Rapid intracellular assembly of tenasin hexabrachions suggests a novel co-translational process

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SUMMARY

Tenasin, an extracellular matrix protein that modulates cell adhesion, exists as a unique six-armed structure called a hexabrachion. The human hexabrachion is composed of six identical 320 kDa subunits and the structure is stabilized by inter-subunit disulfide bonds between amino-terminal segments. We have examined the biosynthesis of tenasin and its assembly into hexabrachions using pulse-chase labeling of U-138 MG human glioma cells. Newly synthesized tenasin hexamers are secreted within 60 minutes of translation initiation. Intracellularly, as early as full length tenasin can be detected in pulse-labeled cell lysates, it is already in hexameric form. No precursors, such as monomers, dimers, or trimers, were identified that could be chased into hexamers. This lack of assembly intermediates suggests that nascent tenasin polypeptides associate prior to completion of translation. In contrast, fibronectin monomers in the same lysates are gradually formed into disulfide-bonded dimers. Although hexamer assembly is rapid, the rate-limiting step in secretion appears to be transport to the medial Golgi as endoglycosidase H-resistance was not detected until after a 30 minute chase. These results provide evidence for a novel co-translational mechanism of tenasin assembly which would be facilitated by its length and by the amino-terminal location of the assembly domain.

Key words: tenasin, oligomerization, disulfide bond, extracellular matrix, glioma cell

INTRODUCTION

Most extracellular matrix proteins are multimeric. A variety of subunit combinations have been described including multimers composed of identical subunits (tenasin (TN), cartilage oligomeric matrix protein, and thrombospondin), subunits that differ by alternative splicing (fibronectin), or subunits derived from different genes (many collagens, fibrinogen, and laminin) (Engel, 1991). These multimeric complexes are stabilized by disulfide bonds between subunits. For example, fibronectin dimers are formed by a pair of disulfide bonds at the very carboxy terminus of the chains. Disulfide bonds between the non-collagenous domains of collagen chains initiate triple helix formation (Vuorio and de Crombrugghe, 1990). The Aβ, Bβ, and γ chains of fibrinogen are held together via an amino-terminal knot which is stabilized by disulfide bonds (Zhang et al., 1993). Each of these covalently-linked protein complexes is assembled post-translationally in the lumen of the endoplasmic reticulum (ER). Similarly, the luminal domains of influenza hemagglutinin and other transmembrane proteins associate post-translationally to form multimers (Hurtley and Helenius, 1989). TN, cartilage oligomeric matrix protein, and thrombospondin differ from the other classes of multimeric matrix proteins in that identical subunits are assembled using amino-terminal inter-subunit disulfide bonds. No information is yet available on the mechanism of assembly of these three large multimeric proteins.

One of the most striking features of the extracellular matrix molecule TN is its unusual hexameric structure, called a hexabrachion. As first observed by Erickson and Inglesias (1984), the human TN hexabrachion appears to consist of two trimers joined at their centers by short linker arms and a central knob. Chicken TN has also been shown to adopt this novel hexabrachion structure (Vaughan et al., 1987; Hoffman et al., 1988; Spring et al., 1989) and structurally similar molecules have been isolated from leech and sponge (Masuda-Nakagawa et al., 1988; Humbert-David and Garrone, 1993). Like many extracellular matrix proteins, the TN monomer exhibits a repeating structure and is composed of four types of modules, the so-called heptad repeat domain, the epidermal growth factor-like repeats, the fibronectin type III-like repeats and the fibrinogen-like domain (reviewed by Erickson, 1993). Similar repeating patterns of these modules are present in TN from mouse (Saga et al., 1991; Weller et al., 1991) and pig (Nishi et al., 1991) and in other members of the TN family which include human gene X (Bristow et al., 1993), Drosophila ten (Baumgartner and Chiquet-Ehrismann, 1993), rat janusin (Fuss et al., 1993) and chicken restrictin (Nörenberg et al., 1992). Restrictin and janusin form trimers resembling half hexamers, suggesting that multimeric form is functionally important to this protein family.

