

## Molecular, biochemical and functional analysis of a novel and developmentally important fibrillar collagen (Hcol-I) in hydra

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### SUMMARY

The body wall of hydra (a member of the phylum Cnidaria) is structurally reduced to an epithelial bilayer with an intervening extracellular matrix (ECM). Previous studies have established that cell-ECM interactions are important for morphogenesis and cell differentiation in this simple metazoan. The ECM of hydra is particularly interesting because it represents a primordial form of matrix. Despite progress in our understanding of hydra ECM, we still know little about the nature of hydra collagens. In the current study we provide a molecular, biochemical and functional analysis of a hydra fibrillar collagen that has similarity to vertebrate type I and type II collagens. This fibrillar collagen has been named hydra collagen-I (Hcol-I) because of its structure and because it is the first ECM collagen to be identified in hydra. It represents a novel member of the collagen family. Similar to vertebrate type I and II collagens, Hcol-I contains an N-terminal propeptide-like domain, a triple helical domain containing typical Gly-X-Y repeats and a C-terminal propeptide domain. The overall identity to vertebrate fibrillar collagens is about 30%, while the identity of the C-terminal propeptide domain is 50%. Because the N-terminal propeptide domain is retained after post-translational processing, Hcol-I does not form thick fibers as seen in vertebrates. This was confirmed using transmission electron microscopy to study rotary shadow images of purified Hcol-I. In addition, absence of crucial lysine residues and an overall reduction in proline content, results in reduced crosslinking of fibrils and increased

flexibility of the molecule, respectively. These structural changes in Hcol-I help to explain the flexible properties of hydra ECM. Immunocytochemical studies indicate that Hcol-I forms the 10 nm fibrils that comprise the majority of molecules in the central fibrous zone of hydra ECM. The central fibrous zone resides between the two subepithelial zones where hydra laminin is localized. While previous studies have shown that basal lamina components like laminin are expressed by the endoderm, in situ hybridisation studies show that Hcol-I mRNA expression is restricted to the ectoderm. Hcol-I expression is upregulated during head regeneration, and antisense studies using thio-oligonucleotides demonstrated that blocking the translation of Hcol-I leads to a reversible inhibition of head morphogenesis during this regenerative process. Taken in total, the data presented in this study indicate that Hcol-I is required for morphogenesis in hydra and represents a novel fibrillar collagen whose structural characteristics help to explain the unique biophysical properties of hydra ECM. Interestingly, the structure of Hcol-I mimics what is seen in Ehlers-Danlos syndrome type VII in humans; an inherited pathological condition that leads to joint and skin abnormalities. Hcol-I therefore illustrates an adaptive trait in which the normal physiological situation in hydra translates into a pathological condition in humans.

Key words: Hydra, ECM, Collagen, Morphogenesis, Ehlers-Danlos syndrome type VII evolution, Hydra

### INTRODUCTION

Hydrozoans such as *Hydra vulgaris* are considered one of the most ancient multicellular animal groups. Hydra are organised as a gastric tube with a tentacle ring and mouth (hypostome) at their apical pole and a foot process (composed of a peduncle and basal disk) at their basal pole. The entire body wall is formed from an epithelial bilayer with an intervening acellular extracellular matrix (ECM) termed mesoglea that has been

shown to play a crucial role in morphogenesis and cell differentiation in this simple metazoan (Sarras et al., 1991b, 1993, 1994; Zhang et al., 1994; Yan et al., 1995; Leontovich et al., 2000). Despite this simple body structure, hydra have competed successfully throughout evolution for more than 600 million years. Early ultrastructural and functional studies indicated similarities between the mesoglea of hydra and vertebrate basement membranes (Fawcett, 1961; Hausman, 1973; Day and Lenhoff, 1981). Recent biochemical studies

have presented some biochemical evidence for the presence of basement membrane molecules such as type IV collagen, heparan sulfate proteoglycan and fibronectin (Schlage, 1988; Sarras et al., 1991a), although to date only laminin chains (Sarras et al., 1994) and type IV collagen chains (Fowler et al., 2000) have been successfully cloned. Recent ultrastructural, cytochemical and immunological studies have shown that hydra ECM is actually composed of two subepithelial zones that appear similar to vertebrate basal lamina, and a central fibrous zone that appears more similar to interstitial matrix (Sarras et al., 1993, 1994). As indicated, a laminin B1-like chain has been cloned and its role in head regeneration has been investigated (Sarras et al., 1994). Immunocytochemical studies have shown that hydra laminin is localized to both subepithelial zones of hydra ECM and is not associated with the central fibrous zone (Sarras et al., 1994). This supports the proposal that the subepithelial zones equate to basal lamina. In contrast, the molecular composition of the central fibrous zone is largely unknown and therefore no definite proof exists to classify it as an interstitial-like matrix. Indirect evidence suggest the presence of interstitial collagens in hydra ECM (Hausman and Burnet, 1971; Barzansky and Lenhoff, 1974), but biochemical investigations and sequence data to reveal the precise nature of these molecules are still lacking. With the huge complexity and functional diversity of the collagen family, such data are crucial for an understanding of the biological role of the extracellular matrix of hydra and for comparison of its function with higher animals, especially the vertebrates.

If the central fibrous zone of hydra ECM represents an interstitial-like matrix, then it should contain fibrillar collagen molecules. A common feature of all collagens is a triple helical domain with the characteristic sequence repeat (Gly-X-Y)<sub>n</sub>, in which X and Y are frequently proline. This proline can be modified to hydroxyproline in the Y position. The high diversity of collagens originates from differences in the size of the triple helical domains, imperfections in the triple helical structures, and the presence of additional globular domains. These factors result in a wide variety of self assembly patterns and biological functions for collagens (Prockop and Kivirikko, 1995; Olsen and Ninomiya, 1999).

Best characterised are the vertebrate collagens. In man and mouse, 19 different collagens are known, including the classical fibrillar collagens type I, II, III and V and the basement membrane collagen type IV, and less abundant molecules like the membrane-associated collagen type XVII (Olsen and Ninomiya, 1999). Some of these collagens are also found in invertebrates. Collagen type IV has been found in all species investigated so far, including *Caenorhabditis elegans* and *Drosophila*, reflecting its requirement for the proper structure of basement membranes and its function in regulating the behaviour of adhering cells (Kuehn, 1994). Fibrillar collagens have been reported to occur in worms (Gaill et al., 1991, 1995) and sponges (Esposito and Garrone, 1990), but full-length sequences have only been obtained for two collagen chains from sea urchin (Esposito et al., 1992a,b). One of these chains is remarkably similar to vertebrate  $\alpha 2(I)$  while the other chain contains a large N-terminal globular domain, which is rather atypical for fibrillar collagens. Interestingly, fibrillar collagens have not been found in *C. elegans* or *Drosophila*.

In addition, invertebrates also contain unusual collagens

with specialised functions (Engel, 1997). These include the highly crosslinked mini-collagens in hydra nematocytes (Kurz et al., 1991), the giant cuticle collagen of deep sea worms (Gaill et al., 1991, 1995) and the small cuticle collagens of *C. elegans* (Kramer, 1994) – the latter being encoded by some 100 genes.

Most of the information on invertebrate collagens has been obtained from cDNA sequencing. Therefore the nature of post-translational modifications and assembly are largely unknown as is their contribution to the establishment of body structure and their function in development. We report for the first time the full-length sequence of a lower invertebrate fibrillar collagen together with structural and functional data. We name this collagen, hydra collagen-I (Hcol-I). We show that the central fibrous zone of hydra ECM contains Hcol-I and that this molecule is related to vertebrate fibrillar collagens based on amino acid sequence and structure. We also show that Hcol-I is processed in a distinct manner from that seen with vertebrate fibrillar collagens. The relatively low proline content of the Hcol-I triple helical domain together with retention of the N-terminal non-collagenous domain during assembly into fibrils helps to explain the flexible nature of hydra ECM. Finally, we show that this fibrillar collagen is the major structural component of hydra ECM and that it is essential for morphogenesis in this simple metazoan.

