A cysteine-rich protein in the *Theromyzon* (Annelida: Hirudinea) cocoon membrane

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Abstract **The aquatic leech, *Theromyzon rude*, secretes a flexible, proteinaceous cocoon that is resistant to a broad range of denaturing conditions (e.g. heat, denaturing chemicals). We have partially solubilized the *Theromyzon* cocoon membrane in 10% acetic acid and identified two major protein fragments. Microsequencing of both *Theromyzon* cocoon protein (Tcp) fragments generated an identical stretch of the amino-terminal sequence that was used to clone the corresponding gene. The predicted linear amino acid sequence of the resulting cDNA contained an unusually high cysteine content (17.8%). Sequence analysis identified six internal repeats, each comprising 12 ordered Cys residues in a ~62 amino acid repeating unit. Sequence comparisons identified homology with undescribed, Cys-rich repeats across animal phyla (i.e. Arthropod, Nematoda).**

Key words: Glossiphoniid leech; Annelid; Cysteine-rich; Development; Cocoon; Egg capsule

1. Introduction

Cocoons provide microenvironments necessary for pupal and embryonic development in several metazoan phyla (e.g. Arthropoda, Annelida, Platyhelminthes). Protein components of invertebrate cocoons often have unique physical properties (e.g. high tensile strength, protease resistance) and some have proven commercially valuable (e.g. silk fibroin [1]). While cocoons are most commonly associated with arthropod taxa (e.g. Arachnids, Lepidoptera, Trichoptera), more than 3000 known species of clitellate annelids (i.e. oligochaetes, leeches) secrete cocoons that protect and often nurture eggs during the critical stages of early development [2,3]. Components of annelid cocoons are released from specialized glands situated within the clitellar sex segments, forming a sheath around the clitellum into which fertilized eggs are deposited. The cocoon membrane is then passed over the head and sealed at both ends forming 'plugs' at either end [4–6].

Previous investigations have determined that annelid cocoons are proteinaceous structures, comprising multiple layers of fibrillar arrays stacked upon each other at various angles [7,8]. But unlike the well-described protein constituents of arthropod cocoons and their related structures (e.g. fibroin, dragline silk), no proteins have previously been isolated from annelid cocoons, despite their clear potential as a biomaterial (e.g. flexible, highly resilient membrane). In this report, we present the full-length sequence of a major protein component in the *Theromyzon rude* cocoon and identify a cysteine-rich repeating unit that represents a novel protein domain likely to contribute to the unusual physical properties (e.g. heat, chemical resistance) of the cocoon membrane.

2. Materials and methods

2.1. Leech specimens

T. rude specimens were collected in ponds at Golden Gate Park, San Francisco, CA, USA. Leeches were stored at 12°C in glass bowls filled with 0.3% Instant Ocean (PETsMART).

2.2. Cocoon isolation

Cocoons were detached from glass bowls with a cuticle pusher and transferred into a shallow Petri dish containing distilled water. Under a stereomicroscope, fine tweezers were used to tear the cocoon wall and release embryos. Isolated cocoons were pooled and washed repeatedly in distilled water before conducting subsequent analyses.

2.3. Amino acid composition analysis

Standard acid hydrolysis analysis followed by cation exchange chromatography was conducted on ~10 intact cocoons at two independent research facilities: The Scientific Research Consortium (St. Paul, MN, USA) and Core Labs LSU Medical Center (New Orleans, LA, USA).

2.4. Cocoon solubilization

For each solubilization attempt, ~five cocoons were transferred to a 1.7 ml microfuge tube with 100 μl of solvent. For large-scale acetic acid digestions, 1 ml of 10% acetic acid was added to ~100 cocoons and incubated at 50°C for 3 h. Aliquots (20 μl) were taken every ~40 min. Each aliquot received an equal volume of 2× sodium dodecyl sulfate (SDS) buffer [9]; after boiling for 5 min tubes were stored at −20°C until further use.

