Tenascin-C is a large, multimeric extracellular matrix protein that is found in a variety of tissues and can have profound effects on cell adhesion. It is secreted from cells as a hexamer of six identical chains called a hexabrachion. Disulfide bonding among tenascin subunits mediates intracellular assembly into hexamers. The amino-terminal assembly domain consists of heptad repeats and at least six cysteine residues (Cys-64, -111, -113, -140, -146, -147) that could be involved in multimerization. We have now determined the requirements for these cysteine residues during hexamer assembly. Our results show that only Cys-64 is required to form the hexameric structure. Mutation of Cys-64 to glycine resulted in release of trimer intermediates, which probably form via the heptad repeats, but no hexamers were secreted. In contrast, individual or pairs of mutations of each of the other cysteines had no effect on tenascin hexamer formation, and inclusion of any other cysteine mutations along with C64G did not further disrupt the multimer pattern. However, when all six cysteines were mutated, monomers were the major extracellular form. Together, these results show that trimers are an intermediate of tenascin-C assembly and that Cys-64 is essential for formation of hexabrachions.

Tenascin-C is a large extracellular matrix glycoprotein that functions during embryogenesis, in the nervous system, and in tumors (1–4). Tenascin is able to interact with cell surface receptors including integrins (5–10) and also binds to extracellular matrix proteins such as fibronectin (11, 12). It is secreted as a large hexabrachion composed of six identical subunits disulfide-bonded together at their amino termini, yielding a hexamer as large as 2 MDa. Subunits range in size from 180 to 320 kDa depending on species and on alternative splicing within the type III repeats. Each subunit consists of an amino-terminal assembly domain containing heptad repeats and multiple cysteine residues, a domain of epidermal growth factor like repeats, fibronectin type III repeats, and a terminal knot that is homologous to fibrinogen (13–17). Tenascin-C is a member of a family of related proteins containing varying numbers of type III and epidermal growth factor repeats. All family members, TN-C, TN-R, TN-X, and apparently TN-Y (18) are multimeric but can differ in the numbers of subunits per multimer (2).

The conservation of the amino-terminal assembly domain among all family members suggests that the multimeric structure of the tenascin family is important for function. During tenascin-C biosynthesis, the hexamer is detected very rapidly without accumulation of intermediates, suggesting that tenascin multimers form by association of nascent polypeptides during translation (19). Within the assembly domain reside at least six cysteine residues that are available for interchain disulfide bonding in the hexabrachion. Some or all of these cysteines are likely to function in maintaining the disulfide-bonded structure. To determine which cysteines are required for hexamer assembly and to identify any disulfide-bonded assembly intermediates, we have mutagenized these cysteine residues and have used a transient expression system based on COS-1 cells to analyze the requirements for each of these cysteines and for combinations of cysteines in the formation of tenascin hexamers. Our results clearly show that only Cys-64 is essential for hexamer formation. Mutant recombinant tenascins lacking Cys-64 are assembled into trimers but not hexamers, demonstrating that trimers are an intermediate in hexamer assembly. In addition, mutation of four cysteine residues flanking the heptad repeat region does not prevent hexamer formation, indicating that trimers might form by noncovalent interactions mediated by the heptad repeats, and these trimer intermediates are then assembled into hexamers by disulfide bonding via Cys-64. The disulfide bonds surrounding the heptad repeats probably function to stabilize the trimeric intermediate structure.

