Tenascin-C Hexabrachion Assembly Is a Sequential Two-step Process Initiated by Coiled-coil α-Helices*

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We have investigated the oligomerization process of tenascin-C using a variety of recombinant wild-type and mutant polypeptide chain fragments produced by heterologous gene expression in Escherichia coli. Biochemical and biophysical analyses of the structures and assemblies of these fragments indicated a sequential two-step oligomerization mechanism of tenascin-C involving the concerted interaction of two distinct domains and cysteines 64, 111, and 113. First, the sequence between alanine 114 and glutamine 139 initiates hexabrachion formation via a parallel three-stranded coiled coil. Subsequently, the tenascin assembly domain, which is unique to the tenascins, is responsible for the connection of two triplets to a hexamer. The oligomerization of the tenascin assembly domains by the three-stranded coiled coil increases their homophilic binding affinity and is an important prerequisite for tenascin-C hexamerization. Although formation of the characteristic hexabrachion structure involves the covalent linkage of the six subunits by cysteine residues, mutational analysis indicates that hexamer formation is not dependent on intermolecular disulfide bonds. Most interestingly, substitution of glutamate 130 within the coiled-coil domain by leucine or alanine resulted in the formation of parallel four-stranded helix structures, which further associated to dodecamers. Aside from supporting a sequential process of tenascin-C assembly, this finding provides experimental evidence that non-core residues can have profound effects on the oligomerization states of coiled coils.

Cyttotactin/hexabrachion/tenascin-C (TN-C)1 exists as a large disulfide-bonded hexamer in extracellular matrices (1). Depending on the species analyzed and the variant generated by alternative splicing, each subunit has a molecular mass in the range of 190~300 kDa (2). Like many other extracellular matrix components, TN-C is a modular glycoprotein that contains five types of domains (3). The N-terminal stretch of ~110 residues is unique to the TNs. This segment is followed by a short heptad repeat region (putative coiled-coil domain), 13 domains that are homologous to the epidermal growth factor, and a series of fibronectin type III domains. The C-terminal end is well conserved, with homology to the C-terminal domains of β and γ chains of fibrinogen. Similar patterns of domains are present in the other members of the TN family, including TN-R (4), TN-X (5), and TN-Y (6). Although also TN-R protein isolated from tissues has been found in the form of dimers and trimers (4), the biological importance of the multimeric form to this protein family has not been elucidated.

TN-C is highly expressed in a large number of developing and regenerating tissues, such as sites of wound healing (7, 8), whereas it is generally present at low or undetectable levels in the corresponding intact regions in adults. Together with numerous cell culture experiments, these findings implied important roles of TN-C in embryonic development as well as tissue growth and remodeling. In vivo functions of TN-C, however, remain elusive and may be subtle since knockout mice lacking TN-C were reported to develop normally (9).

One of the most impressive characteristics of TN-C is its radially arranged six-armed structure called hexabrachion. As first observed by Erickson and Iglesias (10), the TN-C hexabrachion appears to consist of two trimers joined at their centers by short linker arms and a central knob. Biochemical and electron microscopic evidences located the oligomerization domains of TN-C at the N-terminal end of the molecule and suggested that flanking Cys residues are involved in hexabra- chion formation (11). Consistent with these observations, sequence analysis of TN-C revealed the presence of three complete heptad repeats within the N-terminal segment (11, 12). Heptad repeats of seven amino acid residues denoted a–g (13, 14) are the hallmark of proteins with a potential for coiled-coil formation (15, 16). The residues at positions a and d are mostly apolar, forming a 3,4-hydrophobic repeat, with charged resi- dues occurring frequently at the e and g sites. Residues at these four positions form the hydrophobic interface between α-helices and can participate in interhelical electrostatic interactions (17). Since the coiled-coil structural motif is widely used and well suited for the assembly of oligomeric proteins (18, 19), it has been speculated that TN-C hexabrachion assembly might involve the formation of a trimer intermediate whose oligomerization is mediated by coiled-coil α-helices (11). However, uncertainty existed about whether the short stretch of α-helix in TN-C actually could form a coiled coil since three to four heptad repeats were reported to be the minimum size requirement of amphipathic peptides to fold into stable coiled-coil structures (20–22). In addition, pulse-chase experiments have demonstrated that TN-C assembly is surprisingly rapid and suggested that nascent TN-C polypeptide chains assemble to hexamers prior to completion of translation since no intermediates such as monomers, dimers, or trimers could be identified (23).