2.5. Protein analyses

Protein fragments resolved by 10% SDS–polyacrylamide gel electrophoresis (PAGE) [10] were transferred onto polyvinylidene difluoride (PVDF) membrane (Millipore) by standard procedures [9]. Membranes were stained in Coomassie brilliant blue (Sigma) for ~1.5 h followed by methanol/acetic acid destaining until bands were distinguished from background. Protein bands were excised from PVDF membrane with a razor blade and microsequenced commercially using Edman degradation chemistry (Warren Medical Research Institute, OK, USA).

2.6. Degenerate polymerase chain reaction (PCR) and cloning

For 3′ RACE-PCR (rapid amplification of cDNA ends by PCR), first strand cDNA was synthesized with a SMART cDNA synthesis kit according to the manufacturer's specifications (Clontech). The degenerate oligonucleotide GCNATHGAYGARGCNATHAARAT (coc3′R.4) was used in conjunction with CDSIII primer (Clontech)

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Table 1
Solubility of the *Theromyzon* cocoon membrane

Reagent	22°C	37°C	50°C	Autoclave
Distilled water	1	–	1	1
8 M guanidinium isothiocyanate	1	–	–	1
1–10% β-mercaptoethanol (β-ME)	1	–	1	1
8 M urea	1	–	–	1
4 M GIT, 2 M urea, 2% β-ME	–	–	–	1
8 M urea, 2% β-ME	–	–	–	1
Formamide	–	–	–	1
Dimethylsulfoxide (DMSO)	1	–	1	1
NaOH (10 N)	2	–	3	3
KOH (5 N)	3	–	–	–
HCl (6 N)	3*	–	–	–
Acetic acid (10%)	1	–	2	–
Trifluoroacetic acid	2*	–	–	2*
Chloroform	1	–	–	–
Cyclohexane	1	–	–	–
Hexane	1	–	–	–
1,4-Dioxane	1	–	–	–
N-octane	1	–	–	–
Xylene	1	–	–	–
Proteinase K	–	–	3	–
Chymotrypsin	–	1	–	–
Trypsin	–	1	–	–

Categories: 1, insoluble; 2, partially soluble (fragmented or fibrous consistency); 3, soluble. Asterisks indicate solution color changes to brown.

to amplify the 3′ end of *Tcp* cDNA under the following PCR conditions: 94°C (10 s), 53.5→50°C (−0.1°C each cycle; 30 s), 72°C (1.5 min) for 35 cycles. The 5′ end of *Tcp* was amplified with the anchor-specific primer AGTGGTATCAACGCAGAGT (5′partial) and *Tcp*-specific oligonucleotide CGTAATGCTCGCAAGGATC (5′R.3) using PCR conditions: 94°C (10 s), 58°C (30 s), 72°C (1.5 min). PCR fragments were cloned into pGemT-Easy (Promega) and sequenced commercially (Northwoods DNA, Inc.).

2.7. RNA isolation and Northern blot analysis

Clitellum segments and tail suckers were independently excised from adult leeches with a razor blade and immediately homogenized in denaturing solution (Total RNA Isolation System; Promega) using a Polytron (Brinkmann Instruments). Pelleted total RNA was resuspended in 80% formamide and quantitated by agarose gel electrophoresis. Between 5 and 15 μg total RNA was electrophoresed at ∼1 V/cm on an agarose/formaldehyde (0.8%/3.0%) borate gel by standard procedures [9]. Transfer onto nylon membrane (GeneScreen^{+}; NEN) was conducted as described [9]. Probes were prepared by incorporating [α-^{32}P]deoxycytidine triphosphate (dCTP) (Perkin-Elmer) into a standard PCR amplification of the 3′ *Tcp* fragment (see below) and used at a concentration of ∼10^6 cpm/ml. Hybridization conditions were as described for high stringency [9]. Membranes were exposed to film (Biomax MS; Kodak) for 2–4 days and developed by standard procedures.