As is common among proteins of the extracellular matrix, transcripts from a single TN gene are alternatively spliced to yield two or more polypeptides that differ only in their number
of fibronectin type III-like repeats (Erickson, 1993). The so-called ‘small’ and ‘large’ splice forms are differentially located in developing embryos, both spatially and temporally (Chiquet-Ehrismann et al., 1991; Mackie and Tucker, 1992; Saga et al., 1991; Tucker, 1993; Weller et al., 1991). Although cultured cell lines often produce both splice variants of TN, mixed hexamers containing both small and large monomers have never been reported (Erickson and Lightner, 1988; Taylor et al., 1989). The segregation of small and large splice variants could suggest that the roles of these two forms are distinct.

Hexabrachion distribution in vivo is consistent with TN’s role as adhesion modulator, as TN is localized primarily to regions of active cell migration. TN is widely expressed at epithelial-mesenchymal boundaries during development (Erickson and Bourdon, 1989). Expression in the adult is limited to areas of tissue growth or remodeling, such as areas of epithelial cell shedding (Probstmeier et al., 1990), hematopoietic cell migration (Ocklind et al., 1993), wound healing (Mackie et al., 1988), and tumor formation (Chiquet-Ehrismann, 1993). In vitro, TN can have vastly different effects on cell adhesion, depending on how it is presented to the cells. Many cell types are able to adhere to immobilized TN (for examples, see Chiquet-Ehrismann et al., 1986; Friedlander et al., 1988; Lotz et al., 1989), yet soluble TN can prevent cell adhesion to fibronectin (Chiquet-Ehrismann et al., 1988). Recombinant fusion proteins containing TN fragments have been used to confirm that TN has adhesive as well as anti-adhesive properties in vitro (Spring et al., 1989; Murphy-Ullrich et al., 1991; Prieto et al., 1992; Joshi et al., 1993). The significance of TN’s hexameric structure in modulating cell adhesion has not been elucidated.

The conservation of the hexabrachion from invertebrates to mammals suggests a functional role for the unique structure of TN. Aside from the observations that the majority of TN purified from cell-conditioned medium is hexameric, little is known about the biosynthesis, assembly, and secretion of TN. We have determined the process by which TN is assembled into hexamers. Although it has been proposed that TN is first formed into trimers and these subsequently assemble into hexamers (Spring et al., 1989), hexabrachion assembly is surprisingly rapid and no assembly intermediates are detectable. The data indicate that, unlike all other multi-subunit proteins analyzed to date, including transmembrane proteins such as influenza hemagglutinin and extracellular matrix proteins such as fibronectin, TN hexamers can be assembled from ribosome-associated monomers, a novel multimerization mechanism. A model is presented explaining how TN’s structure could facilitate assembly of six nascent polypeptide chains.

**MATERIALS AND METHODS**

**Anti-TN antiserum preparation**

Mouse TN cDNA clones spanning all 13 type III repeats were isolated from a mouse bone marrow stromal cell cDNA library kindly provided by Thor Lemischka (Princeton University). A 1053 base pair fragment encoding repeats III 2–5 (nucleotides 2329 to 3381 as numbered by Weller et al., 1991) was inserted into the bacterial expression vector pMALcRI (New England Biolabs, Beverly, MA). The maltose-binding protein-TN fusion protein was purified according to the manufacturer’s protocol. Peak fractions from an amylose resin column were pooled and dialyzed into 10 mM Tris-HCl, pH 8, 50 mM NaCl, 1 mM EDTA. A New Zealand White rabbit was immunized with 0.2 µg fusion protein in complete Freund’s adjuvant (Life Technologies, Inc., Gaithersburg, MD) and was boosted two weeks later with 0.2 µg of fusion protein in incomplete Freund’s adjuvant (Life Technologies, Inc.). Immune bleeds were collected at intervals after the boost. Each bleed was assayed by efficiency of immunoprecipitation of TN from U-138 MG conditioned medium (see below) and bleeds of similar titer were pooled. This serum is referred to as R759. R759 and commercially available anti-human TN antiserum (Life Technologies, Inc.) were compared for their ability to immunoprecipitate TN from both conditioned medium and Nonidet P-40 (NP-40) (Calbiochem, La Jolla, CA) lysates and no differences were observed in the immunoprecipitates. The data presented here were obtained with the R759 anti-serum.