Aside from these observations, nothing is known about TN-C assembly, raising the question about the mechanism of this process. We have addressed this issue by generating a variety...
of recombinant TN-C polypeptide chain fragments by heterologous gene expression in Escherichia coli. We have analyzed the structures and assemblies of these fragments by SDS-PAGE, CD spectroscopy, and analytical ultracentrifugation. In addition, we have identified by mutational analysis the Cys residues involved in the stabilization of TN-C hexamers. The functional significance of disulfide bond formation in TN-C assembly was assayed by analytical ultracentrifugation measurements of the mutant polypeptide chain fragments. Furthermore, we have investigated by site-specific mutagenesis the role of amino acid residues within the coiled-coil domain that are conserved among known species and members of the TN family. Our data indicate that formation of the TN-C hexabrachion structure is a two-step process involving the concerted interaction of the three-stranded coiled coil and the domain unique to the TNs. Furthermore, we provide evidence that a conserved Glu is a key residue in modulating the oligomeric state of the three-stranded coiled-coil domain, which is essential for the specific formation of TN-C hexabrachions.

EXPERIMENTAL PROCEDURES

Construction of Expression Plasmids—Plasmid pCTN230 was kindly provided by Dr. Ruth Chiquet-Ehrismann (Friedrich Miescher Institute, Basel, Switzerland). It was used as a template for polymerase chain reaction amplification of DNA fragments coding for Glu-34 to Ala-110 (p1), Ala-110 to Glu-34 (p3), and Ala-114 to Glu-34 (p4) of chicken TN-C (11). Oligonucleotide primer sets were designed as (p1: p1-5'-GCCCGGATCCACTTCCCG-3' and p1-3' (5'-CCGGAGGG-3'); p2: p1-5' and p2-3' (5'-TATGATTTCTTATTAG-3'); p3: p3-5' (5'-CCGGAGGG-3'); and p3-3' (5'-CCGGAGGG-3'); p4: p4-5' (5'-CCCCGACGGGC-3') and p4-3' (5'-CCGGGATTCTTATTACTGCTC-3').

The designed mutant polypeptide chain fragments pC64S and pE130L were generated by polymerase chain reaction as described by Ho et al. (25) using the sets of primers for each fragment (pC64S: pC64S-5' (5'-GGGAGGATACCAGCCCAGCCAGCTCCTCCAGTCTGCTCAG-3') and pC64S-3' (5'-CCGGAGGG-3'); and pE130L: pE130L-5' (5'-CTGTGCGGC-3') and pE130L-3' (5'-CCGGAGGG-3').

Mutant p1C/S was constructed by the same method from pC64S using the following primer sets: p1C64S: p1-5' and p1-3' (5'-GGGAGGATACCAGCCCAGCCAGCTCCTCCAGTCTGCTCAG-3'); and p1C64S: p2-5' and p2-3' (5'-CCGGAGGG-3'); and p2C64S: p2-5' and p2-3'. The designed mutant polypeptide chain fragments p1C64S and p2C64S were generated by polymerase chain reaction as described by Ho et al. (25) using the sets of primers for each fragment (p1C64S: p1-5' and p1-3' (5'-CCGGAGGG-3'); and p2C64S: p2-5' and p2-3').

Circular Dichroism Spectroscopy—CD spectra were acquired on a Jasco J720 spectropolarimeter. Far-ultraviolet spectra (200–250 nm) were measured in a 1-mm path length quartz cell and represent averages of 10 accumulations. Helix content was calculated by the method of Chen et al. (28). CD melting profiles were recorded on a Cary 61 spectropolarimeter equipped with a thermostated 1-mm path length quartz cell. Thermal stability was determined by monitoring the change in the mean molar residue ellipticity at a fixed wavelength of 221 nm [(θ)221] as a function of temperature. A ramping rate of 1 °C/min was used for all experiments. Data reduction analysis was performed with the Jasco (Japan Spectroscopic Co.), LABView (National Instruments), and Sigma Plot (Jandel Scientific) software packages.

RESULTS

Design and Production of N-terminal TN-C Polypeptide Chain Fragments—Biochemical and electron microscopic data suggest that the oligomerization sites of TN-C are located at the N terminus of the molecule and that flanking Cys residues are involved in hexamer formation (11). Consistent with these observations, sequence analysis of TN-C revealed the presence of three complete heptad repeats within the N-terminal part of the protein (Fig. 1). To dissect the mechanism of TN-C assembly, repurification of recombinant N-terminal polypeptide chain fragments was generated by heterologous gene expression in E. coli (Figs. 2A and 4A).