2.8. Sequence analyses and homology searches

Nucleotide sequences were assembled and translated using Chromas

Table 2
Amino acid composition analysis of the *Theromyzon* cocoon membrane and structurally or chemically related proteins

Amino acid	*Theromyzon* cocoon protein (Tcp)			*E. octoculata* cocoon	*S. purpuratus* (fibropellin-3)	*C. tentans* sp185	*B. mori*		*N. clavipes* (dragline)
	CL	SRC	Cloned				Sericin	Fibroin	
Asp	10.8	13.2	7.3	11.1	7.7	6.4	16.8	1.9	2.6
Thr	9.2	8.6	4.6	5.5	6.8	4.9	8.5	1.2	1.6
Ser	11.1	9.6	4.2	8.3	4.7	5.7	30.1	14.7	4.4
Glu	8.0	9.7	12.9	10.2	5.7	5.8	10.1	1.7	9.2
Pro	9.6	10.9	8.8	15.6	4.5	7.8	0.5	0.6	4.3
Cys	–	–	17.8	–	12.3	17.9	0.3	0.1	0.1
Gly	11.8	8.1	5.9	9.1	11.8	6.9	8.8	42.8	36.9
Ala	5.6	3.2	5.6	7.0	6.4	4.8	4.0	32.4	21.2
Val	5.4	4.7	10.3	4.4	7.5	3.3	3.1	3.0	1.7
Met	4.3	–	0.2	0.1	1.9	1.3	0.1	0.2	0.2
Ile	3.9	4.2	3.0	3.0	4.8	2.3	0.6	0.9	1.0
Leu	4.6	5.4	4.4	3.5	3.6	2.2	0.9	0.7	3.7
Tyr	0.1	4.1	3.4	5.4	4.2	0.6	4.9	11.8	3.2
Phe	2.4	2.8	2.0	2.4	2.9	1.7	0.6	1.2	0.7
His	0.3	–	1.0	0.8	0.2	0.3	1.4	0.3	0.4
Lys	7.6	7.6	5.1	7.4	3.6	12.2	5.5	0.5	1.0
Arg	5.5	5.6	2.9	4.2	1.9	2.1	4.2	0.9	7.7
Asn	–	–	2.7	–	8.2	6.5	–	–	–
Glu	–	–	5.6	–	3.4	4.9	–	–	–
Trp	–	–	0.0	–	0.7	2.9	0.5	0.5	–

Numbers represent amino acid molar percent based on hydrolysis analysis or cloned sequences (Tcp, *S. purpuratus*, *C. tentans*). CL, Core Labs LSU Medical Center (New Orleans, LA, USA); SRC, Scientific Research Consortium, Inc. (St. Paul, MN, USA). Note that Cys was not represented by CL and SRC methodology. *Erpobdella* [7]; fibroin, sericin [21]; *Strongylocentrotus purpuratus* [25]; *Chironomus tentans* [26]; *Nephila clavipes* [32].

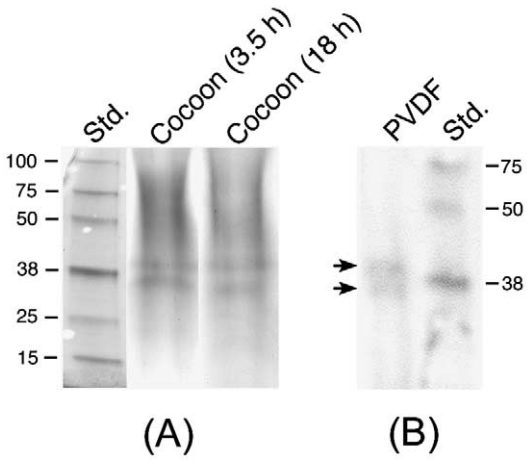

Fig. 1. Acetic acid-soluble protein fragments from the *Theromyzon* cocoon membrane. A: SDS–PAGE analysis of cocoons incubated in 10% acetic acid at 50°C for 3.5 and 18 h. Distinct bands consistently appeared at ~35 and 40 kDa. B: Electrophoretic transfer of an independent gel onto PVDF membrane. Arrows identify protein bands that were excised and microsequenced. Protein standard bands are in kDa (Bio-Rad).