**Metabolic labeling and cell lysis**

U-138 MG cells (ATCC, Rockville, MD, number HTB 16) were grown in minimum essential medium, α medium (Life Technologies, Inc.), supplemented with 10% fetal bovine serum (Hyclone, Logan, UT). For overnight labeling, cells were incubated in methionine-free minimum essential medium, α medium, supplemented with 10% fetal bovine serum, 10 µM unlabeled methionine and 25 µCi/ml [35S]methionine (Protein Express, Dupont-NEN, Boston, MA). Labeled medium was collected and, following centrifugation to remove cells and debris, EDTA and phenylmethylsulfonil fluoride were added to 10 mM and 2 mM, respectively. Four hour continuous labelings were carried out in cysteine and methionine deficient medium supplemented with 10% fetal bovine serum and 100 µCi/ml each of [35S]methionine and [35S]cysteine (1,200 Ci/mmol) (Dupont-NEN). At each of the timepoints, an aliquot of labeled medium was removed and treated with protease inhibitors as above.

Pulse-chase labelings were performed on confluent cells in 35 mm dishes. The cells were first rinsed in cysteine and methionine deficient serum-free medium, then pulse labeled with 100 µCi/ml each of [35S]cysteine and methionine in the same medium. Following the pulse, the cells were rinsed with minimum essential medium, α medium, supplemented with 10% fetal bovine serum and a five-fold excess of unlabeled cysteine and methionine, then incubated in the same medium for the duration of the chase. Where indicated, 0.5 mM cycloheximide (Sigma Chemical Co., St Louis, MO) was included in this chase medium in order to inhibit protein synthesis (Braakman et al., 1991). In experiments where cycloheximide was included in the chase, pulse time was increased to 12 minutes in order to maintain signal intensity. The chase medium was then aspirated and the cell monolayer rinsed with phosphate buffered saline containing 1 mM N-ethylmaleimide. The cells were lysed in 0.5 ml of NP-40 lysis buffer (1% NP-40, 150 mM NaCl, 50 mM Tris-HCl, pH 7.5, 1 mM N-ethylmaleimide, 1 mM iodoacetic acid and 2 mM phenylmethylsulfonyl fluoride) (Bode et al., 1986) on ice and the dishes were scraped with a rubber policeman. The alkylating agents N-ethylmaleimide and/or iodoacetic acid were included in the wash and lysis buffers in order to prevent the formation of disulfide bonds between free cysteines. 1 mM concentrations were shown to be sufficient to alkylate any free cysteines, as ten-fold higher concentrations (10 mM) of N-ethylmaleimide and/or iodoacetic acid in lysis and wash buffers had no effect on the oligomeric forms seen in intracellular lysates. NP-40 insoluble material was removed by centrifugation in a microfuge for 15 minutes at 4°C.

For trichloroacetic acid (TCA) precipitations, small aliquots (20-25 µl) of conditioned medium were spotted onto filter paper and allowed to dry. The filter paper was then incubated in 10% TCA on ice for 30 minutes. This was followed by incubation in 5% TCA on ice for 30 minutes. The filter paper was then washed in two changes of 95% ethanol and allowed to dry. The papers were placed in liquid scintillant (EcoScint, National Diagnostics, Manville, NJ) and counted in a scintillation counter.
Immunoprecipitation and glycosidase treatment

$^{35}$S-labeled tenascin and fibronectin were isolated from equal volumes of NP-40 cell lysates or of conditioned medium. $^{35}$S-labeled fibronectin was purified by binding to gelatin-agarose beads. Following a one hour incubation at 4°C, the beads were washed with phosphate buffered saline and bound proteins were eluted by boiling in sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) sample buffer without reducing agents. $^{35}$S-labeled TN was isolated by immunoprecipitation with R759 anti-TN antiserum. Immunoprecipitations were carried out with an excess of antibody (as determined empirically) for one hour at 37°C in the presence of 0.5% NP-40, 0.5% deoxycholate and 0.1% SDS. This was followed by overnight incubation with Protein A-agarose beads (Repligen, Cambridge, MA) at 4°C. These beads were then washed three times in 50 mM Tris-HCl, pH 8, 2 mM EDTA, including the above detergents. Proteins were eluted by boiling in SDS sample buffer without reducing agents. For glycosidase experiments, the beads were divided into equal portions during the detergent wash and then washed with phosphate buffered saline to remove residual detergent. These aliquots were then digested with N-glycosidase F or endoglycosidase H (endo H) (Boehringer Mannheim, Indianapolis, IN) according to the manufacturer’s suggested reaction conditions. Following these digestions, proteins were eluted from the beads as above.

