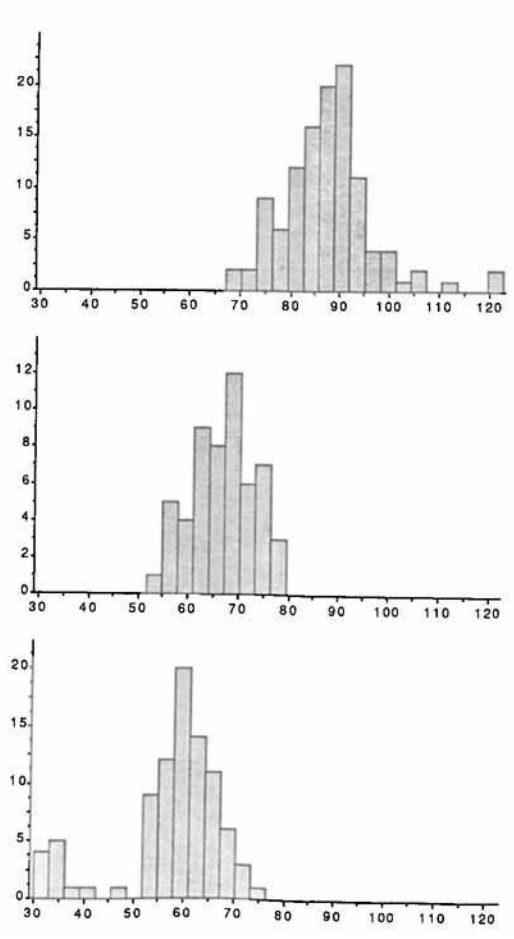
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bicarbonate buffer. A different preparation of human hexabrachions, sedimented in a glycerol gradient containing 0.2 M ammonium formate, 10 mM sodium PIPES, pH 8.0, gave an average arm length of only 80 nm. Thus, the measured arm length may be affected by the buffer from which the specimens are prepared.

CEF tenascin showed smaller subunits than the human protein on SDS-PAGE: a major band at 250 kDa and a pair of bands at 225 and 215 kDa. The arms of CEF hexabrachions (Fig. 1B) were correspondingly shorter, averaging 67 ± 0.8 nm in 0.2 M ammonium bicarbonate buffer, pH 9.5.

Tenascin extracted from adult chicken gizzard and affinity purified on an M1 antibody column (Fig. 1—GIZ) showed prominent bands at 225 kDa, 215 kDa, and 200 kDa as well as a diffuse band at approximately 500 kDa and several diffuse bands between 180 and 120 kDa. Micrographs of the gizzard protein showed hexabrachions with arms of varying length. Most arms, even the shortest ones observed, had an apparently normal distal knob. The distribution of gizzard hexabrachion arm lengths is markedly shifted relative to CEF arms, with an average of 56 nm and some arms as short as 30 nm. In immunoblots (data not shown) of M1 affinity-purified gizzard tenascin, all of the bands visible on silver-stained gels were stained by the polyclonal antibody to chicken myotendinous antigen [1]. However, the monoclonal antibody M1 stained only bands at or above 200 kDa. This indicates that the M1 epitope, which has been localized to a point about one-third of the way from the T-junction, is absent from the shorter arms.

To separate affinity-purified CEF tenascin subspecies on the basis of size, a single fraction eluted from M1-Sepharose was sedimented on a 15–40% glycerol gradient. The tenascin separated into two distinct peaks, a 13-12 S peak in fractions 7 and 8 and a 10-9 S peak in fractions 10 and 11 (Fig. 2A). Electron microscopy revealed that the 13-12 S peak contained almost exclusively intact hexabrachions, while the 10-9 S peak contained three-armed half-hexabrachions. Within each of these peaks, there was a partial separation of the different subunit sizes. In the faster-sedimenting fraction of each peak, the 250 kDa band was very prominent, while the 215–225 kDa doublet was weak. These two bands were of nearly equal intensity, however, in the trailing fractions of each peak. Measurements of arm length from electron micrographs showed that the hexabrachions in fraction 7 had more arms in the upper range of the length distribution (Fig. 2B). The average arm length in fraction 7 (Fig. 2), which showed almost exclusively the 250 kDa subunit, was 72 nm. The average arm length in fraction 8, which was approximately 50:50 in small and large subunits, was 66 nm, so the average length of the small subunit arms must be less than this.

Human hexabrachions sedimented at 14 S, slightly faster than the chicken protein. There were very few half-hexabrachions in the protein prepared from the U-251 MG glioma cells.

Fig. 1. A comparison of tenascins by reducing SDS-PAGE and electron microscopy. **A:** Tenascins and human plasma fibronectin (pFN) on silver-stained 5% reducing PAGE: GLM-gr = gradient-purified human glioma tenascin; GLM-af = human glioma tenascin affinity purified on 81C6-Sepharose; CEF = M1 affinity-purified chicken embryo fibroblast tenascin; GIZ = affinity-purified rooster gizzard tenascin. Lines on the left indicate the position of standard proteins used as molecular mass standards: myosin, 212 kDa; laminin, 225 kDa; plasma fibronectin, 255 kDa; and laminin, 400 kDa. **B:** Electron micrographs of rotary-shadowed hexabrachions from human glioma, CEF, and rooster gizzard. Scale bar represents 100 nm. **C:** Distribution of arm lengths measured from well-spread hexabrachions in these samples.