## MATERIALS AND METHODS

### Culture of polyps and isolation of collagens

Hydra polyps were grown in 20 × 20 cm plastic dishes to a density of about 6000 hydranths per tray. For isolation of hydra ECM, polyps from 20 dishes were typically harvested and suspended in 100 ml 10 mM Tris buffer, pH 7.5 containing 1% Triton X-100 and 10 µg/ml of the protease inhibitors PMSF and p-chloromercuribenzoate (buffer A). Polyps were then shock frozen in aliquots of 30 ml at −196°C, and stored at −78°C until further use.

Extraction of collagen was carried out at 4°C starting with polyps from 20–40 dishes. After thawing, the material was centrifuged for 15 minutes at 1000 g. The pellet was suspended in 60 ml of buffer A, shaken for 10 minutes and centrifuged again for 15 minutes at 3000 g. This procedure was repeated until the supernatant was clear and colourless. Following the washing procedure, the pellet was suspended in 50 mM Tris buffer, pH 7.5, containing 1 M NaCl and 0.5% Triton X-100, and the same protease inhibitors as above, homogenised briefly and shaken for 16 hours. Following centrifugation, the pellet was extracted with 10 ml of 50 mM Tris buffer, pH 7.5, 1 M NaCl and 10 mM EDTA (+ 10 µg/ml PMSF) for 5 hours. This fraction contained most of the collagens. Finally the pellet was extracted for several hours at room temperature with 10 ml TBS, containing 2% SDS and 2% SDS+0.5% mercaptoethanol.

To investigate the insoluble residue remaining after these extraction steps, the pellet was washed twice with water to remove detergents and then repeatedly suspended in ethanol to remove contaminating pigment granules that arose from feeding with brine shrimps. These extractions were performed until the pellet became white. To remove traces of alcohol, the pellet was washed twice with water and lyophilised. To solubilise proteins, the pellet was digested either with pepsin or BrCN. Pepsin digestions were carried out overnight at 4°C at a protease concentration of 10 µg/ml in water. BrCN digestions were performed in 70% formic acid containing 10 mg/ml of the reagent. Cleavage was done at 45°C for 2 hours followed by overnight incubation at 4°C.

### Separation of hydra col-I and hydra col-IV under non-denaturing conditions

To isolate macromolecular structures of Hcol-I and to separate the two collagens identified in our preparations (see Results section), the EDTA extract was made 1% in mercaptoethanol and centrifuged for 1 hour at 15,000 *g*. The pellet contained large Hcol-I structures exclusively, whereas the supernatant contained a mixture of Hcol-I oligomers and hydra type IV collagen (Hcol-IV). The supernatant was centrifuged again at 35,000 *g* and then 45,000 *g* in a Beckman 50 TI rotor. By this treatment the residual Hcol-I (together with some hydra col-IV) was removed and was found in the pellet. The supernatant of the 45,000 *g* centrifugation step contained pure hydra col-IV molecules (see the flow chart in the left panel of Fig. 1 and lanes 8 and 9, respectively in the right panel of Fig. 1).

To isolate oligomeric Hcol-I structures, the EDTA extract (10 ml) was dialysed overnight against 20 mM Tris buffer pH 7.4 and separated on a HiTrap Q column (1 ml) using a gradient of 0–1 M NaCl within 40 minutes. Hcol-I and hydra col-IV eluted as broad peaks in the range of 0.1–0.2 and 0.2–0.4 M NaCl, respectively.

### Pyroglutamase digestion

RP-HPLC purified Hcol-I was lyophilised, solubilised in 1.0 ml digestion buffer (0.1M sodium phosphate buffer, pH 8.0, containing 10mM EDTA, 5 mM DTE, 5% glycerol, and digested for 18 hours at 4°C with 2.5 µg of pyroglutamase (Boehringer Mannheim, sequencing grade), followed by 4h at 25°C. The reaction mixture was separated by RP-chromatography as described below and eluting peptides were subjected to amino acid sequencing.

### Preparation of peptides for Edman degradation

To isolate denatured collagen chains for sequence analysis, the EDTA extract was made 1% in mercaptoethanol and separated by reversed phase chromatography on a Vydac C-18 column (Separations Group, 250 × 2.2 mm) at a flow rate of 0.2 ml/minute using a gradient of 5–70% acetonitril in 50 minutes. Two major peaks at 21% and 35% of the organic modifier were obtained, the former of which contained Hcol-I chains. After lyophilisation, the isolated chains were dissolved in 70% formic acid or 0.2 M ammonium acetate for digestion with BrCN and trypsin, respectively.

Alternatively, peptides were generated by in gel digestion. EDTA extracts were separated on 4–10% SDS gels. The Coomassie stained bands were excised, cut in small pieces with a razor blade, and washed successively with 0.2 M NH<sub>4</sub>HCO<sub>3</sub>, 50 mM NH<sub>4</sub>HCO<sub>3</sub> in 25% acetonitril, 25% acetonitril, 50% acetonitril and pure acetonitril. The gel pieces were air dried for 2 h and then rehydrated in 0.2% NH<sub>4</sub>HCO<sub>3</sub>, containing 2 µg trypsin per 100 µl gel pieces. Only as much liquid was used as was necessary to restore the original gel volume. Protease digestions were performed overnight at 37°C, the peptides were extracted twice with 5% trifluoroacetic acid and once with 2.5% trifluoroacetic acid in 50% acetonitril.

The peptides were separated by reversed phase chromatography and sequenced on a Procise 492A sequencer (PE Biosystems) with on-line detection of the PTH amino acids, according to the manufactures instructions.

### Electron microscopy

For electron microscopy, samples (about 50 µg/ml) were mixed with an equal volume of glycerol and, after spraying onto mica discs, coated with platinum/carbon either from one direction or by rotary shadowing. The replicas were viewed with a Philips CM 12 electron microscope.

### Cloning of hydra col-I

Initial selection and isolation of the Hcol-I cDNA clones was achieved using two convergent approaches. One approach involved expression screening using antibodies to hydra ECM and the second involved degenerate oligonucleotide screening. Both approaches used hydra

cDNA libraries that were enriched for ECM components. Hydra enriched for ECM components were generated by surgically segmenting polyps and then isolating RNA 24 hours after segmentation. Under these conditions, ECM components are upregulated (data not shown; Sarras et al., 1993). The libraries were generated using either random primers or oligo-dT primers. For expression screening a polyclonal antibody was used that had been generated against isolated hydra ECM (Sarras et al., 1993). This antibody reacted with a broad spectrum of hydra ECM components as determined using western blot analysis (Sarras et al., 1993). Expression screening was performed as previously described (Sarras et al., 1994) and replicated clones were isolated using tertiary screening techniques. In the second approach, degenerate oligonucleotides were designed from peptides cleaved from a 155 kDa hydra ECM protein (Hcol-I). Sequence of these peptides indicated similarity to a variety of vertebrate and invertebrate fibrillar collagens.