2.21 (Techneysium Pty. Ltd.). Homology searches were conducted with BLAST software [11] and the Sanger Institutes Pfam domain organization retrieval program [12]. Linear amino acid sequences were aligned with Clustal X software [13] and viewed on GeneDoc [14]. Dotter software [15] was employed to identify internal repeating units. Sequence comparisons and evolutionary distance analysis were conducted with NJ plot software [16].

3. Results

The composition of the *Theromyzon* cocoon was judged to be proteinaceous based upon its complete solubilization in proteinase K (20 μg/ml) and 6 N HCl (Table 1). It was surprising, therefore, that the cocoon was insoluble in strong denaturing agents (e.g. guanidium isothiocyanate, β-mercaptoethanol, urea) even when autoclaved, and was resistant to proteases listed in Table 1. Nonetheless, its solubility in acid permitted an amino acid composition analysis presented in Table 2. Unlike proteins in arthropod cocoons (e.g. fibroin, sericin), the *Theromyzon* cocoon displayed an abundance of Asp, Glu, Gly Lys, Pro, Ser, Thr (note that cysteine was not represented by this analysis), similar to that reported for the leech, *Erpobdella octoculata* [7].

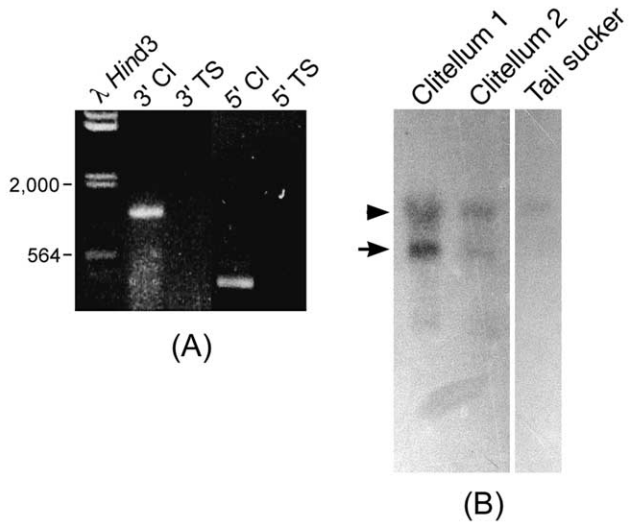

Fig. 3. Clitellum-specific expression of the *Tcp* gene. A: In conjunction with cDNA anchor primers, *Tcp*-specific oligonucleotides coc3′R.4 and 5′R.3 amplified cDNA (1200 and 198 bp, respectively) derived from clitellar (Cl; midbody segments ~5–10) segments, but not cDNA from tail segments (TS; midbody segments ~20–25 and tail sucker). B: Northern blot analysis of clitellum-specific total RNA isolated from leeches in the process of egg laying (clitellum 1) and ~12 h after egg laying (clitellum 2); total RNA from the tail sucker lane was spliced from the same blot. The arrow identifies a ~1500 bp band hybridizing with the 3′ *Tcp* fragment in A, in both clitellum lanes; the arrowhead indicates rRNA.

It was hypothesized that a hypersensitive site(s) within a putative structural domain of the *Theromyzon* cocoon may permit the cleavage and release of a peptide fragment that could be monitored by SDS–PAGE. To test this idea, cocoons were incubated with weak solutions of HCl (1 N), acetic acid (10%) and proteinase K (20 μg/ml) over the course of several hours. While HCl- and proteinase K-treated cocoons failed to produce detectable protein bands, cocoons incubated in 10% acetic acid at 50°C generated two distinct fragments at ~35 and ~40 kDa, after ~30 min incubation (Fig. 1A). Both fragments persisted for at least 48 h and in most experiments represented an estimated >90% of the total protein in each lane (no other distinct bands were apparent). Following acetic acid treatment, the cocoon membrane was notably less rigid than untreated cocoons and often had a fibrous-like consistency, but never went fully into solution.