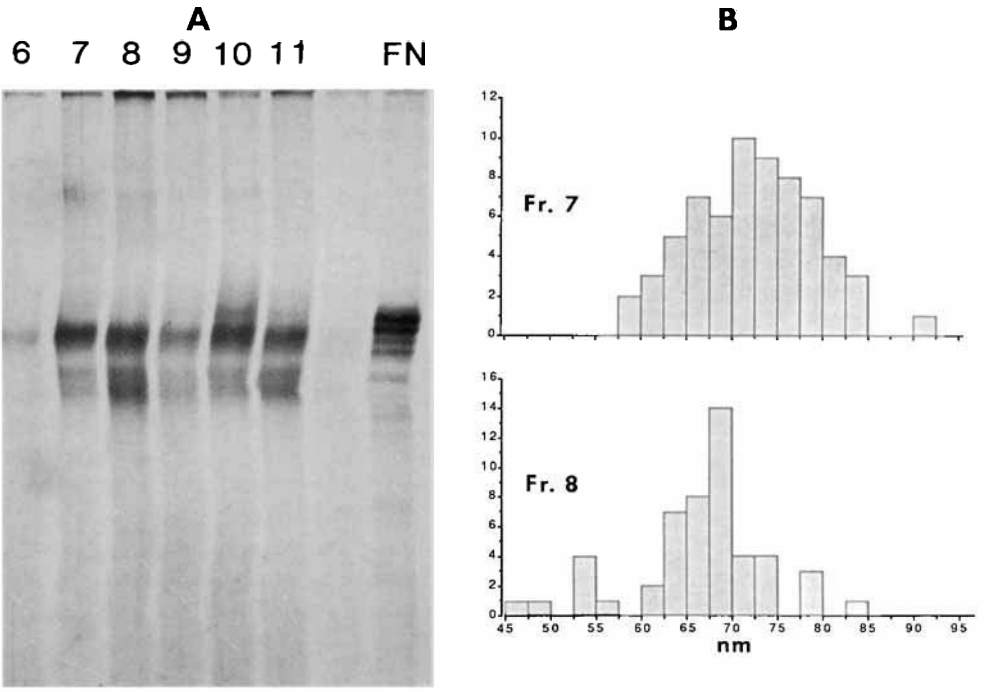

Fig. 2. Glycerol gradient sedimentation of affinity-purified chicken embryo fibroblast tenascin. **A:** Reducing SDS-PAGE of gradient fractions. Lane numbers correspond to gradient fraction numbers (fraction 7 is about 13 S, fraction 10 about 10 S). FN = human plasma fibronectin. Fractions 7 and 8 contained over 90% hexabrachions as judged by EM and fractions 10 and 11 contained predominantly trimers. There is a partial separation of "heavy" and "light" hexabrachions and trimers. In fractions 7 and 10 the 250 kDa band is stronger, while in fractions 8 and 11 the 225/215 kDa doublet is stronger. **B:** Distribution of arm lengths measured from well-spread hexabrachions in several micrograph fields of gradient fractions 7 and 8, demonstrating a small but significant difference in length.

Circular Dichroism Spectroscopy

The ultraviolet CD spectrum of purified tenascin is very similar to that of fibronectin (Fig. 3). The spectrum shown here is of Mono-Q tenascin, but that of affinity purified tenascin is virtually identical. Most notably absent from these spectra are the double minima at 222 and 209 nm. This indicates an absence of appreciable α helix in these proteins. The spectrum of fibronectin is similar to those previously published [28,29]. Both fibronectin and tenascin exhibit a minimum centered at 213 nm, indicating a substantial degree of β structure. The magnitude of the molar ellipticity values reported here may be underestimated due not only to our lamp condition, but also to possible binding by small amounts of proteoglycan [29].

The Molecular Mass of the Native Hexabrachion

Human and chicken tenascin were run on 0.75% non-reducing agarose gels by using von Willebrand protein oligomers, laminin, and fibronectin as high molecular weight standards (Fig. 4). These studies indicated an M_r of 1.9×10^6 for native human tenascin. This corresponds to a hexamer of 318 kDa subunits, in good agreement with the subunit size estimated on reducing SDS–polyacrylamide gels. Agarose gel electrophoresis of a sample of chicken tenascin, containing predominantly trimers (half-

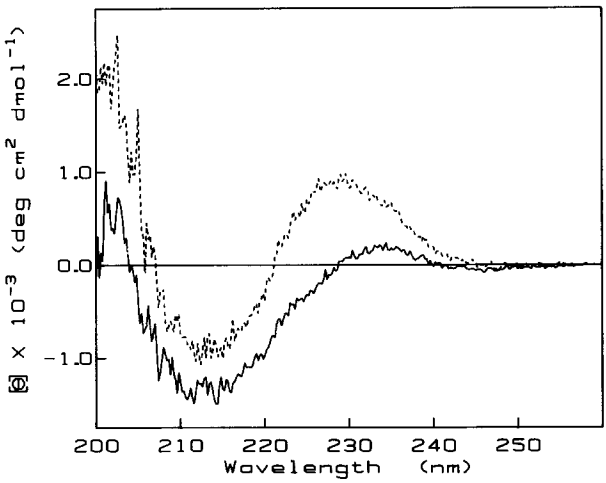

Fig. 3. Ultraviolet circular dichroism spectra of fibronectin and tenascin. The dotted line (upper curve) is bovine plasma fibronectin, and the solid line is human tenascin. Proteins were in phosphate-buffered saline, pH 7.4.

hexabrachions) as seen by EM, presented a more complex band pattern, apparently related to the diversity of subunit molecular masses. In this preparation, the hexamer band is relatively weak and diffuse, but the trimers appeared as a sharp doublet with M_r 770 kDa and 640 kDa. If each trimer were composed of subunits of one size, the "large" trimers would contain subunits of 250 kDa and the smaller trimers would have subunits of 215 kDa.