EXPERIMENTAL PROCEDURES

Cysteine Mutations and Plasmid Preparation—Mouse tenascin cDNA encoding the large isoform and containing the alternatively spliced type III repeats A–D was isolated from a bone marrow stromal cell cDNA library (provided by Dr. Ihor Lemischka, Princeton University) using a human tenascin cDNA probe (20). The tenascin cDNA encoding the large isoform was inserted into the vector pcDNA1 (Invitrogen). Restriction and other enzymes used in the construction of these plasmids were purchased from New England Biolabs or Boehringer Mannheim. The 5’ end of the cDNA is a BamHI site engineered at the NcoI site at position 125 of mouse tenasin, and a XhoI site was engineered within the 3’-untranslated region at an ApaI site, position 6,222 (17). Polymerase chain reaction (PCR) amplification was used to generate the small splice variant (TN-S). Two fragments were amplified, one ending at the 3’ end of repeat III, and the other beginning with the 5’ end of IIIa. These fragments were ligated together to exclude the alternatively spliced repeats and then inserted into tenascin cDNA in pcDNA1, yielding a plasmid encoding TN-S.

All cysteine mutations were made by site-directed mutagenesis using primers with the desired nucleotide substitutions in conjunction with appropriate flanking primers to amplify segments of tenasin by PCR. Below is a list of mutant primers with nucleotide changes underlined. Each primer contained a nearby restriction site (in italics) that was

acrylamide gel electrophoresis; BHK, baby hamster kidney.
used in combination with a site on the other side of the mutation in the tenasin cDNA to generate a short fragment for insertion into the TN-S cDNA in pcDNAI. The pairs of restriction sites are listed at the right with the first of each pair being the site within the mutant primer. An asterisk indicates an antisense orientation for the primer.

'TNC64G' CCAGATCCACTGAGCCGTGGAGGA, BstXI to PvuMI TNC111A GCCCGGCGCTGCTGGTGTCGACG, NcoI to ApaLI TNC113A GCCGGGCGCTGCTGGTGTCGACG, NcoI to ApaLI TNC114A GGTTACAGGGCCTGGCTCCACCTGTC, RsaI to XcmI TNC140A ACCATGCTGGCTGGCTCCCTTT, NcoI to BstXI TNC111G ACCGGGGCTGGCGGCTGTCGACG, NcoI to ApaLI

PCR amplification with mutant primers was carried out for 30 cycles under the following conditions: 94 °C, 30 s; 37 °C, 1 min; 72 °C, 30 s. The DNA sequences of all PCR products were verified using Sequenase (U. S. Biochemical Corp.).

For construction of multiple mutant combinations, convenient restriction sites were used to prepare fragments containing the desired mutations, and these were ligated into the tenasin cDNA. In those cases where the complexity of the construction precluded using convenient restriction fragments, mutant tenasin cDNA was used as a template for PCR amplification using primers containing additional mutations (from the list above). To confirm the presence of each of the mutations within the cDNA before transfection, the assembly domain sequence in pcDNAI was determined for each mutant construct.

Cell Culture, Transfections, and Metabolic Labeling—BHK-HxB.S cells (provided by Dr. Harold Erickson, Duke University) (21) were cultured in Dulbecco's modified Eagle's medium (Life Technologies, Inc.) plus 10% fetal calf serum (Hyclone Laboratories). COS-1 cells (provided by Dr. Ihor Lesischka, Princeton University) were grown in Dulbecco's modified Eagle's medium supplemented with 10% calf serum. DEAE-dextran transfections were performed essentially as described (22) using 3.3 μg of plasmid DNA with 4 × 10^5 COS-1 cells/6-cm dish.

Cells in 35-mm culture dishes were labeled for 24 h with [35S]methionine (NEN Life Science Products) at 25 μCi/ml in Dulbecco's modified Eagle's medium minus methionine supplemented with 10% calf serum (or fetal calf serum for BHK cells) and unlabeled methionine at one-tenth the normal concentration. Labeled conditioned medium was collected and stored at −20 °C with protease inhibitors (19).