To gain a stretch of the linear amino acid sequence, both

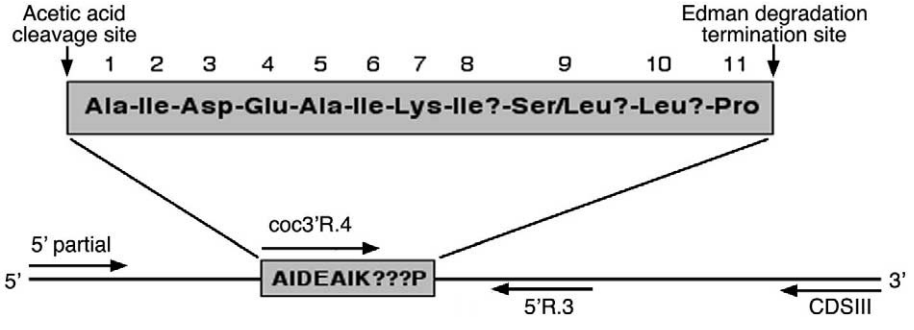

Fig. 2. Cloning strategy for *Tcp* cDNA. The degenerate oligonucleotide coc3′R.4 designed from the deduced peptide sequence (gray box) was used with anchor CDSIII to amplify the 3′ end by RACE-PCR. *Tcp*-specific primer 5′R.3 obtained from the 3′ sequence was used with anchor 5′partial to amplify the 5′ end of *Tcp* cDNA. Question marks indicate sequence ambiguity in the Edman-derived amino acid sequence; the reaction terminated abruptly at position 11.

protein fragments were transferred to PVDF membrane (Fig. 1B) and their respective amino-termini were microsequenced by Edman degradation (Warren Medical Research Institute; [17]). Two surprising results were obtained by this analysis; first, both protein fragments contained the identical amino acid sequence at their amino-terminus, and second, the clear signal obtained in the first seven amino-terminal residues (AIDEAIK) thereafter became ambiguous and stopped abruptly at position 11 (Fig. 2), suggesting an atypical, and perhaps tightly wound protein configuration.

Utilizing the short amino acid sequence available, a degenerate oligonucleotide (coc3′R.4) was designed to amplify the 3′ end of the putative *Theromyzon* cocoon protein (*Tcp*) gene by RACE-PCR (Fig. 2). In cDNA pools derived from various segments, a ~1200 bp fragment was amplified in clitellar segments of an adult leech in the process of egg laying (Fig. 3A). DNA sequencing of the candidate fragment permitted the design of a gene-specific primer (5′R.3) that was employed to amplify the 5′ end of *Tcp* (Fig. 2). Importantly, the amplified 5′ fragment contained the previously determined peptide sequence (i.e. from Edman degradation), thus confirming the identity of the *Tcp* cDNA. The full-length nucleotide sequence of *Tcp* cDNA (1548 bp) is available at GenBank (accession number AY536890); the predicted amino acid sequence is presented in Fig. 4. Unexpectedly, the Cys content of Tcp was unusually high (17.8%), but otherwise abundant amino acids (e.g. Pro, Glu, Asp) were generally consistent with amino acid composition analyses of the *Theromyzon* cocoon membrane, with some exceptions (cf. Table 2; see Section 4). Northern blot analysis complemented segment-specific PCR experiments, suggesting that *Tcp* is expressed in clitellar segments during and shortly after cocoon production (Fig. 3B).

Analysis of the predicted Tcp amino acid sequence using Dotter software [15] revealed the presence of six repeating units, each comprising 12 ordered cysteine residues in a ~62 amino acid repeat (Fig. 4). It is noteworthy that the abrupt termination of the Edman-derived sequence occurred at the boundary between the N-terminal leader sequence and the first cysteine repeat. BLAST comparisons [11] against the GenBank protein database and domain searches in Pfam [12] identified related, Cys-rich repeating units in hypothetical proteins from *Caenorhabditis elegans*, *Drosophila melanogaster* and *Anopheles gambiae*, and more distant relatedness with antistasin homologs (e.g. leech anticoagulants) and EGF-like repeats (e.g. sea urchin fibropellin family, papilin-3) (Fig. 4). Relative evolutionary distances among the Cys-rich proteins were examined by NJ plot matrix analysis (Fig. 5) and suggest a pre-Cambrian origin of a Tcp-like repeat based on close relationships between repeating units across metazoan phyla.