Three degenerate oligonucleotides were synthesised with the following sequences: (1) HCOL1, 5'-GG(AGCT)CA(AG)-CA(AG)GG(AGCT)GZ(AG)CA(AG)GG-3'; (2) HCOL4, 5'-GG(AGCT)CA(AG)GG(AGCT)AC(AGCT)GC-3'; and (3) HCOL5, 5'-GG(AGCT)GA(AG)CA(AG)GG(AGCT)AC(AGCT)CC-3'. The hydra cDNA libraries were subsequently screened using the tetramethylammonium chloride procedure described by Wood et al. (1985) and replicated clones were isolated using tertiary screening techniques. Purified clones from both screening approaches were excised *in vivo*. The DNA sequence was determined using the T7 sequenase chain-termination DNA sequencing method (Amersham) or using an ABI PRISM XL 377 DNA sequencer in combination with a Big Dye terminator chemical kit (Perkin-Elmer, Applied Biosystems, Foster City, CA). The DNA clones were sequenced in both directions using a primer-walking strategy. Comparisons of DNA sequences were conducted using the Blastn and Blastx programs and the GenBank database (NCBI, NIH). Protein alignments were obtained using the Blastp, GCG, and MacVector 5.0.

### Immunocytochemical studies

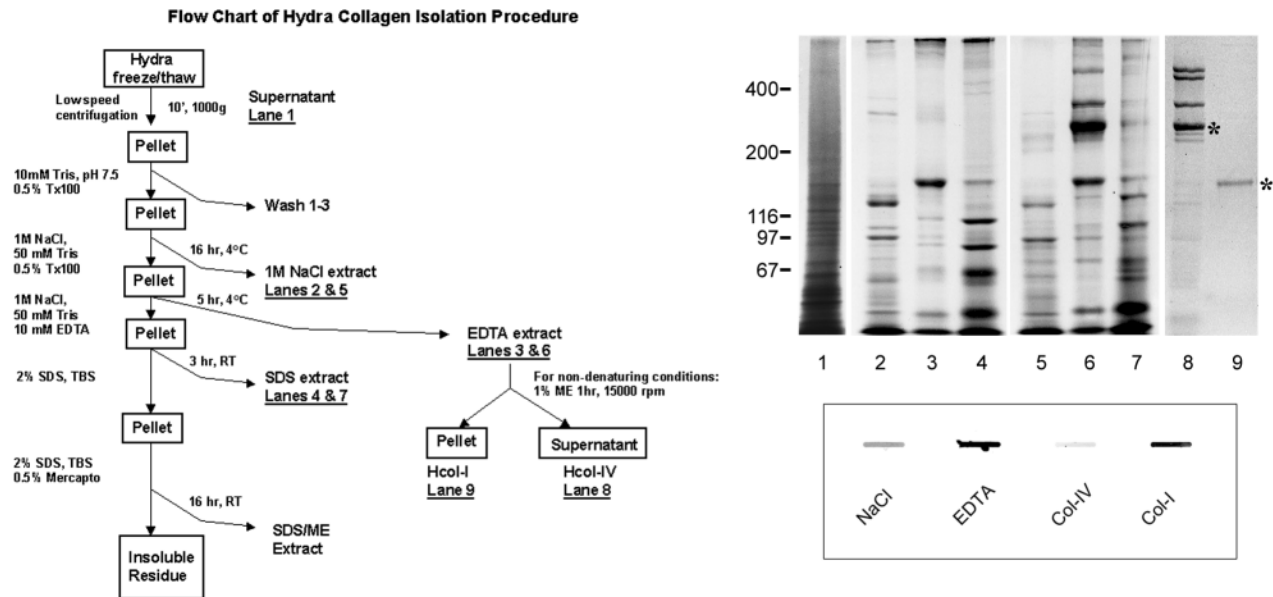
Whole-mount and frozen section immunofluorescence was conducted as previously described (Sarras et al., 1991a, 1993). Hydra ECM was isolated and used to generate a battery of monoclonal and polyclonal antibodies as described previously (Sarras et al., 1993). Initial characterization of monoclonal antibody mAb39 and immunogold ultrastructural localisation procedures were conducted as previously described (Sarras et al., 1993).

### In situ hybridisation

Whole-mount *in situ* localisation of mRNA was performed using a digoxigenin-labeled RNA probe generated from a 2.1 kb clone corresponding to the 5' ORF of Hcol-I.

Fixation, processing, hybridisation and visualisation of the riboprobe in whole-mount preparations was performed as previously described by Grens et al. (1995), Martinez et al. (1997) and Grens et al. (1999). Briefly, hydra were fixed with 4% paraformaldehyde after relaxation of the polyps in 2% urethane. Specimens were subsequently treated with ethanol and proteinase K to facilitate diffusion of the probes into the epithelial bilayer. To stabilise digested tissues, specimens were re-fixed with 4% paraformaldehyde and then prehybridised in hybridisation solution (50% formamide, 5× SSC, 1× Denhardt's, 200 mg/ml tRNA, 0.1% Tween 20, 0.1% CHAPS, 100 mg/ml heparin) to block nonspecific binding. This was followed by a 48 hour hybridisation with the digoxigenin-labelled RNA probe and a subsequent wash in hybridisation solution and 2× SSC. Specimens were washed in MAB (100 mM maleic acid, 150 mM NaCl, pH 7.5) and pre-blocked in MAB with 20% of sheep serum and 1% BSA. This was followed by 16 hours incubation at 4°C in the same solution with anti-digoxigenin antibody (1:2000). Animals were washed eight times with MAB and then briefly in alkaline phosphatase buffer (100 mM Tris HCl, pH 9.5, 50 mM MgCl<sub>2</sub>, 100 mM NaCl, 0.1% Tween-20).





**Fig. 1.** Purification of hydra collagens. Left panel, flow chart of hydra collagen isolation procedure. The numbering of the lanes refers to the SDS gel in the upper right panel. Upper right panel, monitoring the isolation of hydra mesoglea collagens by SDS gel chromatography under reducing (lanes 1-4, 8, 9) and non-reducing (lanes 5-7) conditions. Molecular masses of marker proteins in kDa are shown at the left side. The asterisk in lane 8 indicates the position of hydra type IV collagen (290 kDa) while the asterisk in lane 9 indicates the position of hydra collagen I/II (155 kDa, Hcol-I). Lower right panel, monoclonal antibody mAb39 that stains the central fibrous zone of hydra ECM (see Fig. 5) recognises Hcol-I. Slot blots of various hydra fractions (1M NaCl and NaCl/EDTA extracts, supernatant (containing hydra col-IV) and pellet (containing Hcol-I) of the EDTA fraction after centrifugation). Only fractions containing Hcol-I give a positive signal.

Specimens were then stained with BM purple AP substrate (Boehringer Mannheim), dehydrated with ethanol and mounted in euparal (Asco Laboratories).

### Functional analysis of the role of hydra col-I in head regeneration

To determine if Hcol-I is required for head regeneration, functional studies were performed using antisense thio-oligonucleotides. These oligonucleotides were designed from the following sequence regions of hydra fibrillar collagen: 5'UTR, AGCCAGGAAGTAAAGGATTA; Initiation region, ATGGGAGTACTGGTAATCC; Coding region, GACCTACCGGACCTGATGGT; 3'UTR, AACAGACTAGTATT-ATACGT. Antisense oligonucleotides were introduced into the ectoderm of the head pole of hydra using a localized electroporation (LEP) technique previously describe in detail by Yan et al. (2000a,b) and Leontovich et al. (2000). Shortly after LEP (2-4 hours, see previous studies for details of the technique), hydra were decapitated and head regeneration was monitored over a five- to seven-day period to monitor if blockage of head regeneration occurred and if blocked hydra subsequently recovered from blockage. Blockage of head regeneration was defined as a complete lack of tentacle eruption as compared to mock LEP controls or animals in which mismatch or sense thio-oligonucleotides were used.