Immunoprecipitations and SDS-PAGE—Equal volumes of conditioned medium from transfected cells were used to isolate [35S]-labeled normal and mutant tenasins by immunoprecipitation with R759, an anti-tenasin antiserum (19). Protein samples were separated in SDS-polyacrylamide gels either reduced with 100 mM dithiothreitol or non-reduced. Nonreduced samples were separated in 2–5% polyacrylamide gradient SDS gels with a 2.5% stacking gel. Reduced samples were electrophoresed in a 5% polyacrylamide-SDS gel. Prestained molecular mass standards (Sigma), fibronectin isolated from COS-1 cell medium, and purified laminin-1 (provided by Dr. Peter Yurchenco, Robert Wood Johnson Medical School, Piscataway, NJ) were used as markers. Two-dimensional nonreduced/reduced gels were performed as described (23). After electrophoresis, gels were fixed and stained with Coomassie Brilliant Blue to visualize laminin and dried and exposed to a storage phosphor screen (Molecular Dynamics) for 15–72 h. PhosphorImager counts per band were quantified using ImageQuant NT software. In addition, gels were exposed to Kodak XAR-5 film at −80 °C for 2–4 weeks. Autoradiographs were scanned using a UMAX flatbed scanner with transparency adapter, and scans were used to prepare these figures.

RESULTS

Construction of Cysteine Mutations and Expression in COS-1 Cells—The amino-terminal assembly domain of tenasin-C spans the first 150 residues of the protein. Several interesting structural features within this region include a set of three to four heptad repeats composed of residues 118–142, cysteines that can participate in multimerization, as well as two N-linked glycosylation sites. The locations of six cysteines and of the heptad repeats are shown in Fig. 1. Mutations of cysteines at positions 64, 111, 113, 140, 146, and 147 were prepared and expressed individually or in combinations in the tenasin-C small isoform (TN-S).

To analyze the multimers formed by tenasin chains lacking one or more cysteine residues, reasonable quantities of recombinant proteins were needed. Therefore, we tested transfected COS-1 cells for the ability to assemble tenasin into hexamers. The full-length tenasin cDNA encoding the small splice variant was inserted into the vector pcDNAI, which has an SV40 origin for increased expression in COS cells with a cytomegalovirus promoter to drive tenasin transcription. The pcDNAI-TN-S plasmid was transfected into COS-1 cells. 24 h after transfection, cells were metabolically labeled with [35S]methionine for 24 h, and tenasin was isolated from labeled cell-conditioned media by immunoprecipitation with an anti-tenasin polyclonal antiserum. The level of secretion of tenasin from COS-1 transfectants was compared with a BHK cell line expressing HxB.S, the small isoform of human tenasin-C, which has previously been shown by electron microscopy to secrete recombinant tenasin hexabrachions (21). Fig. 2 shows that transient expression of recombinant tenasin in COS-1 cells results in secretion of tenasin multimers with an identical pattern to the HxB.S tenasin. The major band represents hexamers. A minor band is observed migrating at the predicted size for trimers, about 700 kDa, as determined by comparison to laminin-1 (800–850 kDa) and fibronectin (500 kDa). Thus COS-1 cells are able to assemble and secrete tenasin hexamers and can be used to examine the behavior of mutant tenasins.

Effects of Cysteine Mutations on Tenasin Assembly—To determine the roles for the individual cysteines, we have used PCR-directed mutagenesis to mutate each of the six cysteines surrounding the heptad repeats. Cysteines were mutated to either glycine or alanine codons by changing appropriate bases within oligonucleotide primers used for PCR amplification. PCR-amplified products were then inserted into the tenasin-C small splice variant cDNA in the vector pcDNAI for transient expression in COS-1 cells. Each mutation was tested individually except for C146,147G, which was synthesized as a double mutation. Secreted recombinant tenasins containing each of the cysteine mutations were electrophoresed under nonreducing and reducing conditions. All transfectants secreted recombinant tenasins that migrated as single bands of about 230 kDa upon reduction (Fig. 3B). In all but one case, the proteins were present mainly as hexamers (Fig. 3B) and there was no difference in the multimer patterns between mutants and TN-S. The one exception is C64G, which was secreted largely as trimers with variable amounts of dimer-sized material. Two-dimensional nonreduced/reduced SDS-PAGE was used to show that these multimers are composed of tenasin subunits (Fig. 3C). Clearly, trimers are a major intermediate in an assembly pathway that is dependent on Cys-64 for formation of hexamers.