For sedimentation equilibrium studies purified tenascin was brought to equilibrium at three rotor speeds. The resulting protein gradients at each rotor speed were analyzed by plotting ln A_{280} against R^2 (the distance from the center of rotation). Curved lines were obtained at each rotor speed. Further analysis indicated the presence of a major species with $M > 10^6$ and a minor species with $M \sim 10^5$. Correcting the data at 3,600 and 4,000 rpm and using $\bar{v} = 0.70$ ml/g (see Methods), the molecular weight of the large component was calculated to be $1.90 \pm 0.05 \times 10^6$. This same value was obtained for the two samples of tenascin, one purified by antibody affinity chromatography and the other by gradient sedimentation and Mono-Q chromatography. Assuming that our estimate of $\bar{v}$ might be in error ± 1.5% ($\bar{v} = 0.69$–0.71 ml/g), we could have an additional error of $\pm 0.07 \times 10^6$. A value of $1.9 \pm 0.1 \times 10^6$ is an overall estimate of the molecular weight and error range for the human hexabrachion.

Exoglycosidase Treatments of Tenascin

Digestion of CEF tenascin with 1 U/ml of neuraminidase decreased the apparent subunit size by approximately 10 kDa and sharpened all three bands (see Fig. 5, lanes 1–3). A similar and perhaps larger shift was obtained with neuraminidase-treated human tenascin (lanes 4–6): the apparent molecular weight of the 320 kDa band was decreased by more than 10 kDa and the resulting band was perceptibly narrower than that of the untreated protein. The 640 kDa band was also decreased slightly in apparent molecular mass. We observed no change in the characteristic hexabrachion structure in EM after treatment with neuraminidase. Even after 24 h of incubation of human

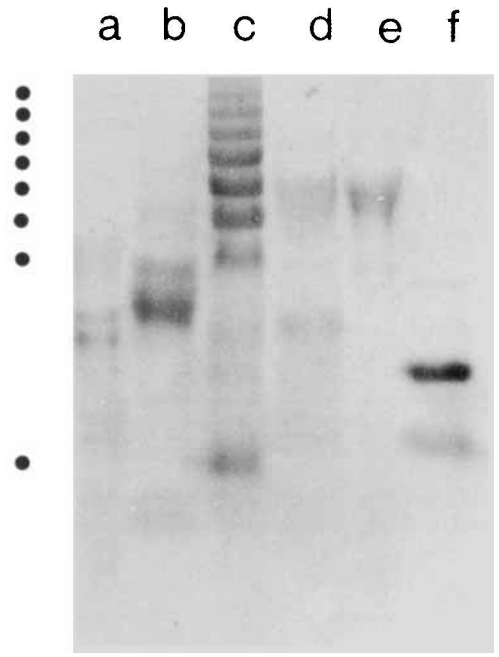

Fig. 4. Agarose gel analysis of native tenascin relative molecular weights. Proteins were electrophoresed on a 0.75% agarose gel, transferred to nitrocellulose, and stained with India ink as described in Methods. **Lane a:** Affinity-purified CEF tenascin (containing predominantly trimers). **b:** Mouse laminin (MW = 825 kDa). **c:** von Willebrand protein (multimers are indicated by dots to the left of the figure. Starting from the bottom, the dots represent [14]: 0.25×10^6, 1×10^6, 1.5×10^6, 2×10^6, 2.5×10^6, 3×10^6, 3.5×10^6, 4×10^6 Da). **d:** gradient-purified human glioma tenascin. **e:** Affinity-purified human glioma tenascin. **f:** Human plasma fibronectin (MW = 510 kDa, 255 kDa).

tenascin with α-mannosidase, no change in the migration of the protein on SDS-PAGE was observed (data not shown).

Reduction of Human Tenascin

Human tenascin was treated with dithiothreitol and iodoacetamide in the presence of 4M urea in order to dissociate the disulfide-bonded subunits. After treatment, hexabrachions and reduced fragments were separated by glycerol gradient sedimentation. Gradient fractions were analyzed on non-reducing 3–15% SDS-PAGE and by electron microscopy (Fig. 6). The disulfide reduction of the protein was apparently incomplete, producing a range of hexabrachion fragments. Various oligomers were found in fraction 11′. Fraction 14′ (6 S) contained most of the reduced protein in a strong, broad band at 320 kDa. Electron microscopic examination showed a homogeneous sample of single hexabrachion arms, averaging 80 nm in length, complete with distal knobs.