## RESULTS

### Isolation of hydra ECM (mesoglea) enriched in collagen

Hydra ECM was prepared according to a previously described procedure with some modifications (Sarras et al., 1991a). To prevent cleavage of disulphide bonds, reducing agents were omitted in all purification steps, even though nematocyte capsules were not removed completely from the mesoglea by

this treatment (see below). The isolation procedure is outlined in Fig. 1 (left panel). Frozen hydras were thawed and washed several times with hypotonic Tris buffer containing Triton X-100 to remove soluble cytosolic proteins. Subsequently, the material was extracted with 1M NaCl to solubilise cell surface proteins still bound to the ECM and attached cytoskeletal components. This was followed by an extraction step with the same buffer, but containing 20 mM EDTA, since the aggregation of many ECM proteins is dependent on divalent cations (Maurer et al., 1996). Finally, the residue was extracted with SDS- and SDS/mercaptoethanol containing buffers. After this treatment some insoluble material was left. Amino acid analysis of the insoluble material revealed a high amount of glycine, proline and hydroxyproline, indicating the presence of collagenous proteins. Using pepsin digestion and BrCN digestion, it was possible to isolate peptides that proved to be derived from spinalin (Koch et al., 1998) and minicollagens (Kurz et al., 1991), indicating the presence of nematocyte capsules that could also be seen by light microscopical inspection. No evidence for the presence of mesoglea collagens was obtained (data not shown).

The results of the extraction procedure were monitored by SDS-PAGE (Fig. 1, upper right panel). Although the pools containing the cytosolic proteins showed almost a continuum of bands, the other fractions revealed unique protein patterns with little carry over. Comparison of the reduced (Fig. 1, upper right panel, lanes 2-4) and nonreduced (Fig. 1, upper right panel, lanes 5-7) samples showed characteristic differences. This holds especially for the EDTA extract. In the nonreduced sample (lane 3) only one major band at 155 kDa was observed. After reduction (lane 6) the mobility of this band was not changed but in addition high molecular weight bands were visible, a band of 290 kDa being the most intense.

**Table 1. Peptides isolated from the 155 kDa chain (Hydra col-I)**

<b>G</b> <b>Q</b> <b>X</b> <b>G</b> <b>E</b> <b>P</b> <b>G</b> <b>S</b> <b>X</b> <b>G</b> <b>L</b> <b>M</b> <b>G</b> <b>V</b>
<b>G</b> <b>S</b> <b>D</b> <b>G</b> <b>Q</b> <b>Q</b> <b>G</b> <b>T</b> <b>A</b> <b>G</b> <b>L</b> <b>A</b> <b>G</b> <b>E</b> <b>S</b> <b>G</b> <b>D</b> <b>P</b>
<b>G</b> <b>L</b> <b>P</b> <b>G</b> <b>P</b> <b>V</b> <b>G</b> <b>L</b> <b>P</b> <b>G</b> <b>A</b> <b>P</b> <b>G</b> <b>P</b> <b>V</b> <b>G</b> <b>E</b> <b>R</b> <b>G</b> <b>E</b> <b>P</b> <b>G</b> <b>K</b>
<b>G</b> <b>F</b> <b>P</b> <b>G</b> <b>Q</b> <b>Q</b> <b>G</b> <b>E</b> <b>Q</b> <b>G</b> <b>S</b> <b>P</b> <b>G</b> <b>I</b> <b>P</b> <b>G</b> <b>V</b> <b>V</b> <b>G</b> <b>E</b> <b>A</b> <b>G</b> <b>D</b> <b>K</b>
<b>G</b> <b>L</b> <b>P</b> <b>G</b> <b>P</b> <b>P</b> <b>G</b> <b>Q</b> <b>A</b> <b>G</b> <b>L</b> <b>R</b>
<b>G</b> <b>Y</b> <b>S</b> <b>G</b> <b>E</b> <b>Q</b> <b>G</b> <b>T</b> <b>P</b> <b>G</b> <b>S</b> <b>P</b> <b>G</b> <b>V</b> <b>I</b> <b>G</b> <b>E</b> <b>P</b>

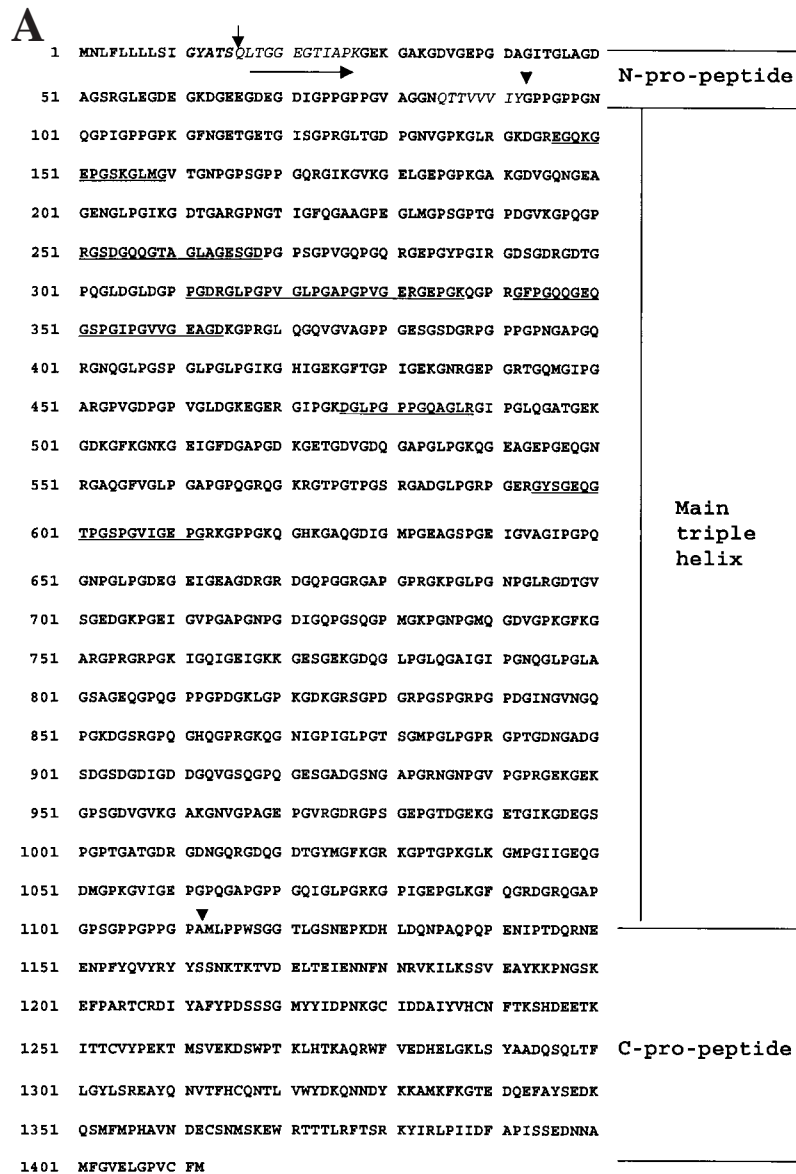
Glycine residues are shown in bold. P denotes hydroxyproline residues.

Partial sequence analysis revealed that the two bands represent two different hydra ECM collagens. The 290 kDa band (level of asterisk shown in lane 8) represents a hydra type IV collagen based on peptide sequence analysis and is described in another manuscript now in press (Fowler et al., 2000), whereas the 155 kDa component (level of asterisk shown in lane 9) represents the hydra fibrillar collagen, Hcol-I, as shown by a comparison of the peptide sequences from the peptide fragments (Table 1) with the cDNA sequence of Hcol-I (compare Table 1 with Fig. 2). These protein results demonstrate that by simple salt extraction in the presence of chelating agents, collagens can easily be extracted from hydra ECM under non-denaturing conditions.