To determine whether multiple cysteine mutations might have an effect on tenasin assembly, the mutations were expressed in combination. C64G in combination with any other cysteine mutation always gave a pattern of multimers identical to C64G alone (Fig. 4). The pattern of secreted multimers was unaffected by the presence of additional mutations. We conclude that the inclusion of additional cysteine mutations along
with C64G does not further affect the types of multimers that form.

Two pairs of cysteines, Cys-111, -113 and Cys-146, -147, flank the heptad repeats. It is possible that these cysteines are involved in forming disulfide bonds that stabilize tenascin subunit interactions within a trimer intermediate structure. Mutation of all four of these residues might result in a further reduction in multimer size. A recombinant tenascin cDNA with mutations in the codons for these four cysteines (4×C) was constructed, and secreted products from transfected COS-1 cells were examined. A number of additional intermediates appeared consisting of between one and five subunits (Fig. 5). However, the majority of multimers were composed of four or more subunits. Therefore, in the absence of disulfide bonds flanking the heptad repeats, hexamers are still able to form. Significantly fewer large multimers were seen with the addition of a fifth mutation at Cys-140 (5×C).

Mutation of All Six Cysteines Yields Monomers—As Cys-64 is required for hexamer formation and the other cysteines participate in disulfide bonding to stabilize the hexamer and trimer intermediates, then mutation of all six cysteines should result in release of tenascin monomers. Transient expression of a mutant lacking all six cysteines (6×C) yielded monomers (Fig. 5), demonstrating that the hexamers and other multimers present in 4×C and 5×C mutants are held together by disulfide bonds involving Cys-64. Quantification revealed that 80% of the 6×C subunits were in the monomer band. The remainder of the subunits were present in dimer-sized material, which could result from disulfide bonds involving the two conserved cysteines that lie 14 and 25 residues downstream of Cys-147 at positions 161 and 172, respectively.

The results of our mutational analyses are summarized in Table I. Recombinant tenascin-C expressed in either BHK or COS-1 cells showed the same pattern of multimers, which is largely hexameric with variable small amounts of trimer-sized complexes. Each individual mutation, Cys-111 through Cys-147, yielded the same multimeric pattern as intact tenascin. Mutation of Cys-64 either alone or in combination with any of the other cysteine mutations ablated hexamer formation and resulted in secretion of trimers. With Cys-64 intact, the absence of cysteines flanking the heptad repeats did not eliminate hexamers but caused the appearance of monomer, dimer, and trimer intermediates (4×C, 5×C). Mutation of all six cysteines gave mainly monomers. Together, these results show that Cys-64 is essential for hexamer formation.

DISCUSSION

The multimeric structure is conserved among the tenascin family of proteins. Each type of tenascin has a set of heptad repeats within the amino-terminal assembly domain, and these are predicted to form a triple coiled-coil structure. We have analyzed the roles of each of the six cysteines in and around the heptad repeats and have found that only one cysteine, Cys-64,
is essential for hexamer formation. Mutation of the other cysteines did not prevent the secretion of hexamers, and mutation of Cys-64 resulted in assembly and secretion of trimers but no higher multimers. Our results also demonstrate that tenascin is assembled through a trimer intermediate. Apparently, tenascin hexamers \( (H) \), trimers \( (T) \), dimers \( (D) \), and monomers \( (M) \) are essential for hexamer formation. Mutations of Cys-64G (plus/minus other mutations) did not prevent the secretion of hexamers, and mutation of the cysteine residues into a favorable position for disulfide bridge formation. Using the Multicoils2 program to predict disulfide bonding partners. In the case of the five-stranded coiled-coil of cartilage oligomeric matrix protein, there are two cysteines at the carboxyl terminus of the heptad repeats, and these form the interchain disulfide bonds (31). Although these residues lie at the end of the heptad repeats, they are not part of the coiled-coil structure. In fact, the coiled-coil is disrupted just before these residues, and they actually reside within a type III \( \beta \) turn. Thus, other determinants bring the cysteine residues into a favorable position for disulfide bridge formation. Using the Multicoils2 program to predict heptad repeats in the assembly domain of tenascin indicated