Polyacrylamide gel electrophoresis and data analysis

Equal volumes of protein samples were applied to SDS polyacrylamide gels (Laemmli, 1970) either in the absence of reducing agents or in the presence of 100 mM dithiothreitol. In order to examine the 1,900 kDa hexamer under non-reducing conditions, a 2-5% polyacrylamide gradient-SDS resolving gel with a 2.5% polyacrylamide-SDS stacking gel was used. In addition to pre-stained molecular mass standards (Sigma Chemical Co.), myofibrillar proteins (kindly provided by Isaac Peng and Donald Winkelmann, Robert Wood Johnson Medical School, Piscataway, NJ) and purified laminin (kindly provided by Peter Yurchenco, Robert Wood Johnson Medical School, Piscataway, NJ) were used as marker proteins. Following electrophoresis, the gels were fixed, stained with Coomassie Brilliant Blue, dried and exposed to a storage phosphor screen (Molecular Dynamics, Sunnyvale, CA) for 15 to 72 hours. Additionally, gels were exposed to Kodak (Rochester, NY) XAR-5 film at ~80°C for 3 to 4 weeks. All figures presented herein are photographs of those films.

The storage phosphor screen was scanned using a model 400E PhosphorImager (Molecular Dynamics). Bands were identified and band intensities quantitated using ImageQuant software (Molecular Dynamics) as recommended by the manufacturer. Briefly, a grid was drawn across all of the lanes of a gel such that the band of interest was completely enclosed by a separate rectangle in each lane. Background was calculated for each rectangle within the grid by measuring the number of counts in the outline of the rectangle and using this as an average for the entire area of the rectangle. The total number of counts for each rectangle in the grid was calculated and the background was subtracted. The number of counts above background per rectangle was then normalized to TCA precipitable counts in each sample in order to correct for differences in total protein and labeling efficiency in the various samples. The data were then plotted using Cricket Graph version 1.3.2 (Cricket Software, Malvern, PA).

The synthesis time of TN was estimated using the method of Horwitz et al. (1969). Briefly, TCA-precipitable counts in total protein and TN-specific PhosphorImager counts were plotted versus labeling time. The linear portion of each of these curves was extrapolated to the abcissa in order to determine the lag time before linear incorporation of label into total protein and TN. The difference between these lag times gives an estimated synthesis time for TN of 6 minutes.

RESULTS

Secretion of TN by U-138 MG cells

In order to examine the biosynthesis and assembly of hexabrachions, a cell line that produces easily-detectable amounts of TN was required. Examination of numerous mesenchymal cell lines led to the selection of U-138 MG human glioblastoma cells for use in this study. As previously reported by Bourdon et al. (1983), these glioblastoma cells secrete relatively high levels of TN, even when compared with other mesenchymal cells, such as fibroblasts.

A TN-specific polyclonal antiserum was prepared against a bacterial fusion protein containing type III repeats 2-5 from mouse TN fused to maltose binding protein. This antiserum was used to immunoprecipitate TN from U-138 MG conditioned medium (Fig. 1). Hexamers and monomers were examined simultaneously by using a 2-5% polyacrylamide gradient SDS gel. Under reducing conditions, TN migrates as a 320 kDa monomer (right lane), while non-reduced TN contains a major hexamer band at 1,900 kDa (left lane). The sizes of the TN bands were confirmed using purified laminin (850 kDa) (Martin and Timpl, 1987) and the myofibrillar proteins titin (3,000 kDa) and nebulin (700 kDa) (Granzier and Wang, 1993). In addition, contaminating fibronectin in the TN immunoprecipitations served as internal size markers of 250 kDa reduced and 500 kDa non-reduced. Fibronectin could be removed by pre-incubating samples with gelatin-agarose before immunoprecipitation. However, this step was generally omitted because the easily-identified fibronectin was well-separated from the TN under the electrophoresis conditions used. Non-reduced immunoprecipitates also contained two larger-sized bands and material which did not enter the separating gel. This high molecular mass (>3,000 kDa) material probably represents higher order multimers of hexabrachions and other secreted molecules.