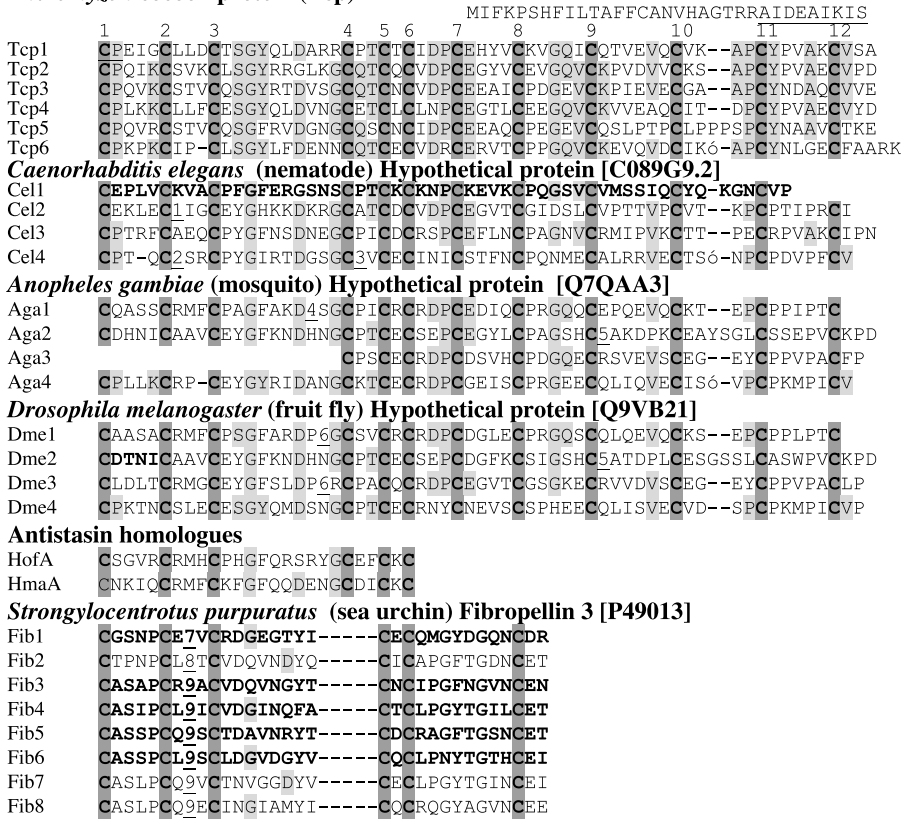

Fig. 4. Amino acid alignment of *Theromyzon* Tcp Cys-rich repeats and homologous sequences. The Tcp sequence is shown in its entirety with Cys-rich repeats (Tcp 1–6) aligned; underlined amino acids identify the position corresponding to the Edman-derived sequence (AIDEAIKISCP; boundary between the N-terminal leader and first Cys-rich repeat). The total length of cloned cDNA is 1552 bp (GenBank accession number AY536890) encoding a predicted protein of 408 amino acids (~44 kDa). Ordered Cys residues in all sequences are numbered, aligned vertically and highlighted dark gray; other conserved residues are light gray. Antistasin homologs are *HofA* (*Haementaria officinalis* [27]); *HmaA* (*Hydra magnipapillata*; [28]). Note that *HofA* antistasin contains four ordered, amino-terminal Cys residues (i.e. follistatin domain [31]) not included in the comparison. Dashes indicate gaps introduced to maximize the sequence alignment (Clustal X). Underlined numbers correspond to the following amino acid sequences: 1, GT; 2, IG; 3, PANG; 4, PA; 5, EV; 6, AR; 7, NGS; 8, NGA; 9, NGG.