### cDNA-cloning of hydra col-I and sequence analysis

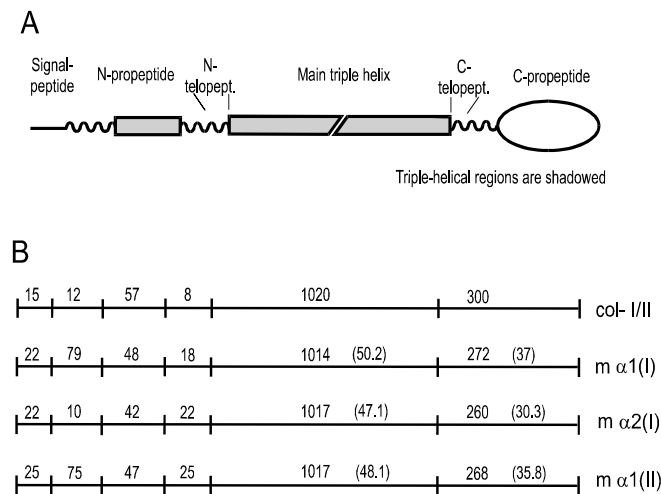
Expression screening using polyclonal antibodies to hydra ECM and degenerate oligonucleotide screening using primers designed from the peptides obtained from purified hydra fibrillar collagen resulted in the identification of five cDNA clones (2.1 kb, 4.0 kb, 2.0 kb, 2.2 kb and 2.6 kb) that represented overlapping sequences of a single transcript of 4460 bp. The sequence contained an open reading frame of approximately 4236 bp, encoding a protein of 1412 amino acids. Comparison of DNA sequences using the Blastn and Blastx programs and the GenBank database (NCBI, NIH) indicated the deduced amino acid sequence of this cDNA had greatest similarity to fibrillar members of the collagen family based on the presence of numerous Gly-X-Y tripeptide repeats (Fig. 2). Peptides that were isolated from purified Hcol-I and then sequenced (Table 1 and shown underlined in Fig. 2) show that

proline residues in the Y position of the Gly-X-Y repeats are often hydroxylated (prolines that are underlined in Table 1). Interestingly, the overall proline content is considerably lower in hydra fibrillar collagen compared with the vertebrate collagens (15% versus some 24%). A closer inspection of the sequence revealed that the Hcol-I exhibited the same gross structural features as vertebrate fibrillar collagen chains (Kuehn, 1987), especially the type I and type II collagens: amino acids 1-15 form the signal sequence, as predicted by the program signal peptide (GCG) and also verified experimentally (see below); (2) amino acids 16-92 form an N-terminal pro-peptide-like domain; (3) amino acids 93-1112 comprise the major triple helical region with no interruptions of the Gly-X-Y repeat. (This domain is the same length as in vertebrate



**Fig. 2.** Sequence of Hcol-I. (A) Sequence of Hcol-I derived from overlapping cDNA clones from a lambda cDNA library. The signal peptide cleavage site is marked by a vertical arrow and the arrowheads flank the main triple helical region. Sequences of the peptides obtained by Edman degradation and listed in Table 1 are underlined. The N-terminal sequence obtained by Edman degradation after deblocking is marked by a horizontal arrow. (B) Northern blot analysis of adult hydra RNA showing a single transcript with the size of about 5kb. The small bars at the left side denote the position of ribosomal RNAs.





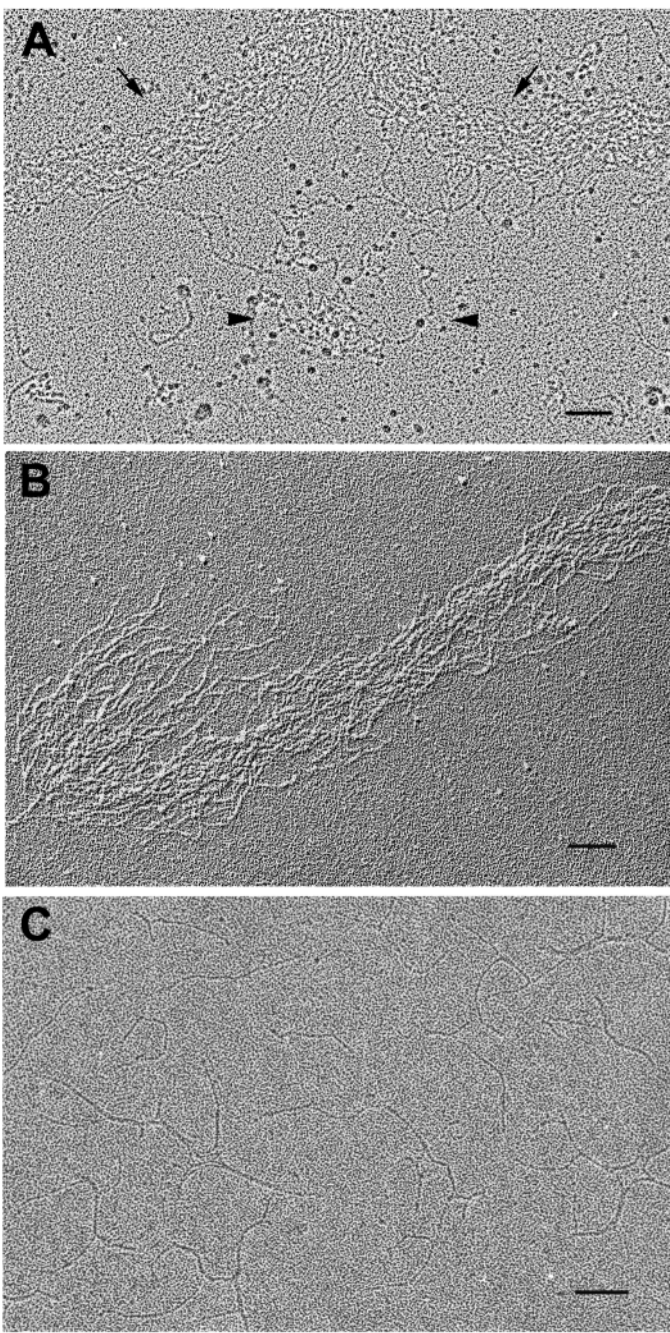
**Fig. 3.** Comparison of the domain structures of Hcol-I with mouse  $\alpha 1(I)$ ,  $\alpha 2(I)$  and  $\alpha 1(II)$  collagen chains (Accession numbers U08020, Q01149 and P28481). (A) Schematic drawing of the structure of fibrillar collagens (the triple helical domains are shaded), (B) Comparison of the sizes (number of amino acids) of the various domains. The sequence identity (%) of Hcol-I triple helical region and C-propeptide to the vertebrate collagen  $\alpha$ -chains is given in parentheses.

fibrillar collagens I to V); and (4) amino acids 1113-1412 form the non-collagenous C-propeptide.