Based on similarities to the coiled-coil domains of cartilage matrix protein/matrixin 1 and the members of the thrombospondin gene family, which all are flanked by a pair of closely

polypeptide chain fragments contained two additional N-terminal residues, Glu and Ser, which originated from the expression plasmids and are not part of the TN-C coding sequence. If not stated otherwise, recombinant TN-C fragments were analyzed at room temperature in 5 mM sodium phosphate buffer (pH 7.4) supplemented with 150 mM NaCl. Disulfide Bridge Exchange and Shuffling in Redox Buffer—Cys sulfhydryl groups were completely reduced by incubation of the proteins with 100 mM dithiothreitol for 30 min at 37 °C. After precipitation by 75% ammonium sulfate saturation, proteins were redissolved and incubated at room temperature in redox buffer (0.2 M Tris-HCl (pH 8.0), 0.2 mM EDTA, 5 mM reduced glutathione, and 5 mM oxidized glutathione) under anaerobic conditions to allow disulfide bond formation and exchange of monomers. Disulfide bond formation was monitored by collecting aliquots at various times and subsequent SDS-PAGE or Tricine/SDS-PAGE analysis.

Geel Electrophoresis—SDS-PAGE and Tricine/SDS-PAGE (27) were performed on 12 × 13-cm slab gels. Proteins were visualized by staining with Coomassie Brilliant Blue R-250. Apparent molecular masses were obtained by comparison with low molecular mass markers (Amersham Pharmacia Biotech and Sigma).

Circular Dichroism Spectroscopy—CD spectra were acquired on a Jasco J720 spectropolarimeter. Far-ultraviolet spectra (200–250 nm) were measured in a 1-mm path length quartz cell and represent averages of 10 accumulations. Helix content was calculated by the method of Chen et al. (28). CD melting profiles were recorded on a Cary 61 spectropolarimeter equipped with a thermostatted 1-mm path length quartz cell. Thermal stability was determined by monitoring the change in the mean molar residue ellipticity at a fixed wavelength of 221 nm [(θ)221] as a function of temperature. A ramping rate of 1 °C/min was used for all experiments. Data reduction analysis was performed with the Jasco (Japan Spectroscopic Co.), LABView (National Instruments), and Sigma Plot (Jandel Scientific) software packages.

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spaced Cys residues (19), fragment p1 is expected to contain all the structural information required for the formation of a disulfide-linked hexamer. Cys-111 and Cys-113 are candidate residues to covalently connect three subunits by interchain disulfide bonds, and Cys-64 possesses the putative function of linking two triplets to the characteristic hexabrachion structure. To confirm the proposed roles of Cys-64, Cys-111, and Cys-113, mutant polypeptide chain fragments were generated in which either Cys-64 (p2C64S and p1C64S) or all three Cys residues (p1C/S) were replaced by Ser (Fig. 2A).

To assay the functional significance of conserved residues within the coiled-coil segment, mutant proteins were designed based on the alignment of known TN sequences (Fig. 1). Specifically, Gln-139 was changed to Leu (p1C64S/Q139L and p3Q139L), and Glu-130 was replaced by either Leu or Ala (p1C64S/E130L, p1C64S/E130A, p3E130L, and p3E130A) (Fig. 4A).

The homogeneity of the affinity-purified recombinant TN-C polypeptide chain fragments was assessed by Tricine/SDS-PAGE and SDS-PAGE under reducing conditions. Single bands migrating with mobilities corresponding to their respective molecular masses and with no degradation products were detected (Figs. 2C and 4C). Notably, peptides p3 and p4 could only be visualized as faint bands after transfer to nitrocellulose membranes followed by staining with Amido Black (data not shown).

TN-C Assembly Is Mediated by Two Autonomous Folding Domains Involving Cys-64, Cys-111, and Cys-113—When the wild-type fragments p1 and p2 were analyzed by SDS-PAGE under nonreducing conditions after oxidation with glutathione (see “Experimental Procedures”), the p2 polypeptide chain fragment essentially migrated as a disulfide-linked dimer (Fig. 2B, lane 4), whereas p1 revealed a less defined band corresponding in size to a hexamer (lane 1). In addition, SDS-PAGE analysis revealed the presence of higher disulfide-linked p1 aggregates, which did not enter the resolving gel (Fig. 2B, lane 1). For the TN-C mutant p1C64S, disulfide-linked trimers were the largest species observed (Fig. 2B, lane 3). As expected from the absence of Cys residues, p1C/S and p2C64S migrated with mobilities corresponding to their apparent monomer molecular masses (Fig. 2B, lanes 2 and 5).

CD spectroscopy was used to test for the secondary structure of the recombinant TN-C fragments (Fig. 3). CD spectra recorded from p4 and disulfide-linked p3 at total chain concentrations of 100 and 36 μM, respectively, were characteristic for α-helical structures showing minima at 208 and 222 nm (Fig. 3A). [θ]222 values of −21,000 degrees cm2 dmol−1 indicated degrees of α-helicity of 60–70% for both p3 and p4, assuming that values of −32,600 and −33,200 degrees cm2 dmol−1 correspond to a helix content of 100% for 28- and 32-residue peptides, respectively, and correcting for the length dependence of [θ]222 (28).