Fig. 1. Tenascin from U-138 MG conditioned medium. Immunoprecipitated TN was electrophoresed in a 2-5% polyacrylamide gradient SDS gel non-reduced (left) or reduced (right). TN monomers and hexamers are indicated by arrowheads marked TN or H, respectively. Fibronectin monomers and dimers are indicated by arrows marked FN or D, respectively. Positions of titin (3,000 kDa), laminin (850 kDa), and nebulin (700 kDa) are indicated. The 700 kDa species in the reduced TN lane is probably equivalent to the TN-dimer-sized band reported by Erickson and Lightner (1988) and Taylor et al. (1989). Two-dimensional (non-reducing/reducing) SDS-PAGE analysis revealed that this 700 kDa band is a component of the hexabrachion band (not shown).
U-138 MG cells rapidly secrete TN into the culture medium. TN was detectable in U-138 MG cell conditioned medium as early as 45 minutes after the addition of labeling medium (Fig. 2) and this TN was secreted in hexameric form (data not shown). This rate of secretion is comparable to that of fibronectin dimers which appear in cell-conditioned medium approximately 30 minutes after initiation of labeling of U-138 MG cells.

**Appearance of TN in intracellular fractions**

The rate of synthesis of TN was examined by monitoring detergent lysates of labeled U-138 MG cells. Labeling medium contained both $[^{35}S]$cysteine and $[^{35}S]$methionine which, given the cysteine-richness of the epidermal growth factor-like repeats of TN, should yield nascent chains labeled to relatively high specific activities. Full length TN appeared in detergent lysates following a 5 minute labeling and steadily increased in abundance throughout the course of the 20 minute labeling period (Fig. 3A). At the 5 minute timepoint and throughout the labeling, TN migrates as a hexamer (Fig. 3B). Significantly lower levels of monomer were observed.

**Assembly of TN into hexamers**

To monitor the synthesis and assembly of TN, pulse-chase labeling experiments were performed using a 10 minute labeling pulse followed by varying periods of chase. This pulse time, based on the estimated rate of TN synthesis, was short enough to label a single pool of newly-synthesized molecules but sufficiently long to allow easy detection of TN. SDS-PAGE of anti-TN immunoprecipitates under non-reducing conditions revealed that the vast majority of TN was hexameric following the 10 minute labeling pulse and throughout the 60 minute chase (Fig. 4A). No clear precursor/product relationship was seen during TN assembly. Other minor bands are visible in detergent lysates of the U-138 MG cells. However, their levels do not vary relative to total labeled protein. Therefore, none of these bands represents intermediates that are chased into TN hexamers.

In contrast, assembly of fibronectin dimers from monomer subunits can be followed using the same lysates. Fibronectin was isolated from these pulse-chase labeled cell lysates using gelatin-agarose affinity and examined by SDS-PAGE. Following the 10 minute pulse, the majority of labeled fibronectin migrates as a monomer, with a minor fraction migrating as a dimer (Fig. 4B). During the chase, the monomer band decreases and the dimer band accumulates with a halftime of dimer formation between 5 and 10 minutes.

A quantitative assessment of the levels of each of the species of TN and fibronectin was made using arbitrary PhosphorImager counts normalized to TCA-precipitable, labeled protein (see Materials and Methods). A graphic representation of the radioactivity (measured as PhosphorImager counts) in the fibronectin monomer and dimer bands shows that the maximal level of monomer was reached after a 5 minute chase (Fig. 5B). An additional 20 to 25 minutes were required before the monomer band disappeared. As the proportion of fibronectin monomer steadily declined (Fig. 5B, inset), the amount of dimer in the cell lysate increased and then decreased as dimers were secreted. Similarly, the amount of labeled TN hexamers first increased and then declined, consistent with the labeled molecules being completed and then secreted into the culture medium. While the level of TN hexamer shows the expected variation, the level of monomer remained constant (Fig. 5A). Unlike fibronectin, there is no clear precursor/product relationship between monomer and hexamer. Therefore, the monomeric TN polypeptides detected in cell lysates are not assembly intermediates for hexamers. The constant low level of monomers indicates that they are either abortive assembly products or artifacts generated by experimental manipulation.
The lack of detectable assembly intermediates suggests that TN can be assembled into hexamers co-translationally. This process could involve six nascent chains or one or more nascent chains and a large pool of intermediates residing in the ER. If this pool of intermediates were sufficiently large, the fraction of intermediates labeled during the 10 minute pulse might be so small that it would not be detected by the means used here. We therefore used a 60 minute labeling followed by varying periods of non-radioactive chase. Anti-TN immunoprecipitates of these lysates failed to reveal the presence of a pool of TN monomers or other precursor molecules to which nascent chains could be added.