Proteolysis of Tenascins

Fragments produced by incubation of affinity-purified CEF tenascin with 10 μg/ml of α-chymotrypsin or trypsin were studied by SDS-PAGE and electron microscopy (Fig. 7). Chymotrypsin reduced the apparent size of all three bands on reduced gels by about 10 kDa (Fig. 7A, lane 2). On non-reduced gels (Fig. 7B) native CEF tenascin

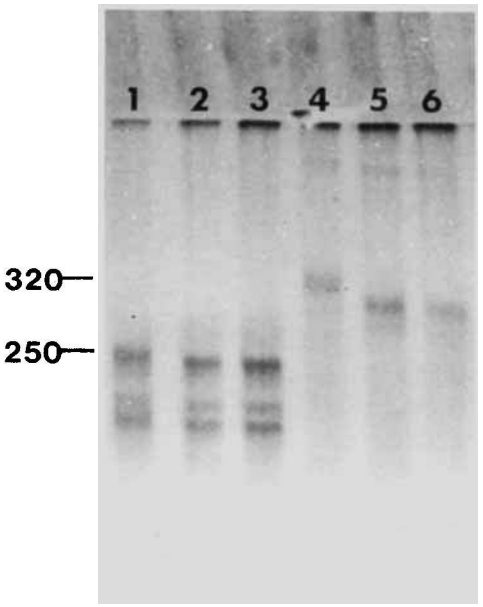

Fig. 5. Neuraminidase treatment of CEF and human tenascin. Gradient-purified proteins were dialyzed into 0.1 M sodium acetate buffer, pH 5.0. Aliquots of CEF (**lanes 1–3**) and human glioma (**lanes 4–6**) tenascin were treated at room temperature with no added enzyme (1 and 4); 1 U/ml neuraminidase for 1 h (2 and 5): 1 U/ml neuraminidase for 3 h (3 and 6).

showed two bands: one that barely enters the running gel, probably the hexamer, and one slightly below it corresponding to the trimer. (These correlations are based on other experiments with fractions enriched for hexamers or trimers.) Chymotrypsin treatment eliminated the hexamer band and left a prominent band running slightly faster than the native trimer. Electron microscopy of this material (Fig. 7C) showed predominantly

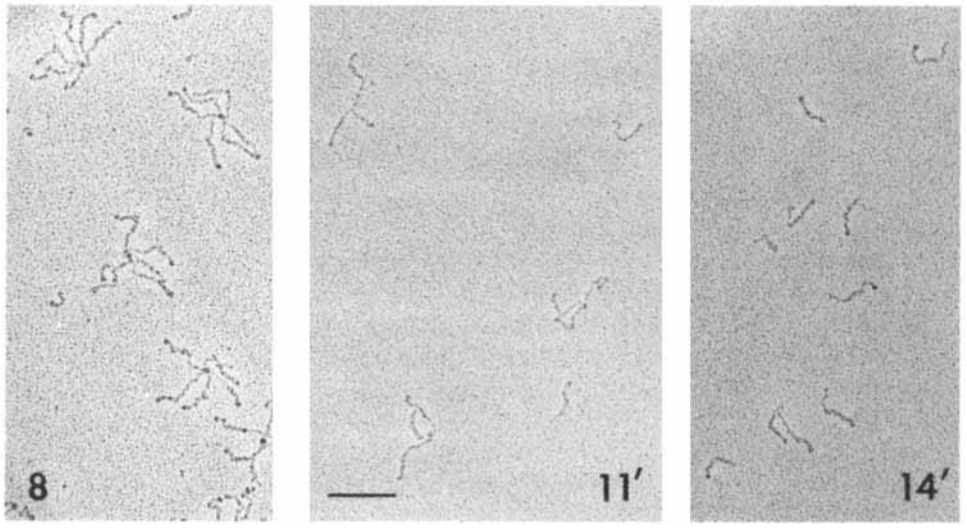

Fig. 6. Reduction of human hexabrachions. Electron micrographs of individual gradient fractions, as labelled. Fraction 8 shows gradient-purified natiive hexabrachions. Fraction 11′ shows incompletely reduced oligomers, and fraction 14′ is almost exclusively single hexabrachion arms. Scale bar = 100 nm.