The highly negative values of the mean molar residue ellipticity recorded for p3 and p4 at 222 nm suggests that both peptides form α-helical coiled-coil structures. This conclusion is further supported by thermal transition profiles recorded for both peptides at 221 nm (Fig. 3B). Fragment p3 exhibited a profile that indicates an incomplete unfolding transition with a half-value at 80 °C, which was independent of concentration (data not shown). In contrast, concentration dependence of the CD spectra of all mutant TN-C polypeptide chain fragments showed a Tm at 51 °C (Fig. 3B). The thermal melting profile exhibited a sigmoid shape and was monophasic and reversible, with >95% of the starting signal regained on cooling. Although analysis by CD of p2 indicated proper folding of the domain, the fragment’s spectrum was difficult to interpret and most likely represents a mixture of types of secondary structure (Fig. 3A). The spectrum of p1 was consistent with the average-weighted sum of the spectra recorded for p2 and p3 (Fig. 3A). Notably, the CD spectra of all mutant TN-C polypeptide chain fragments (data not shown) were identical to those of the wild-type fragments (Fig. 3A), indicating that the amino acid substitutions did not significantly interfere with the proteins’ secondary structure (data not shown).

To assay the functional significance of disulfide bond formation in the assembly process of TN-C subunits, wild-type and mutant chain fragments were analyzed by analytical ultracen-
and 100 mM buffer (pH 7.4) containing 150 mM sodium chloride. Polypeptide chain fragments. Spectra were recorded at 20 °C in 5 mM sodium phosphate monitored by the change in [\(\text{M}\)]221. Polypeptide chain concentrations were 13 mM for p1, 17 mM for p2, 36 mM for p3, and 100 mM for p4. CD spectra of the corresponding mutant peptides were superimposable and could not be distinguished from the spectra of the wild-type fragments (not shown). B, thermal denaturation profiles monitored by the change in [\(\text{M}\)]222. Polypeptide chain concentrations were 36 mM for p3 and 100 mM for p4. The thermal melting profile of p4 exhibited a sigmoid shape. Thermal melting profiles of the mutant peptides p3E130A, p3E130L, and p3Q139L were similar to the profile of p3 (not shown).

The Role of Coiled-coil \(\alpha\)-H helices in Tenascin-C Assembly

![FIG. 3. A](image)

**TABLE I**

Sedimentation coefficients \((s_{20,\text{w}})\) and average molecular masses of recombinant wild-type and mutant TN-C polypeptide chain fragments

| Fragment | \(s_{20,\text{w}}\) (\(\text{cm}^2\text{g}^{-1}\)) | Molecular mass (kDa) |
|----------|-----------------------------------|---------------------|
| A. p1    | 3.2 \(b\)                         | 67                  |
| p1C64S   | 3.2 \(a\)                         | 68                  |
| p1C/S    | 3.2 \(b\)                         | 68                  |
| p2       | 3.1                               | 65 \(15,000 \text{ rpm}\) |
| p2C64S   | 1.1                               | 10.2                |
| p3       | 1.4                               | 9.8                 |
| p4       | ND \(c\)                          | 5.0 \(20^\circ \text{C}\) |
|          |                                    | 3.3 \(32^\circ \text{C}\) |
|          |                                    | 8.9 \(37^\circ \text{C}\) |
| B. p1C64S/Q139L | 3.6                               | 75                  |
| p1C64S/E130L | 4.7                               | 137                |
| p1C64S/E130A | 4.4 \(b\)                     | 127                |
| p3Q139L  | 1.7                               | 10.5                |
| p3E130L  | 1.7                               | 14.5                |
| p3E130A  | 1.6                               | 13.7                |

\(a\) Molecular mass of the monomer based on its amino acid sequence.
\(b\) Low percentage of faster sedimenting material.
\(c\) Additional species of higher molecular mass.
\(d\) ND, not determined.

trifugation (Table I, part A). Sedimentation equilibrium revealed an average molecular mass of 8.9 kDa for p4 at 37 °C, which was consistent with the formation of trimers (calculated molecular mass of 8.7 kDa). Surprisingly, p4 appeared to form a mixture of dimers and trimers at lower temperatures, revealing a temperature dependence opposite to the direction expected. Disulfide-linked p3 chains exhibited an average molecular mass of 9.8 kDa, indicating the formation of trimeric structures (calculated molecular mass of 9.9 kDa). Depending on rotor speed, molecular masses of 43, 55, and 65 kDa were found for wild-type p2. This suggested that disulfide-linked dimers (calculated molecular mass of 17.4 kDa) associate to larger assemblies, most likely representing a mixture of tetramers, hexamers, and octamers. Conversely, an average molecular mass of 10.2 kDa was obtained for the mutant p2C64S, indicative of the predominance of monomers. Molecular masses of 67, 70, and 68 kDa were obtained for disulfide-linked wild-type p1 and the mutant polypeptide chain fragments p1C64S and p1C/S, respectively. These values were in agreement with hexameric structures (calculated molecular mass of 70 kDa).