The pulse-chase data presented above were obtained under chase conditions that allowed completion of nascent chains. Levels of TN, fibronectin, and total protein increased during short chase periods and this accumulation might mask assembly of intermediates into hexamers. Therefore, cycloheximide was included in the chase medium in order to focus on a defined pool of completed TN polypeptides. Since cycloheximide prevents completion of nascent chains, its addition to the chase medium ensures that only those polypeptides completed during the pulse labeling are detected, not a continuing flow of partially labeled nascent chains that are completed during the chase.

Examination of fibronectin from NP-40 lysates showed that protein assembly and secretion were not perturbed by cycloheximide. As expected, the ratio of fibronectin monomer to dimer was relatively high at the end of the pulse labeling, but showed a 4-fold decrease in just 10 minutes of chase in the presence of cycloheximide (Fig. 6). The ratio of TN monomer to hexamer, however, was unchanged throughout the cycloheximide-containing chase. This indicates that full-length monomers are not assembled into hexamers. Similar analyses of the other protein bands in electrophoretograms of these immunoprecipitates failed to reveal any intermediate molecules that were chased into hexamers.

**Glycosylation state of intracellular TN**

To monitor the rate of intracellular transport of TN from the ER and through the Golgi, we used N-glycosidase F and endo H to examine the glycosylation state of the TN immunoprecipitated from pulse-chase lysates. While N-glycosidase F cleaves off all asparagine-linked sugars, endo H is unable to cleave sugars modified in the medial Golgi. Therefore resistance to endo H is a marker for passage into this compartment. Treatment with N-glycosidase F resulted in an increase in
mobility for all the pulse-chase samples, confirming that they have passed into the lumen of the ER where sugar addition begins (Fig. 7A). Additionally, the immunoprecipitated intracellular TN was sensitive to treatment with endo H until the 30 minute chase after which a resistant fraction can be detected, indicating passage through the Golgi. Therefore, although TN hexamers are assembled quickly, passage through the secretory pathway and the accompanying processing events are much slower.

N-glycosidase F treatment of TN from U-138 MG conditioned medium also resulted in an increase in mobility by SDS-PAGE. Before N-glycosidase F treatment, conditioned medium TN migrated slightly slower than TN from intracellular fractions. Following N-glycosidase F treatment, however, conditioned medium and intracellular tenascins co-migrated, indicating that the observed molecular mass differences are due to differences in glycosylation state.

As with other secreted proteins, the rate-limiting step in TN secretion is apparently transport from the ER to the Golgi (Pelham, 1989). Loss of endo H sensitivity is a marker for passage through the medial Golgi. As shown in Fig. 7B, the endo H sensitive pool of TN molecules increased as nascent chains were completed and was then rapidly diminished, as would be expected for a pool of molecules passing out of the ER into the Golgi. The endo H resistant fraction, however, remained at a fairly constant level (Fig. 7B), indicating that there was no accumulation of these molecules. This shows that TN exits the cell rapidly following passage through the Golgi and that passage from the ER to the Golgi is the rate-limiting step in TN secretion.

DISCUSSION

Our results suggest a novel co-translational assembly mechanism for TN. As soon as full length TN is detectable in labeled cell lysates, it exists in disulfide-bonded hexamers. No intermediates in hexamer formation were detected. The lack of intermediates, particularly trimers, was unexpected, since TN hexabrachions in electron micrographs appear to be composed of two trimers joined at a central knob (Erickson and Inglesias, 1984) and TN contains amino-terminal heptad repeats that have been proposed to form a triple coiled-coil structure (Spring et al., 1989). Previously, it has been assumed that the steric constraints of ribosome size precluded the association of multiple nascent chains in the ER lumen (Hurtley and Helenius, 1989). However, the unusual size and structure of TN could facilitate these interactions.
Other similarly-sized extracellular matrix proteins are assembled post-translationally. For example, collagens, fibronectin, and vonWillebrand factor have carboxy-terminal domains for oligomer formation and thus are assembled from completed chains (Vuorio and de Crombrugghe, 1990; Choi and Hynes, 1979; Counts et al., 1978). The rapidity of TN hexamer assembly is dramatically illustrated by comparison to fibronectin dimer formation within the same cell lysates. In contrast to TN assembly, fibronectin monomers remained the predominant species throughout a 10 minute pulse. Several multimeric proteins have amino-terminal assembly domains. Of these, fibrinogen has been shown to be assembled in a stepwise post-translational process (Yu et al., 1983). It remains to be determined whether thrombospondin and cartilage oligomeric matrix protein form multimers as rapidly as TN.