A comparison of the Hcol-I sequence with the mouse  $\alpha 1(I)$ ,  $\alpha 2(I)$  and  $\alpha 1(II)$  chains is shown in Fig. 3. The N-terminal propeptide-like domain contains the same structural elements as the mouse N-propeptide domains, namely, a triple helical segment (57 amino acids long) flanked by two non-triple-helical sequences. Despite having the same features, this domain is the least well conserved, based on sequence comparisons. There is no striking identity at the level of amino acids, neither among the mouse chains nor between the mouse and hydra chains. The sequence identity in the major triple helical domain is about 50%, but this should be interpreted with some care as every third amino acid in a collagen triple helix is glycine. The most conserved region is the C-propeptide that has 30% to 35% identity with mouse counterparts and this identity is especially strong for cysteine residues. The C-propeptide domain has an overall similarity of up to 50%. A similar degree of similarity in this domain was found between Hcol-I and that for invertebrate sea urchin collagen (Esposito et al., 1992a) and a sponge collagen fragment (Esposito and Garrone, 1990) (not shown). Since the C-propeptide domain is known to be important for the assembly of collagen chains into trimeric procollagen molecules, the strong conservation of this domain suggests that major changes in its structure would interfere with the self-assembly process.

### Molecular and supramolecular structure

Collagen molecules are assembled either from three identical chains (homotrimer) or from three non-identical chains arising from different genes (heterotrimer). Our results suggest that Hcol-I forms a homotrimeric molecule. The SDS gel in Fig. 1 (upper right panel) shows only a single band at 155 kDa, no other bands that would match by size and intensity are seen. Furthermore, all peptide sequences obtained from the 155 kDa



**Fig. 4.** Transmission electron micrographs of hydra collagen preparations. (A) Electron micrographs of rotary shadowed collagens in the EDTA fraction. The arrows denote the Hcol-I fibrils and the arrowheads indicate the hydra collagen type IV network, which is characterised by globular structures interrupting the rod like segments. Electron micrographs of Hcol-I polymers and oligomers isolated by ultracentrifugation (B) or ion-exchange chromatography (C). The samples were prepared by coating with platinum/carbon either from one direction to show better the filamentous structure (A) or by rotary shadowing (B). Scale bars: 100 nm.

peptide (see Table 1) are contained in the cDNA sequence of Hcol-I. The three polypeptide chains are not linked by disulphide bridges as the electrophoretic mobility does not shift upon reduction (compare lanes 3 and 6), in contrast to the



290 kDa component (hydra type IV collagen; Fowler et al., 2000).

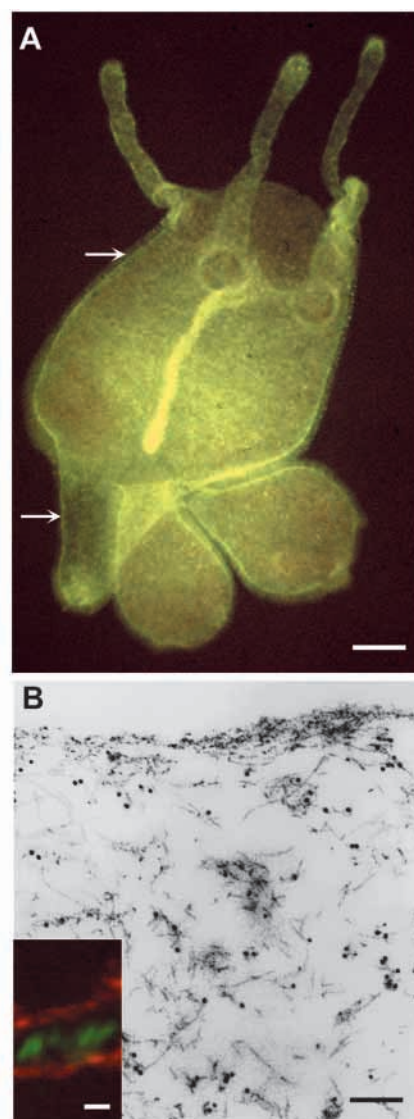
Vertebrate collagens undergo extensive post-translational processing of the N-terminal and C-terminal propeptides with crosslinking of the molecules into insoluble fibres. To investigate whether N-terminal processing also occurs in Hcol-I, we isolated the chains by reversed phase chromatography and determined the N-terminal protein sequence. Edman degradation revealed a blocked N-terminus, but after treatment with pyroglutamase a sequence starting at position 16 of Hcol-I (QLTGGE; first set of amino acids shown in *italic* in Fig. 2) was obtained. This demonstrated that the Hcol-I chain starts with the (cyclised) glutamine residue at position 15, just following the predicted signal cleavage site. Thus, unlike vertebrate fibrillar collagens, the N-terminal propeptide is not cleaved in Hcol-I and, therefore, is not a true 'propeptide'. In marked contrast to the N-terminus, there is cleavage of the C-terminal propeptide as could be shown by electron microscopy (see below).

To investigate the supramolecular structure of Hcol-I we first examined the unfractionated EDTA extract by electron microscopy (Fig. 4A). Surprisingly, despite giving well resolved bands in SDS-PAGE, single molecules were not very abundant and large aggregates were the most prominent structures observed. Two types of polymers were distinguished: (1) large fibrillar structures forming a filamentous network, indicated by the arrows in Fig. 4A; and (2) smaller aggregates consisting of a loose network of molecules containing distinct globular domains interrupting rod like segments, marked by the arrowheads in Fig. 4A. These latter structures could be shown to be formed by the hydra type IV collagen molecules (Fowler et al., 2000), whereas the large fibrillar structures are shown here to be formed by Hcol-I. Taking advantage of the fact that the hydra type IV collagen is crosslinked via disulphide bonds, we could isolate pure Hcol-I polymers. After treatment of the EDTA extract with mercaptoethanol and centrifugation, Hcol-I was precipitated, whereas the 290 kDa hydra col-IV component remained in the supernatant as shown by the SDS-PAGE in Fig. 1 (upper right panel, lane 8 (hydra col-IV) and lane 9 (Hcol-I)). Analysis of the resuspended precipitate by electron microscopy revealed only fibrillar networks that lacked globular domains (Fig. 4B). In another experiment, oligomeric Hcol-I structures were isolated by separating the EDTA extract using anion exchange chromatography (see Materials and Methods). In this manner, pure Hcol-I was obtained as demonstrated by SDS-PAGE (data not shown). Following this protocol mainly small aggregates and some monomeric molecules were obtained (Fig. 4C) since large polymers were not able to penetrate into the column. It is interesting to note that these molecules show the tendency to aggregate laterally but then often leave one thin fibril and join another. As shown in Fig. 4C, these molecules lack globular structures, suggesting that the C-terminal propeptide had been cleaved off.

#### Determination of the epitope recognized by monoclonal antibody 39

Previously, a monoclonal antibody staining the central fibrous zone of hydra ECM was reported (Sarras et al., 1993). In the current study, we determined whether this antibody recognized one of the hydra collagens described above. Unfortunately, the

antibody did not work using western blot analysis of our biochemical preparations (data not shown), although it did work with intact isolated hydra ECM before any extractions were performed (Sarras et al., 1993). The reason for the loss of the epitope in our purified preparations is not clear from these studies, but a reasonable explanation could be that the antibody recognises only a native triple-helical conformation. To circumvent this problem, we tested the antibody in slot blots applying various fractions containing native proteins. The antibody strongly reacted with the EDTA extract that contains



**Fig. 5.** Immunolocalisation of Hcol-I within the ECM along the body axis of hydra using mAb39. (A) Whole-mount immunofluorescence of *Hydra vulgaris* shows that Hcol-I is localized along the entire longitudinal axis of the polyp (arrowheads indicate the ECM between the ectoderm and endoderm). (B) Inset shows that in frozen cross section, Hcol-I (green signal using mAb39 as the primary antibody) localises to the central fibrous zone of hydra ECM, while laminin (red signal using mAb52 as the primary antibody) localises to the two subepithelial zones. At the ultrastructural level (main image in B), mAb39 localises to the 10 nm fibrils that comprise the majority of the structures associated with the central fibrous zone. Scale bars: 100  $\mu$ m in A; 100 nm in B; 0.5  $\mu$ m in inset in B.

the 155 kDa hydra fibrillar collagen and with the purified Hcol-I fraction, but not with the NaCl extract or the cytosolic fractions that contain the 290 kDa hydra type IV collagen, as shown in the lower right panel of Fig. 1. This indicates that mAb39 is directed against an epitope of Hcol-I.