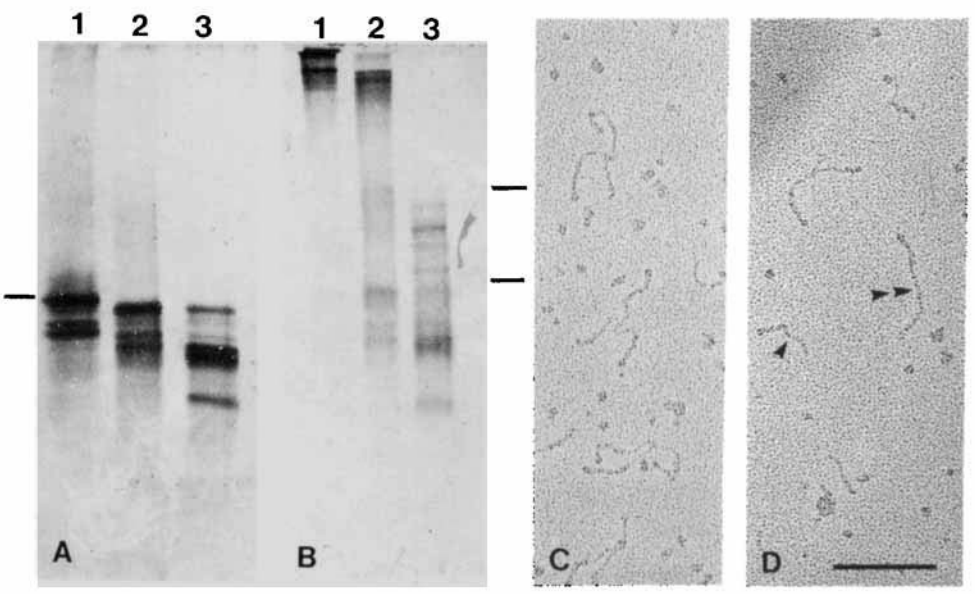

Fig. 7. Proteolysis of chicken tenascin. Affinity-purified CEF tenascin was incubated with 10 μg/ml α-chymotrypsin or 10 μg/ml trypsin in 0.1M Tris buffer, pH 8.0 for 30 min. SDS-PAGE (5%) under reducing (**A**) and non-reducing (**B**) conditions. Control CEF tenascin is shown in **lanes 1A, 1B;** α-chymotrypsin treated in **lanes 2A, 2B;** and trypsin treated in **lanes 3A, 3B.** The lines indicate the position of fibronectin, 255 kDa reduced (A), and 255 and 510 kDa non-reduced (B). Electron micrographs of chymotrypsin-treated tenascin (**C**) show predominantly trimers, missing the central knob. Trypsin-treated tenascin (**D**) shows a mixture of short single arms (average length 62 nm, single arrowhead) and longer arms with a single distal knob (average length 115 nm, double arrowhead). The smaller globular particles were frequently seen in electron micrographs of dialyzed samples and do not correlate with any protein bands that we have identified. Scale bar = 100 nm.

trimers, lacking any central knob. α-chymotrypsin apparently cleaves the CEF hexabrachion at a single site just proximal to the T-junction, cleaving off most of the central knob. We will refer to this site as "C1."

Trypsin treatment of CEF tenascin produced bands of about 215 and 170 kDa on reducing gels (Fig. 7A, lane 3) and a more complex set of bands on non-reducing gels (Fig. 7B, lane 3). Electron micrographs showed single arms of 62 nm average length (single arrowhead, Fig. 7D) and longer fragments averaging 115 nm (double arrowhead) and typically containing only a single terminal knob. Because of the low concentration of the CEF material, these fragments could not be separated for further analysis.

Human glioma tenascin could be obtained at much higher concentrations, permitting separation and recovery of trypsin digestion products. Trypsin-treated human tenascin was sedimented on 15–40% glycerol gradients and analyzed by non-reducing SDS-PAGE and EM (Fig. 8). Undigested tenascin peaked in fraction 7 (14 S), with the intact hexabrachions remaining in the 3% stacker of the non-reducing gel. Trypsin treatment of tenascin produced a variety of oligomeric species. In fraction 11 (9 S), weak bands at or above 10^6 Da probably corresponded to tetramer and trimer fragments. Images of these, shown in Figure 8B, indicate two probable sites of trypsin action. Cleavage at site "T1" (Fig. 9), just proximal to the T-junction, would produce whole trimers. This site is very close to the C1 chymotrypsin site. Cleavage at a site immediately distal to the T-junction, "T2," would remove entire single arms.