Taken together, these findings indicate that TN-C oligomerization is a sequential process involving the concerted interaction of two distinct domains. Considering its oligomerizing function, we propose the term TN assembly (TA) domain for the autonomous folding unit N-terminal to the three-stranded coiled coil. In addition, our results suggest that the heptad repeats are crucial for initiating the specific formation of TN-C hexabrachions. Although Cys-64, Cys-111, and Cys-113 are not necessary for TN-C hexamer assembly, they are most likely involved in the stabilization of the native molecule by interchain disulfide bonds.

Substitution of Glu-130 Switches the Oligomerization State of the TN-C Coiled Coil—Comparison of the p3 fragment to homologous coiled-coil regions of known TN sequences from different species and other family members revealed the presence of six absolutely conserved residues (Fig. 1). The preferences for specific amino acids at the core positions a and d in coiled coils can be explained in terms of packing geometry in these structures (30). Because replacements of these core residues are known to have profound effects on the oligomerization state of coiled coils and have been well studied (30, 31), we focused our attention on non-core residues. Interestingly, two of the residues, Arg-125 and Glu-130, could possibly form an attractive ionic interaction (residue i in chain 2) (31, 32). To assay the functional significance of this putative interchain electrostatic interaction as well as that of the conservation of Glu-139, we generated the mutant polypeptide chain fragments displayed in Fig. 4A. Leu was chosen to eliminate possible ionic and polar interactions with other side chains. Moreover, Leu is the most frequently found hydrophobic residue in coiled-coil sequences (17). To ensure that effects observed for these TN-C mutants were not due to the hydrophobicity of Leu, these results were corroborated by Ala variants. To avoid interference by Cys-64 and to facilitate the interpretation of our results, the mutant

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*Additional information not shown in the text.*
under nonreducing and reducing conditions, respectively. Lane 1
A
p1C64S

p1C64S/ Q139L

p1C64S/ E130L

p1C64S/ E130A

p3

p3Q139L

p3E130L

p3E130A

FIG. 4. Design of mutant polypeptide chain fragments with mutations affecting conserved residues within the coiled-coil domain of TN-C. A, schematic representation of the recombinant mutant fragments. Mutations are indicated by upper-case letters. For numbering, refer to Fig. 2A. B and C, 10–20% gradient SDS-PAGE under nonreducing and reducing conditions, respectively. Lane 1, p1C64S/E130L; lane 2, p1C64S/E130A; lane 3, p1C64S/Q139L; lane 4, p1C64S. The p3 fragments are not visible on SDS-polyacrylamide gels. The migration of marker proteins is indicated.

fragment p1C64S was chosen.

Replacement of Glu-130 by Leu had a dramatic effect on the oligomerization state of p1C64S. When analyzed by SDS-PAGE under nonreducing conditions, p1C64S/E130L revealed the appearance of a new disulfide-linked species corresponding in size to a tetramer (Fig. 4B, lane 1). The same gel pattern was also observed for the p1C64S/E130A mutant (Fig. 4B, lane 2), indicating that this result was not an artifact due the large hydrophobic moiety of Leu. In contrast, substitution of Gln-139 by Ala had no effect on the electrophoretic mobility of p1C64S as disulfide-linked trimers were the largest species detected (Fig. 4B, compare lanes 3 and 4).

These findings are consistent with the results obtained by analytical ultracentrifugation (Table I, part B). Sedimentation equilibrium measurements yielded average molecular masses of 14.5 and 13.7 kDa for p3E130L and p3E130A, respectively, indicating the formation of tetramers (calculated molecular mass of 13.3 kDa). Disulfide-linked p3Q139L exhibited a molecular mass of 10.5 kDa, a value corresponding to trimeric structures (calculated molecular mass of 9.9 kDa). Consistent results were also obtained after reduction and alkylation of the p3 fragments (data not shown). Accordingly, the molecular mass of 75 kDa for p1C64S/Q139L was in agreement with a hexameric structure of the complex (calculated molecular mass of 70 kDa). In contrast, values of 137 and 127 kDa obtained for p1C64S/E130L and p1C64S/E130A, respectively, were significantly larger than those expected for octamers (105 kDa) and indicated the formation of dodecamers (calculated molecular mass of 140 kDa).