### Localisation of hydra col-I within the ECM of hydra

The studies described above show that mAb39 reacts with Hcol-I. We then determined using whole-mount immunofluorescence the distribution of this hydra collagen along the body axis of the organism. As shown in Fig. 5A, Hcol-I is localized along the entire longitudinal axis of the animal. Frozen cross sections of hydra (inset, green signal, Fig. 5B) and immunogold localisation using TEM (Fig. 5B), indicated that Hcol-I was localized in the central fibrous zone of hydra ECM and represented the 10 nm fibrils typically seen in this region of the matrix. The central fibrous zone is positioned between the two subepithelial zones where basal lamina components like laminin are localized (inset, red signal, Fig. 5B).

### In situ analysis of hydra col-I mRNA in intact and regenerating hydra

Whole-mount in situ analysis indicated that Hcol-I was expressed in the ectoderm of adult polyps (Fig. 6). Expression was observed along the entire longitudinal axis of the animal in both *Hydra vulgaris* (Fig. 6A-C) and *Hydra magnipapillata* (Fig. 6D-F). Cross-sectional analysis confirmed that Hcol-I expression was restricted to the ectodermal cell layer (inset, Fig. 6B). During head regeneration, an upregulation of expression was observed within 3-4 hours of decapitation (Fig. 7A,B) and continued throughout the regeneration process (Fig. 7C-F). An apparent increase in expression was observed during tentacle morphogenesis as shown in Fig. 7E,F.

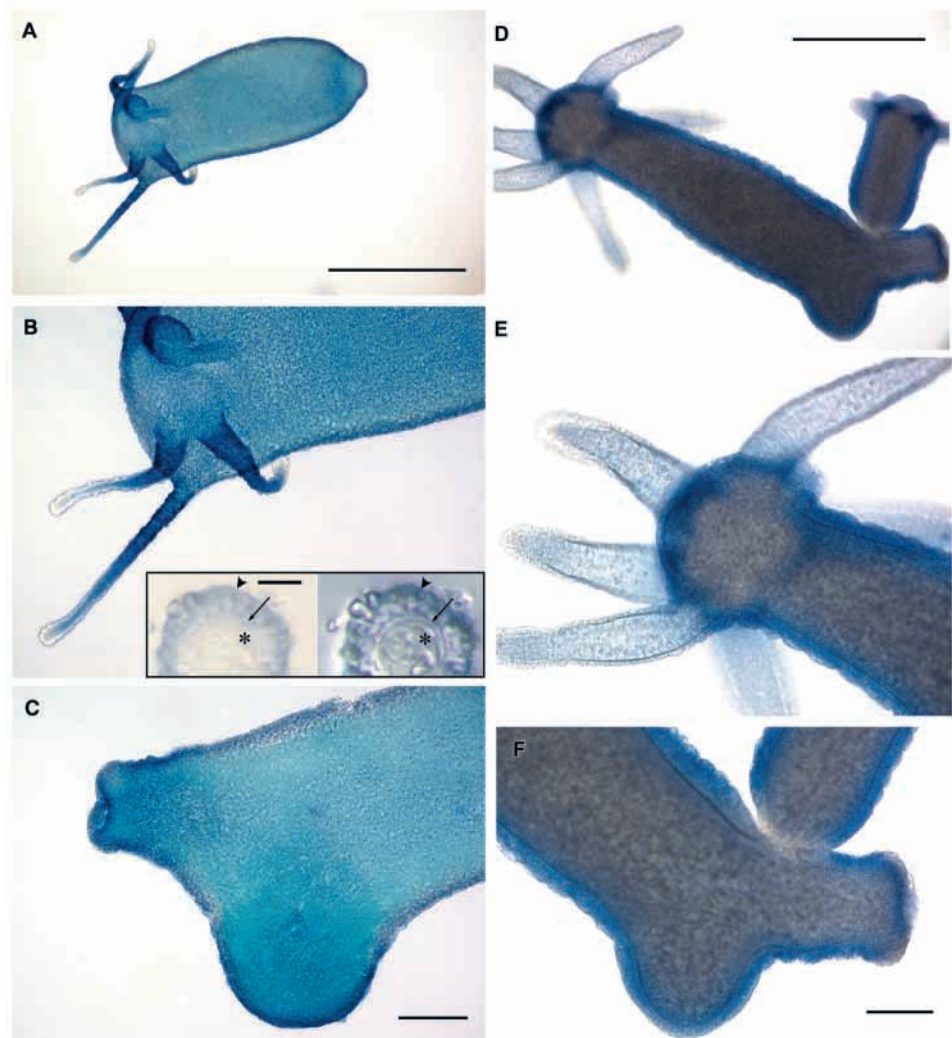
### Functional analysis of the role of hydra fibrillar collagen in head morphogenesis

As shown in Fig. 8, head regeneration was blocked using antisense oligonucleotides to Hcol-I. Blockage (ranging from 30-100%) was observed with all thiol-oligonucleotides tested as compared to mock, mismatch, or sense controls (control blockage levels ranged from 10-13%). Recovery from blockage was observed by five to seven days following initial decapitation (data not shown). The introduction of

antisense oligonucleotides into the regenerating head pole of hydra prevented the deposition of Hcol-I in the newly forming ECM at 24h post decapitation (right panel over graph) in comparison with control sense oligonucleotides (left panel over graph).

### DISCUSSION

Collagens are essential to metazoan systems and have a fundamental role in developmental, adult physiological and disease processes. In contrast to the vertebrate collagens however, very little is known about invertebrate collagens (Engel, 1997). To further understand the structure and function of collagens we used hydra as a model developmental system



**Fig. 6.** In situ localisation of Hcol-I in intact hydra. *Hydra vulgaris* is shown in A,B and *Hydra magnipapillata* is shown in D-F. The intense blue reaction product is associated with the ectodermal layer along the entire longitudinal axis of the polyp. In A and B, Hcol-I expression appears greater at the base of the tentacles. A bud is shown in C. An ectodermal expression pattern for Hcol-I is confirmed in cross sections of whole mounts as shown in the insets in B. A bright-field image is shown in the left panel and a DIC image is shown in the right panel. The arrowhead points to the apical border of the ectoderm and the arrow points to the ECM. The asterisk shows the endoderm layer. Scale bars: 500  $\mu$ m in A; 50  $\mu$ m in B; 100  $\mu$ m in C; 500  $\mu$ m in D; 100  $\mu$ m in F.



because of its simplified morphology (an epithelial bilayer with an intervening ECM) and high regenerative capacity. The current study presents a detailed analysis about the structure and developmental function of a major collagen of this simple metazoan. From an evolutionary standpoint, given the fact that as a Cnidarian, hydra belongs to the second oldest phylum of the animal kingdom (with sponges being the oldest), information gained from an analysis of Hcol-I provides insight into the structural and functional properties of this important family of proteins. We have therefore divided the following discussion into two parts. The first will involve a detailed discussion of the structural and biochemical characteristics of Hcol-I, especially as related to the unique properties of hydra ECM and the second will involve a discussion of the role of hydra ECM and Hcol-I in hydra morphogenesis.