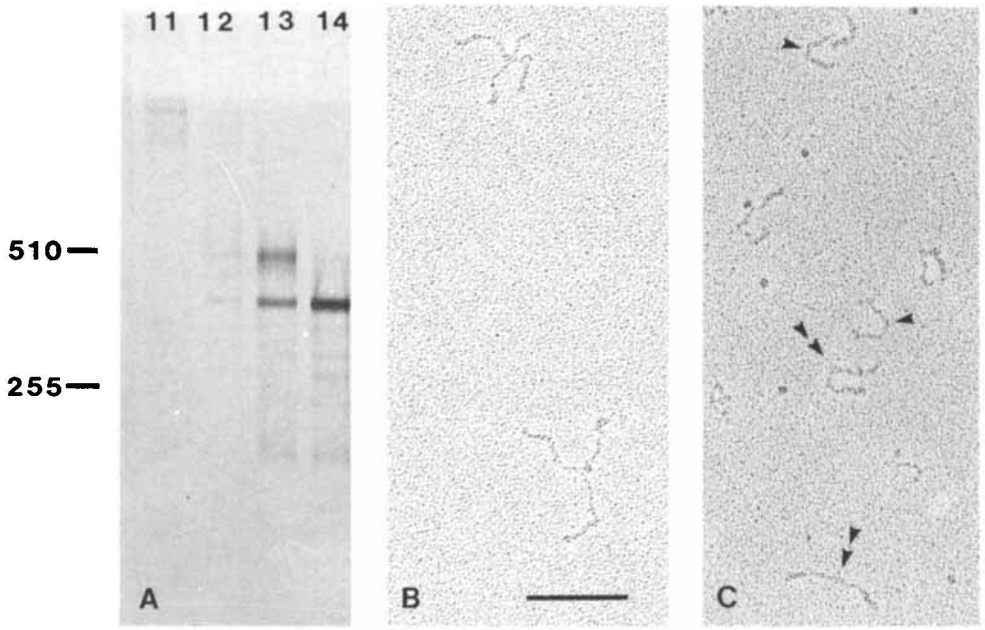

Fig. 8. Proteolysis of human tenascin. Human tenascin was digested with trypsin and fragments were separated by glycerol gradient sedimentation. Non-reducing SDS-PAGE in **A** shows a faint high molecular weight band probably corresponding to trimers in fraction 11 and a prominent 400 kDa band in fraction 14. Electron micrographs of samples from fractions 11 (**B**) and 14 (**C**) are shown. Single arrowheads indicate almost complete single arms, and double arrowheads indicate linear fragments about 1 ¼ times the length of a single arm, apparently disulfide-bonded dimers of arms cleaved at T3. Scale bar = 100 nm.

A third trypsin site, "T3," is indicated by the fragments observed in fraction 14 (Fig. 8C). Cleavage at this site would remove about 100 kDa from the distal end of hexabrachion arms. Depending on how many arms were also cleaved at T1 or T2 a wide variety of oligomers would be produced with one or more short arms, as well as single-arm fragments lacking part of the thickened distal segment and distal knob. The prominent 400 kDa band in fraction 14 probably represents disulfide-bonded dimers of two short arms. (A prominent band is seen on reducing gels at 200 kDa.) The symmetrical strand shown at the bottom of Figure 8C (double arrowheads) is such a double-arm fragment, of average length 100 nm, with some of the thickened distal portion visible at each end. Single-arm fragments averaging 67 nm in length in length (indicated in Fig. 8 by single arrowheads) have apparently been cleaved at sites T2 and T3. The 510 kDa band seen in fraction 13 could represent double-arm fragments with a single T3 cut, but we were unable to see well-spread tenascin fragments in this fraction. Oligomers with short arms, cleaved at T3 and perhaps another minor site, were also seen in fractions 11–13. (E.g., the lower oligomer in Fig. 8B has two normal arms and one cut at T3; the upper oligomer appears to have three arms cut near T3 and one shorter fragment.)

DISCUSSION

We present a summary of the biochemical data for human and chicken tenascin in Table I. The molecular mass of the human hexabrachion was estimated to be 1.9×10^6

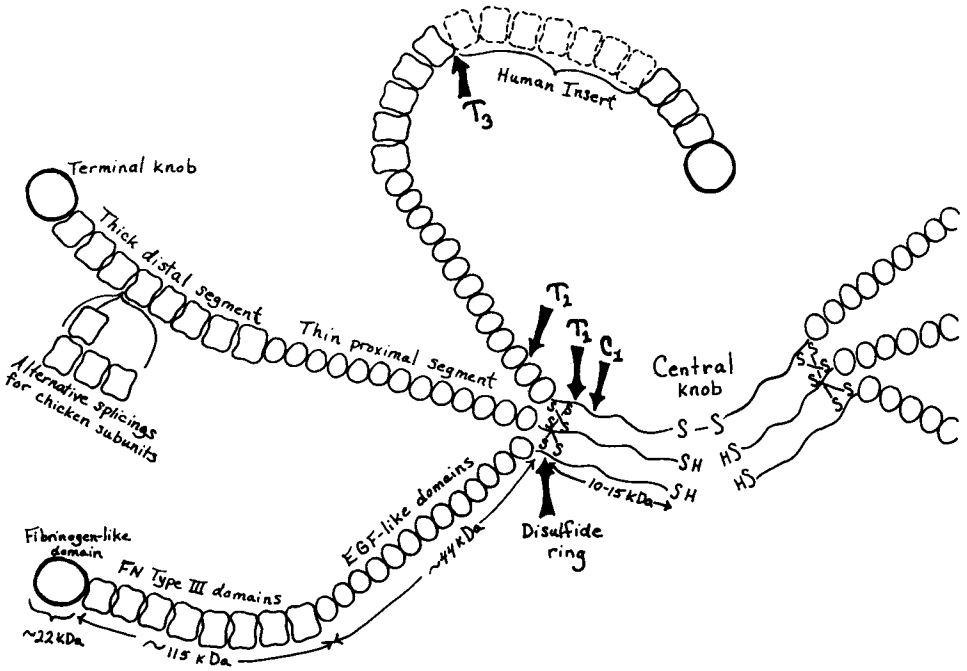

Fig. 9. Model of hexabrachion, showing domain structure, disulfide bonds, and proteolysis sites. This model is based on EM studies and the cDNA sequence of chicken tenascin [29–32]. The lower arm shows the minimum-sized arm for both the chicken and human protein, with eight FN-III domains. The insertions of additional FN-III domains by alternative RNA splicing [31,32] are indicated in the two upper arms. The chymotrypsin cleavage site (C1) was determined for CEF hexabrachions, while the trypsin cleavage sites (T1, T2, and T3) and the locations of disulfides are based on experiments performed with human hexabrachions. Note that these cleavage points are only approximately indicated and do not signify exact locations in the domain structure of tenascin.