Taken together, these findings indicate that Glu-130 plays a key role in imparting structural uniqueness to TN-C hexamers. Moreover, our results demonstrate that a single non-core residue can specify and influence the oligomerization state of a coiled-coil structure.

**DISCUSSION**

The data presented in this study clearly establish that TN-C hexabrachion assembly is a sequential two-step process mediated by the concerted interplay of two autonomous folding domains (Fig. 5). The organization of TN-C into a functional hexamer includes the formation of a trimer intermediate by a short parallel α-helical coiled-coil domain followed by the connection of two such triplets to a hexamer by the clustered TA domains N-terminal to the α-helical segment (Fig. 5). Three Cys residues flanking the N-terminal end of the heptad repeats are involved in the stabilization of the structure by disulfide bonds, but they are not required for the formation of hexamers.

Oligomerization by a three-stranded coiled-coil domain is an essential prerequisite for TN-C hexabrachion formation, and its principal function is the clustering of three individual TA domains. This oligomerization process provides a high local concentration of homophilic weak affinity sites, consequently resulting in an increase in the binding affinity between TA domains. Likewise, the globular heads of complement component C1q and C-type lectin domains, for example, reveal increased avidity upon multivalent binding to their respective ligands, antibody and carbohydrate (33). The finding that clustering of TA domains is essential for TN-C hexamerization is also supported by the observation that disulfide-linked TA domains assembled in an unspecified manner into a mixture of tetramers, hexamers, and octamers (Table I, part A). In contrast, as evidenced by the mutant polypeptide chain fragment p2C64S, individual TA domains did not reveal a significant tendency to form homophilic assemblies at a concentration of 40 μM (Table I, part B). Consistent results were also obtained with alkylated TA domains, indicating that the prevalence of
monomers was not an artifact of the Cys substitution by Ser (data not shown). Although these findings indicate a sequential formation of hexabrachions, the strongest evidence supporting a two-step mechanism of TN-C assembly is provided by mutational analysis of conserved amino acid residues within the coiled-coil domain (Fig. 1). The occurrence of dodecameric mutant structures can only be explained by the formation of four-stranded coiled-coil intermediates and the subsequent connection of three such tetrads to dodecamers (Table I, part B).

In addition, previous observations support our results that assembly of TN-C involves a two-step mechanism. Electron micrographs of native TN-C purified by biochemical means sometimes reveal the presence of nine-armed molecules in these preparations (34), indicating that the number of chains in native TN-C molecules are multiples of three. This suggests that TN-C assembly is initiated by the formation of trimeric intermediates that further associate to hexamers and, in rare cases, to nonamers. Supporting results were also obtained by Fischer et al. (35, 36), who approached the problem of elucidating the function of this complicated multidomain protein in cell and matrix interactions by expressing recombinant chicken TN-C with deletions and mutations in HT1080 human fibrosarcoma cells. Analysis of the recombinant proteins by electron microscopy revealed occasional hybrid molecules between the sarcoma cells. Analysis of the recombinant proteins by electron microscopy revealed occasional hybrid molecules between the sarcoma cells.

Consistent with our findings, Beck et al. (42) provided evidence that Arg-487 forms an interhelical salt bridge to Glu-492.

Due to the similarity of the coiled-coil oligomerization domains in different TNs (Fig. 1), we may speculate that all family members are assembled into trimers (TN-X and TN-Y) or hexamers via trimer intermediates (TN-R). Moreover, like in laminins (43) and probably also in thrombospondins (44, 45), the existence of homologous coiled-coil domains may be important for the formation of tissue-specific isoforms. Comparison of conserved among known species and members of the TN family (Fig. 1), by Ala or Leu had a profound effect on the oligomerization specificity of the three-stranded parallel coiled-coil domain inasmuch as it resulted in a switch to a parallel four-helix structure (Table I, part B). This switch in oligomerization state can be explained by the fact that the e and g residues of the heptad repeat flank the a and d residues, forming the hydrophobic interface of coiled-coil structures, and hence, the type of amino acid may influence the oligomerization state of coiled coils. For example, sequence analyses of two- and three-stranded coiled-coil proteins have revealed that, compared with the corresponding sites in dimers, the e and g positions of trimers are enriched in hydrophobic residues and depleted of hydrophilic residues (12, 17). These patterns are consistent with the extension of the hydrophobic interface of trimers, relative to that of dimers, to partially include the e and g sites (30). Accordingly, the hydrophobic interface in the tetramer is even more extended than in the trimer (30, 31). The e and g residues in the tetramer are almost as buried as the a and d residues in the dimer (30). With the exception of Glu-130 in the middle heptad repeat, all e and g positions within the three-stranded TN-C coiled coil are occupied by either large hydrophobic Leu residues or amino acids whose side chains contain large aliphatic regions (Arg and Gln). Hence, removal of the negatively charged Glu-130 will almost certainly change the hydrophobic contacts in the hydrophobic core. Due to their tendency to arrange their apolar side chains/side chain regions away from the aqueous interface, replacement of Glu-130 by Leu or Ala may allow for a more favorable packing of these hydrophobic or in part hydrophobic e and g residues in the tetramer conformation. In addition, the tetramer also exhibits several types of ion pairs that are less prominent in the trimer (30, 31). For the mutant four-stranded TN-C coiled coils, this should allow for attractive ionic interchain interactions between Asp-118 and Lys-120 and between Arg-125 and Glu-127 (both g to b').