Given the rapidity and specificity of TN multimer formation, it seems likely that a molecular chaperone facilitates hexamer formation. Several chaperones have been implicated in folding and assembly of secreted proteins. HSP47 binds specifically to collagen and apparently plays a role in the formation of collagen hetero-trimers, preventing secretion of incomplete or misfolded collagen (Nakai et al., 1992). Calnexin associates with incompletely folded or misfolded monomeric glycoproteins, presumably aiding folding and oligomerization (Helenius, 1994) and protein disulfide isomerase has been shown to catalyze the formation and rearrangement of disulfide bonds in newly synthesized proteins within the ER (Bulleid and Freedman, 1988). Additionally, BiP is involved in translocation across the ER membrane (Sanders et al., 1992) as well as in folding and assembly in the ER (Bole et al., 1986). Whether these or other ER proteins are involved in TN assembly remains to be determined.

In order for six ribosome-associated chains to multimerize, the arrangement of ribosomes must bring nascent chains near enough to assemble. Free polysomes have been shown to adapt a helical conformation with approximately 5 to 6 ribosomes per turn (Ross and Benditt, 1964). Similar conformational constraints apparently govern the arrangement of the membrane bound ribosomes of the rough ER. En face views of rough ER reveal ribosomes arranged in a variety of rosettes, spirals, loops, and double rows (Palade, 1975). These circular, spiral, and looping membrane-bound polysomes place neighboring ribosomes and their associated nascent chains in close proximity. A variety of electron micrographs of both thin section and freeze-fractured cells have been examined and the average center-to-center distance between adjacent ribosomes is estimated to be 35 nm (Fig. 8A) (Shelton and Kuff, 1966; Ojakian et al., 1977; Tanaka et al., 1989). In addition to the 35 nm, estimates of the minimum length needed for six nascent chains to contact each other must include the 12 nm length of the TN monomer, dimer or trimer forms, but hexamers have never been isolated (Nörenberg et al., 1992; Pesheva et al., 1989). The 50 nm length taken from electron micrographs could facilitate trimer formation by nascent chains (see dotted lines in Fig. 8A), but might not be sufficient to allow co-translational formation of hexamers. Based on a comparison with TN, the Drosophila tena protein is predicted to be less than 30 nm and, consistent with this model, two other TN family members, restrictin and janusin, have been observed in monomer, dimer or trimer forms, but hexamers have never been isolated (Nörenberg et al., 1992; Pesheva et al., 1989).

Fig. 8. A model for co-translational assembly of a TN hexamer. For simplicity, ribosomes are represented by spheres. (A) Top view (approximately to scale) of a rosette of ribosomes with arrows representing ribosome-bound nascent TN chains in the proposed arrangement necessary for contact in the ER lumen. Solid lines indicate nascent polypeptide chains involved in hexamer formation, dotted lines indicate nascent chains involved in trimer formation. Approximate lengths are indicated. (B) Side view (not to scale) of proposed model of assembly of six nascent TN monomer chains.

Hexabrachions are homo-hexameric, consisting of a single splice variant (Erickson and Lightner, 1988; Taylor et al., 1989). Hetero-hexamers of both small and large alternatively-spliced subunits have never been observed. Furthermore, the small and large hexamers often show different localizations during development, both spatially and temporally (Chiquet-Ehrismann et al., 1991; Mackie and Tucker, 1992; Saga et al., 1991; Tucker, 1993; Weller et al., 1991). This lack of overlapping distribution implies that the two different-sized hexabrachions may have functional differences. Kaplony et al. (1991) speculated that the alternatively spliced region functions in facilitating cell migration, given the tight co-local-
ization of the large splice form to areas of corneal cell migration. Chiquet-Ehrismann et al. (1991) observed that the small splice variant binds more strongly to fibronectin than the large variant, suggesting that the small variant may inhibit cell migration by interfering with cell interactions with fibronectin. Co-translational assembly of nascent TN polypeptides from a single polysome provides a mechanism for assembly of homomultimers and ensures that functionally ambiguous heterohexamers containing both small and large subunits are not formed.

In summary, TN apparently employs a novel oligomerization mechanism in which nascent chains are joined to form a hexamer. Identification of the TN residues and the ER proteins involved in hexabrachion assembly will help to elucidate the specific interactions that mediate this unique assembly process.

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