TABLE I. Biochemical Data for Hexabrachion/Tenascin

	Human	Chicken
Subunit mass (by SDS-PAGE, reducing)	320 kDa and *230, 220 kDa*[a]	250 kDa and *225, 215 kDa*[a]
Native hexabrachion mass		
Agarose gel electrophoresis	1.9×10^6 Da	1,540 kDa and *1,280 kDa*[a]
Sedimentation equilibrium	$1.9 \pm 0.1 \times 10^6$ Da	—
Average arm length (in 0.2 M NH_4HCO_3, pH 9.5)	87 nm	72 nm and *<66 nm*[a]
Sedimentation coefficient	14 S	13 S and *12 S*[a]
Extinction coefficient (A_{277} 1 mg/ml, 1 cm)	0.97	—

[a]The italicized numbers refer to small-subunit hexabrachions.

both by agarose gel electrophoresis under non-reducing conditions and by sedimentation equilibrium of native protein. This is very close to six times the 320 kDa molecular mass of a single subunit, estimated by SDS-PAGE. Although it has been generally assumed that each arm of the hexabrachion corresponded to a single subunit, this determination of the mass of the intact (non-reduced) molecule is an important confirmation.

All of the fibrillar extracellular matrix proteins characterized to date are known to be glycoproteins with a substantial carbohydrate content and varying modes of glycosylation. Vaughan et al. [10] have treated tenascin with endoglycosidase F to remove all amide-linked oligosaccharides and report a 20 kDa decrease in apparent molecular mass. Our neuraminidase treatment, which should remove all terminal sialic acid residues, caused an apparent 10 kDa decrease in all subunits of both human and chicken tenascin. The much sharper bands of the neuraminidase-treated subunits point to a substantial heterogeneity in sialylation of the native glycoproteins. Neither human nor chicken tenascin appears to contain significant terminal mannose, which would be indicative of immature N-linked carbohydrate chains. A substantial carbohydrate content is also suggested by the buoyant density of tenascin in CsCl, which was 2.3% higher than that of fibronectin.

Hexabrachion proteins from several sources consistently display the distinctive structural features discussed in the Introduction but vary significantly in arm contour length and rate of migration on SDS-PAGE gels. Indeed, it is obvious from Figure 1 that there is a direct relationship between hexabrachion arm length and apparent subunit molecular weight of the various tenascins we have purified. In performing contour length measurements on chicken hexabrachions, which show three different subunit sizes on gels, each arm of each hexabrachion was labelled uniquely and separate tallies were maintained for arms appearing at the center and sides of each trimer. We found considerable variation in measured arm length within each hexabrachion, but no difference that could be consistently related to the two positions in the trimer.

The variation in arm length within individual chicken hexabrachions initially suggested to us that hexabrachions were assembled from a random assortment of the three subunit sizes. However, the separation on a glycerol gradient of fractions containing predominantly large or small subunits, and the correlation of longer arms with the larger subunit (Fig. 2), suggest that the different subunits are mostly segregated into two types of hexabrachion: "large" hexabrachions with exclusively or predominantly 250 kDa subunits, and "small" hexabrachions with 215–225 kDa subunits. This conclusion is also supported by the two trimer bands seen on agarose gels, corresponding to M_r of 3×250 kDa and 3×215 kDa (Fig. 4). Further corroboration came from studies using a monoclonal antibody that recognizes only the large subunit of chicken tenascin on Western blots: when used for imunoaffinity purification, this antibody bound hexabrachions that contained exclusively the large subunit [30].

Vaughan et al. [10] reported an average length of 74 nm for hexabrachions purified from chicken embryo cartilage. SDS-PAGE showed these hexabrachions to consist almost exclusively of the small subunit. Chiquet-Ehrismann et al. [12] reported an average length of 75 nm for chicken hexabrachions that should be 50:50 small and large subunits. Both of these values are larger than the values of 60 and 72 nm that we determined for chicken hexabrachion arms. However, our own data suggest that buffer and specimen preparation conditions can affect the length measured. Average arm-length measurements can be reproducible within one laboratory, but comparisons between different groups may be less meaningful.

All arms of CEF hexabrachions are defined at the distal end by a terminal knob and at the proximal end by attachment to the central knob, so it seems unlikely that the 215 and 225 kDa subunits represent either precursors or degradation products of the 250 kDa subunits. Rather, the different-sized peptides probably result from differential splicing of the tenascin mRNA. This has already been demonstrated by cDNAs for both chicken and human tenascin [33–36]. The shorter arms and smaller subunit sizes that

we observed for adult chicken gizzard hexabrachions suggest that additional segments can be spliced out from somewhere in the middle of the arm. Even the shortest arms (about 30 nm) had an apparently normal attachment to the "T" junction at one end and a normal distal knob at the other end. Further, the M1 antibody did not stain any bands smaller than 200 kDa in the gizzard protein, though these lower bands were stained by the polyclonal antibody to chicken tenascin. If these bands were the result of proteolysis, the M1 epitope near the middle of the arms [9,10] would not have been removed by cleaving as little as 15 kDa from a subunit.