In the three-stranded coiled coil, Glu-130 might form a favorable interhelical salt bridge with Arg-125 in the preceding heptad repeat of an adjacent chain. Notably, this Arg/Glu pair is strictly conserved among TNs (Fig. 1). In addition, in TN-R, there exists a second possible ion pair between Glu-147 (Glu-146 in chicken) and Arg-152 (Arg-151 in chicken) (Fig. 1). Interhelical electrostatic interactions between ionizable side chains at positions g and e' were identified in several high resolution x-ray structures (31, 32) and are known to be important determinants of parallel or antiparallel orientation (37). While there is no question that relief of unfavorable interhelical interactions in homodimers substantially contributes to heterodimer specificity (38, 39), the contribution of electrostatic attractive interactions to coiled-coil stability is fiercely debated (40, 41). The suggestion that Glu-130 might be involved in an intramolecular salt bridge is supported by findings recently reported by Beck et al. (42). By replacing Arg-487 with Gln at a heptad position g within the three-stranded coiled-coil domain of cartilage matrix protein/matrielin 1, the authors observed a switch in oligomerization state to four-helix structures. Consistent with our findings, Beck et al. (42) provided evidence that Arg-487 forms an interhelical salt bridge to Glu-492.
the oligomerization domains from TN-C and TN-R, for example, reveals strong homology of the two sequences, with 46% of the amino acid residues being identical. This raises the question of whether these molecules form homotypic assemblies exclusively or whether heterotypic combinations exist, e.g., molecules with chain composition (TN-C)$_2$TN-R or (TN-R)$_2$TN-C. TN-C and TN-R mRNAs are both prominently expressed in the developing brain (46, 47), and both homologs may therefore accumulate at similar locations. Heterohexameric molecules involving both TN-C and TN-R chains could increase the structural diversity and modulate the functional properties of the TNs. Although heterotypic assemblies between TN-C and TN-R have not been identified, the possibility of their existence cannot be ruled out and will be further investigated.

Taken together, our findings clearly establish that assembly of TN-C hexabrachions is a two-step process involving the concerted interaction of two distinct domains. The important functional role of the three-stranded coiled coil is documented by mutational analysis, indicating its importance for specific formation of TN-C hexamers. Moreover, we provide experimental evidence that substitution of a single residue at position e of the heptad repeat can switch the oligomeric state of a coiled coil. High resolution structural studies will now be necessary to clarify the exact structural role of Glu-130 and its possible involvement in the formation of an attractive ionic interchain interaction.