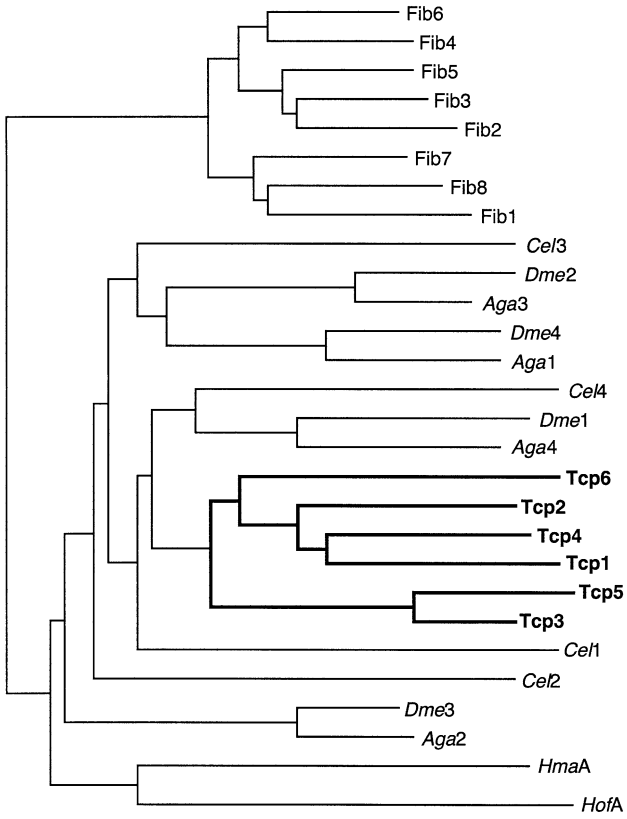

Fig. 5. Evolutionary relationships between Cys-rich repeats. Individual repeats from Fig. 4 were compared by the neighbor-joining matrix method [16], suggesting that Tcp repeats are most closely related to *Aga*4, *Cel*4, *Dme*1 sequences and most distant to antistasin homologs and fibropellin repeats, respectively. The Tcp clade in shown in bold.

4. Discussion

This study identifies a novel, cysteine-rich protein (Tcp) that comprises a major component of the *Theromyzon* cocoon. It remains unclear at this juncture whether additional proteins are present within the cocoon membrane based upon variabilities in amino acid composition between the intact cocoon and the predicted Tcp linear amino acid sequence. But this comparison is at least partially skewed because Cys (comprising 17.8% molar fraction of the linear Tcp sequence) is labile in acid hydrolysis and therefore not represented in the compositional data. Also, Gln and Asn, both significant constituents of the linear Tcp sequence, are converted to their acid forms (Glu and Asp, respectively) during hydrolysis. Nevertheless, factoring in these variables does not fully account for some differences in amino acid composition (e.g. Ala, Val), suggesting that other protein(s) may be present within the *Theromyzon* cocoon. A similar situation involving amino acid discrepancies in dragline silk was resolved when a second cDNA, Spidroin2, was isolated, providing a match between cloned and experimentally determined amino acid composition profiles [18]. In the *Theromyzon* cocoon, one potential source of additional protein(s) is the plug material which seals the cocoon, and which is secreted by clitellar glands distinct from those secreting the cocoon wall [19,20]. Also, a mucous layer is deposited around the clitellum prior to cocoon formation in hirudinids [6], and appears to surround the *Theromyzon* cocoon membrane [8].

Alternatively, multiple proteins may be integrated into the *Theromyzon* cocoon membrane, as observed in most other invertebrate cocoons (e.g. *Bombyx mori*, *Chironomus*; [21,22]) and related structures (e.g. dragline silk [18]). Failure of the *Theromyzon* cocoon to solubilize to completion in 10% acetic acid supports this notion, although there is little evidence from SDS–PAGE gels suggesting an additional protein component. Thus while two protein bands were detected, both had the same amino-terminus and appear to differ in molecular weight by one repeating unit (~62 amino acids; ~6.6 kDa). This may be due to a sequence deviation at the carboxy end of the Tcp5 repeat (i.e. two additional prolines are present between the 10th and 11th Cys residues, respectively). A hypersensitive cleavage site at this position would, in principle, account for the smaller (~35 kDa) fragment observed in SDS–PAGE gels. The observation that the predicted molecular weight of cloned Tcp (44 kDa) corresponds well with the size of the protein fragment (i.e. ~40 kDa) suggests a minor role for post-translation modification (e.g. glycosylation).