\[ \text{Multimers formed with multiple cysteine mutations.} \]
Multimers formed by polypeptides with four \( (4 \times C = C_{111G}, C_{113G}, C_{146G}, C_{147G}) \), five \( (5 \times C = C_{111G}, C_{113G}, C_{146G}, C_{147G}, C_{140A}) \), or all six \( (6 \times C) \) cysteine mutations were analyzed by immunoprecipitation and nonreduced SDS-PAGE. Fibronectin (FN) dimers at 500 kDa are shown as a marker. Tenascin hexamers \( (H) \), trimers \( (T) \), dimers \( (D) \), and monomers \( (M) \) are indicated.

![Diagram](image)

**Table I**

| Proteins | 4,5,6-mer \( ^a \) | Trimer | Dimer | Monomer |
|----------|-----------------|--------|-------|---------|
| HxB.S    | ++++            | +      | –     | –       |
| TN-S     | ++++            | +      | –     | –       |
| C64G (plus/minus other mutations) \( ^b \) | – | ++++ | + | – |
| C111A, C113A, C140A | ++++ | + | – | – |
| C146,147G or C111,113G | ++++ | + | – | – |
| 4 \times C | ++++ | + | + | + |
| 5 \times C | ++ | + | + | + |
| 6 \times C | – | – | – | + + + + |

\( ^a \) ++++, greater than 50%; ++, 25–50%; +, 10–25%; –, negligible; determined using ImageQuant NT software.

\( ^b \) C64G plus and minus other individual mutations or pairs of mutations gave the same pattern of trimers > dimers with no hexamers.

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2 Internet address: ostrich.lcs.mit.edu/cgi-bin/score.
that Cys-111–113 and Cys-146–147 are just outside of the heptad repeats and not likely to be involved in coiled-coil interactions.\(^3\) Therefore, tenasin may resemble cartilage oligomeric matrix protein and use structures in addition to a coiled-coil to bring cysteines into proximity for disulfide bonding.

Whereas tenasin-C is a hexamer, tenasin-R has only been isolated as trimers or dimers (33), and tenasin-X (34) and tenasin-Y (18) contain unknown numbers of chains. Comparison of the assembly domains from these three shows that they are highly conserved. However, there are several differences that could account for the different numbers of subunits in the multimers. We have shown that tenasin-C uses Cys-64 to form hexamers. This cysteine is absent from tenasin-X (35), indicating that this tenasin is probably trimeric. However, a cysteine equivalent to Cys-64 (at position 79) is present in tenasin-R (36–38). Why have only trimers and dimers been observed? In addition to Cys-79, mammalian tenasin-R has an additional cysteine 48 amino acids upstream (37, 38). Perhaps this tenasin can form an intrachain disulfide bond, preventing them from participating in hexamer assembly and thus yielding only trimers. This potential mechanism could also hold for chicken tenasin-R, which has three extra cysteines flanking the 78 amino acids (36). All have cysteines flanking the heptad repeats, suggesting a functional role for these residues. Tenascins probably need to withstand a certain amount of tension within the extracellular matrix, and disulfide bonding around the coiled-coil could serve to prevent unraveling of the trimers in these situations. Clearly, nature has evolved a very complex set of interactions to produce tenasin family members consisting of different numbers of chains and has added extra cysteines for stability, probably to strengthen the hexabrachion for its structural role within the extracellular matrix.

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\(^3\) J. A. Luczak, S. D. Redick, and J. E. Schwarzhauser, unpublished observations.