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REFERENCES
1. Jones, F. S., Hoffman, S., Cunningham, B. A., and Edelman, G. M. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 1905–1909
2. Chiquet-Ehrismann, R. (1995) Experientia (Basel) 51, 853–862
3. Erickson, H. P. (1993) Curr. Opin. Cell Biol. 5, 869–876, and references therein
4. Nörenberg, U., Wille, H., Woff, J. M., Frank, R., and Rathiens, F. G. (1992) Neuron 8, 849–863
5. Bristow, J., Tee, M. K., Gittelman, S. E., Mellon, S. H., and Miller, W. L. (1993) J. Cell Biol. 122, 265–278
6. Hagiya, M., Koch, M., Spring, J., Chiquet, M., and Chiquet-Ehrismann, R. (1996) J. Cell Biol. 134, 1499–1512
7. Whitby, D. J., and Ferguson, M. W. J. (1991) Development (Cam.) 112, 651–668
8. Whitby, D. J., Longaker, M. T., Harrison, M. R., Adzick, N. S., and Ferguson, M. W. J. (1991) J. Cell Sci. 90, 583–586
9. Saga, Y., Yagi, T., Ikawa, Y., Sakakura, T., and Aizawa, S. (1992) Genes Dev. 6, 1821–1831
10. Erickson, H. P., and Igelias, J. L. (1984) Nature 311, 267–269
11. Spring, J., Beck, K., and Chiquet-Ehrismann, R. (1989) Cell 59, 325–334
12. Conway, J. P., and Parry, D. A. D. (1991) Int. J. Biol. Macromol. 13, 14–16
13. Sodek, J., Hodges, R. S., and Jurasek, L. (1972) Proc. Natl. Acad. Sci. U. S. A. 69, 3800–3804
14. McLachlan, A. D., and Stewart, M. (1973) J. Mol. Biol. 86, 293–304
15. Crick, F. H. C. (1953) Acta Crystallogr. 6, 697–697
16. Cohen, C., and Parry, D. A. D. (1990) Proteins Struct. Funct. Genet. 7, 1–15
17. Woolfson, D. N., and Alber, T. (1995) Protein Sci. 4, 1596–1607
18. Lupas, A. (1996) Trends Biochem. Sci. 21, 375–382
19. Kammerer, R. A. (1997) Matrix Biol. 15, 555–565
20. Lumb, K. J., Carr, C. M., and Kim, P. S. (1994) Biochemistry 33, 7361–7367
21. Su, J. Y., Hodges, R. S., and Kay, C. M. (1994) Biochemistry 33, 15561–15570
22. Fairman, R., Chao, H.-G., Lavoie, T. B., Villar, J., Matsueda, G. R., and Novotny, J. (1996) Biochemistry 35, 2824–2829
23. Redick, S. D., and Schwarzbauer, J. E. (1995) J. Cell Sci. 108, 1761–1769
24. Brandenberger, B., Kammerer, R. A., Engel, J., and Chiquet, M. (1996) J. Cell Biol. 135, 1583–1592
25. Ho, S. N., Hunt, H. D., Horton, R. M., Pullen, J. K., and Pease, L. R. (1989) Gene (Amst.) 77, 51–59
26. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
27. Schagger, H., and von Jagow, G. (1987) Anal. Biochem. 166, 368–379
28. Chen, Y.-H., Yang, J. T., and Chau, K. H. (1974) Biochemistry 13, 3350–3359
29. Van Holde, K. E. (1985) Physical Biochemistry, 2nd Ed., Prentice Hall, Englewood Cliffs, NJ
30. Harbury, P. B., Zhang, T., Kim, P. S., and Alber, T. (1993) Science 262, 1401–1407
31. Harbury, P. B., Kim, P. S., and Alber, T. (1994) Nature 371, 80–83
32. O’Shea, K. E., Klemm, J. D., Kim, P. S., and Alber, T. (1994) Science 254, 539–544
33. Dierks, S. E., Bartlett, W. C., Edmeades, R. L., Gould, H. J., Rao, M., and Conrad, D. H. (1993) J. Immunol. 150, 2372–2382
34. Chiquet, M., Vrucinic-Filipi, N., Schenk, S. K., Beck, K., and Chiquet-Ehrismann, R. (1991) Eur. J. Biochem. 199, 379–388
35. Fischer, D., Chiquet-Ehrismann, R., Bernasconi, C., and Chiquet, M. (1995) J. Biol. Chem. 270, 3378–3384
36. Fischer, D., Brown-Ludi, M., Schulthess, T., and Chiquet-Ehrismann, R. (1997) J. Cell Sci. 110, 1513–1522
37. Monera, O. D., Kay, C. M., and Hodges, R. S. (1994) Biochemistry 33, 3862–3871
38. O’Shea, E. K., Rutkowski, R., Stafford, W. F., Ill, and Kim, P. S. (1989) Science 245, 646–648
39. Zhou, N. R., Kay, C. M., and Hodges, R. S. (1994) J. Mol. Biol. 237, 500–512
40. Lavigne, P., Sonnichsen, F. D., Kay, C. M., and Hodges, R. S. (1996) Science 271, 1136–1137
41. Lumb, K. J., and Kim, P. S. (1996) Science 271, 1137–1138
42. Beck, K., Gambee, J. E., Kamawal, A., and Barchinger, H. P. (1997) EMBO J. 16, 3767–3777
43. Timpl, R., and Brown, J. C. (1994) Matrix Biol. 14, 275–281
44. O’Rourke, K. M., Laherty, C. D., and Dixit, V. M. (1992) J. Biol. Chem. 267, 24921–24924
45. Hecht, J. T., Hao, J., Yuan, X.-H., Chen, H., Putkey, J., and Lawler, J. (1997) Matrix Biol. 16, 72
46. Rettig, W. J., Hoffman, S., Su, S. I., and Garin-Chesa, P. (1992) Brain Res. 590, 219–228
47. Fuss, B., Wintergerst, E.-S., Bartsch, U., and Schachner, M. (1993) J. Cell Biol. 120, 1237–1249
48. Carmellotta, B., Leprini, A., Borzi, L., Querze, G., Urbini, S., and Zardi, L. (1996) J. Biol. Chem. 271, 8157–8160