The unusually high Cys content of Tcp implies the presence of intra- and/or intermolecular disulfide cross-linking, yet the cocoon membrane failed to solubilize in the presence of the strong reducing agent β-mercaptoethanol, even when autoclaved. The putative tightly wound configuration of Tcp, as suggested by the abrupt termination of Edman degradation and high Cys/Pro (17.8/8.8%) content, may sterically inhibit the permeation of β-mercaptoethanol, as observed in *C. tentans* silk proteins [23]. The presence of intramolecular disulfide bridges seems likely based on the six Cys residue stretch shared by Tcp repeats and antistasin (see Fig. 4), whose crystal structure identifies three disulfide linkages [24]. But the release of a near full-length Tcp fragment upon 10% acetic acid treatment suggests that at least some Tcp protein does not participate in intermolecular disulfide links.

In the comparisons made here, it appears that Tcp and the sea urchin fibropellin repeats are related only by their Cys-rich nature, and share little if any sequence homology. Nevertheless, a fibrous, fibropellin-based network forms a protective layer around the sea urchin embryo shortly after fertilization that bears a functional/structural resemblance with the leech cocoon membrane [7,8,25]. A similar observation holds for the Cys-rich sp185, sp220 cocoon silk proteins (C-X-C-X-C [26]) from *Chironomus* larvae, which also show little sequence similarity with Tcp repeats. Yet all these proteins have been identified in protective structures from various species, suggesting that cysteine is an inherently favorable amino acid in structures that provide a protective barrier.

The strong sequence homology of the Tcp repeat with proteins from disparate animal phyla (i.e. Arthropoda, Nematoda) suggests that a Tcp-like archetype functioned in an ancestral metazoan, albeit its role is currently difficult to decipher. Significantly, Tcp-related proteins in *C. elegans*, *D. melanogaster* and *A. gambiae* contain a predicted membrane-spanning domain, suggesting that Tcp was co-opted for a very different role in some annelids (i.e. cocoon membrane). Note that other Tcp-like, membrane-associated proteins may also be present in annelids, in which case Tcp may be the result of an independent, annelid-specific duplication event. Nevertheless, the Tcp repeat (12 Cys) appears to be a pre-Cambrian motif based on its presence in diverse animal phyla. It also

seems that Tcp is ancestral to the well-described leech antistasin protein, an anticoagulant containing 10 ordered Cys residues [27]. Although many metazoan proteins share sequence homology with antistasin [28–30], their sequence conservation is limited to six Cys residues that are also contained within Tcp (Cys 1→6; see Fig. 4). Importantly, no other reported metazoan sequences contain a full antistasin repeat (i.e. 10 ordered Cys residues) based on BLAST and Pfam protein database searches [11,12]. Thus a Tcp-like repeat (12 Cys) or the core sequence shared by Tcp and antistasin (Cys 1→6) was likely the ancestral domain. In either case, antistasin appears to have arisen independently in the annelid lineage by fusion of a follistatin domain (four ordered Cys [31]) to the amino-terminus of the six Cys core repeat. The origin of the terminal half of the Tcp repeat (i.e. Cys 7→12) remains unclear.

The unique physical properties of these related Cys-rich proteins (protease resistance; heat, chemical resilience) appear to stem from the common six Cys loop that is cross-linked by three disulfide bonds [24]. Clearly, it will be instructive to learn the structural contribution of the Cys 7→12 in Tcp-like repeats across animal phyla and in *Theromyzon*, particularly with respect to structural features (e.g. fibrillar arrays [7,8]) that have been observed in the *Theromyzon* cocoon membrane.

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