We suggest that there are two types of disulfides involved in the assembly of the native hexabrachion: a "ring" of disulfides connecting the subunits within the trimer and at least one disulfide within the central knob joining the trimers to each other. As the trimer consists of three apparently identical subunits, it is attractive to postulate a structure incorporating three-fold symmetry. Interesting precedents are the "disulfide rings" in fibrinogen, which connect the three chains of the triple α-helix [31], and a similar arrangement of disulfides that apparently connects the three chains of thrombospondin [32]. Proteolysis experiments discussed below suggest that the disulfide ring is outside the major mass of the central knob and close to or at the T-junction. In addition to this ring of disulfides, we hypothesize a second type of cysteine in the central knob of each subunit that can form a disulfide bond between trimers. If the three chains of a trimer are identical and symmetrically distributed, there will be three such cysteine residues in each "half-knob." If all three disulfide bonds were formed, the entire hexabrachion would have three-fold symmetry. However, the ease with which trimers are produced by proteolysis and by disulfide reduction suggests that the trimers may be joined by only a single disulfide. In this case there would be four free sulfhydryls in the central knob of the hexabrachion, which might bind additional trimers. The oligomers with nine and 12 arms, which we find in small numbers but consistently [13], would be explained by this model. A three-dimensional model of the hexabrachion, incorporating these features of symmetry and disulfide bonding, is presented elsewhere [13].

The total contour length of the reduced single arms was found to be the same as the length of native arms from the T-junction to the distal knob. If, as our data suggest, the central globular particle is made up of segments from each arm, we might expect the isolated, reduced arms to be 5–10 nm longer than the native arms as measured from the T-junction. These measurements raise the possibility that the central globular particle is a separate peptide, though we have never seen evidence for this on gels [9,13]. Alternatively, when the arms are dissociated by reduction, the segment that previously extended into the central knob might be too thin to visualize or may refold into a small domain.

The strong, higher molecular weight band (640 kDa), seen on reducing SDS gels of all human glioma tenascins, stains with polyclonal and monoclonal antibodies elicited against the main 320 kDa band [9] (unpublished data), suggesting that this band represents two covalently cross-linked subunits. Longer boiling with reducing agent in Laemmli buffer caused no change in the intensity of the 640 kDa band relative to the principal hexabrachion band. Therefore, we conclude that the upper band is not merely the result of incomplete reduction but rather another type of covalent crosslink, perhaps produced by a transglutaminase. The intensity of this band varied considerably for different preparations but was always less than 20% of the 320 kDa band. Bands corresponding to dimers are not seen in CEF tenascin but are conspicuous in tenascin isolated from chicken brain [9] and adult gizzard (Fig. 1).

Limited proteolysis of chicken hexabrachions by α-chymotrypsin produced a relatively simple set of large fragments. All subunits were reduced by about 10 kDa,

indicating a single cleavage site near one end. Nearly all of the trimers observed in EM specimens of digested CEF hexabrachions were lacking a central knob, indicating a major site of cleavage, C1, proximal to the T-junction (or disulfide ring). Apparently there are one or more minor cleavage sites on the arms of the trimer just distal to the T-junction, as a significant fraction of the chymotrypsin-digested protein was found in a triplet of bands from 400–500 kDa (below dimer molecular mass) and another triplet from 200–240 kDa (below monomer molecular mass).

Trypsin digestion of the chicken hexabrachions produced a more complicated pattern of lower molecular weight bands. The most common hexabrachion fragments in trypsin digests seen by EM were short single arm fragments and linear fragments longer than a single arm with no central nodule. Similar fragments, as well as some fragmented oligomers, were seen in EM analysis of trypsin-digested human hexabrachions. The 100 nm fragments observed are apparently the proximal segments of two arms, joined by a disulfide bond. The human tenascin trypsin fragments could be explained by three cleavage sites, T1 and T2 on either side of the T-junction, and a site T3, which would remove the distal third of an arm.

Our model of the hexabrachion structure, as diagrammed in Figure 9, is a speculative correlation between the structural features observed by EM and published sequence data and is in agreement with previous interpretations of primary structural information [33–36]. A tenascin subunit is interpreted to be a string of small, globular domains, similar to the modular construction of fibronectin [37,38]. The central knob contains the amino terminal segment, with cysteine residues available for inter-chain disulfide bonding. There follows a string of 13 domains of 31 amino acids each, with sequences similar to the EGF-like domains found in several other ECM proteins. These could constitute the thin inner segment of the hexabrachion arm. The thick distal segment then would comprise a series of larger domains (90 amino acids) similar to the type III domains of fibronectin. The number of these FN-III domains depends on alternative RNA splicing. cDNAs have been identified specifying eight or 15 FN-III domains in human [35] and eight, nine, or 11 FN-III domains in chicken [36]. Circular dichroism spectra strongly indicate that tenascin's regions of fibronectin-like sequence are folded into a quite similar secondary structure. Finally, the carboxy terminal segment of the subunit shows similarity to domains in the β- and γ-chains of fibrinogen. This domain probably corresponds to the terminal knob of the hexabrachion arm.

As each of these domains appears to be an independently folded unit, each arm of the hexabrachion could have multiple independent functions. It is reasonable to speculate that these functions will be related to the very large size of the hexabrachion and will utilize its hexavalent structure. Interactions with cells and with other ECM components are currently being studied in several laboratories as potential functional activities [13,16].

ACKNOWLEDGMENTS

We wish to thank Dr. Darrell D. Bigner and his co-workers for their generous gift of the 81C6 monoclonal antibody, for the human glioma cell line U251MG (clone 3), and for helpful and stimulating discussions.

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