### Hydra col-I is a primitive fibrillar collagen whose unique structural features help to explain the special characteristics of hydra ECM

Hcol-I represents a polypeptide of 1412 amino acids that shows striking similarity to vertebrate fibrillar collagens and also exhibits the characteristic modifications such as hydroxyproline in the Y-position of the Gly-X-Y triplet. It is noteworthy that the hydra sequence contains about 40% less proline than the mouse  $\alpha 1(I)$  chain, for example, which should endow the hydra collagen with greater flexibility. As shown in Fig. 3, Hcol-I exhibits the same structural elements as the vertebrate fibrillar collagens: it contains a continuous array of 340 Gly-X-Y repeats, having exactly the same length as in the vertebrate fibrillar collagens. This triple helical domain is flanked by a N-terminal propeptide like domain and a C-terminal propeptide. Sequence comparisons demonstrated a significant similarity at the amino acid level, ranging up to 50% for the C-terminal propeptide. A similar domain structure has also been reported in a collagen from sea urchin, indicating that fibrillar collagens are primordial structural proteins. Unfortunately, in the case of sponges, the oldest animal group, only partial sequence data exist for collagens, but structural similarity to the C-terminal propeptide suggests that a fibrillar collagen does exist in this ancient phylum.

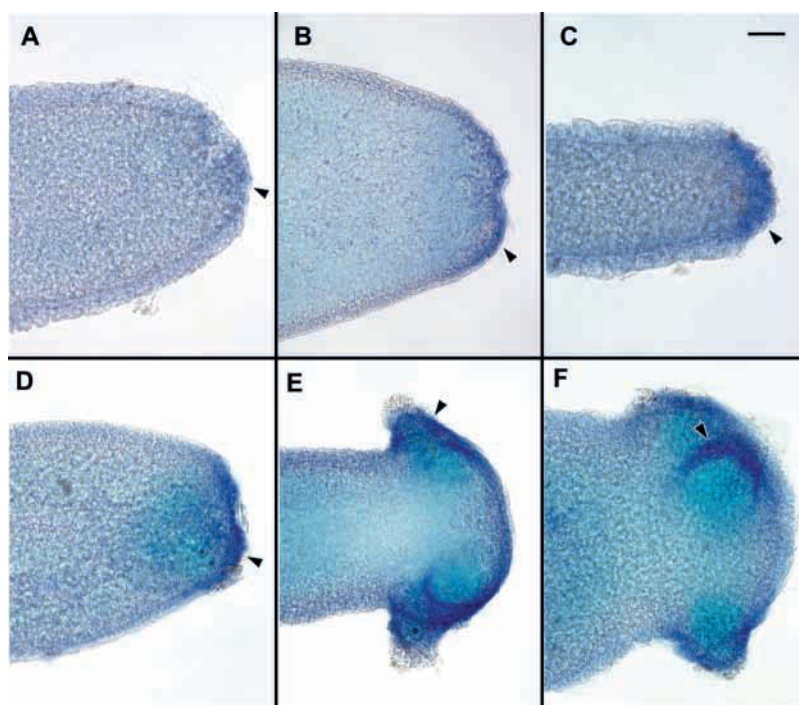
The sequence comparisons, especially the strict conservation of the length of the triple helical domain, would suggest similar fibre forming properties for Hcol-I. Nevertheless, assembly into supramolecular structures is quite different. Looking at the large Hcol-I aggregates shown in Fig. 4B one recognises a network of fine fibrils rather than thicker structures. This is in complete agreement with immunogold-labelling pictures using Hcol-I-specific antibodies (mAb39). Furthermore, EM images of Hcol-I prepared by ion exchange chromatography showed that some molecules aggregate laterally but then turn off one fibril and join a new one (Fig. 4C).

One major reason for this different behaviour is certainly the different post-translational processing of the molecule. Whereas the C-terminal propeptide is absent in the mature homotrimeric collagen, the

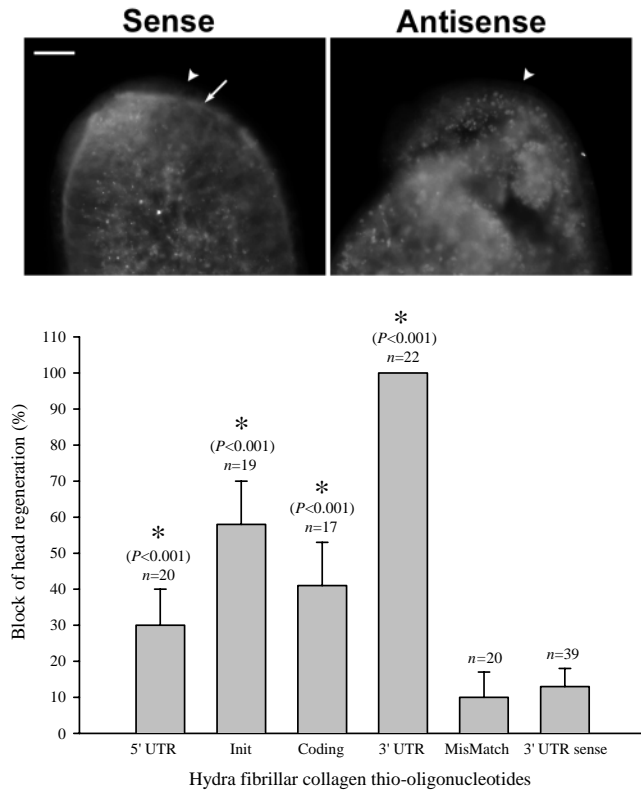
N-terminal propeptide-like domain is not removed, since we could extract at least the major portion of Hcol-I molecules from hydra ECM and all had the same N-terminal sequence, starting right after the signal sequence. The presence of small amounts of nonprocessed molecules is common in vertebrate collagens, too, and is thought to be important for regulation of fibre diameter. Indeed, there is a human disease called Ehlers-Danlos syndrome type VII that is caused by a failure to cleave the N-propeptide from type I collagen (Prockop and Kivirikko, 1995). This drastically alters fibre formation so that the fibrils are thin and highly irregular and this leads to joint and skin abnormalities. Therefore retention of the N-propeptide in Hcol-I should also be important for the formation of its supramolecular structure, in agreement with our findings. It is very interesting to note that a pathological process in humans that leads to joint and skin abnormalities (Ehlers-Danlos syndrome Type VII) is the normal physiological situation in hydra.

An additional surprising result was the fact that Hcol-I could easily be extracted from the mesoglea, even though vertebrate fibrillar collagens needed pepsin digestion to be solubilised. In our experiments, we used EDTA-containing buffers for extraction indicating that aggregation is somehow dependent on divalent cations. But we also tried other methods. Treatment with 0.5M acetic acid was not effective, whereas treatment with 0.5 M formic acid solubilised to the same extent as EDTA (data not shown). This means that the formation of fibrils is at least partially dependant on hydrophilic interactions that are destroyed by protonation of acidic side chains.

Our results show that H-col-I does not exhibit the extensive crosslinking of the vertebrate fibrillar collagens. In SDS-gels



**Fig. 7.** In situ localisation of Hcol-I during head regeneration in hydra. Following decapitation, hydra were fixed and processed for in situ hybridisation at 1h (A), 4h (B), 12h (C), 24h (D), 48h (E) and 72h (F). (A-D) The arrowheads indicate the apical border of the ectoderm. In E and F the arrowheads point to the base of the tentacles where a more intense signal appears. Scale bar: 100  $\mu$ m.



**Fig. 8.** Effect of antisense thio-oligonucleotides to Hcol-I on hydra head regeneration. The number of hydra per group is indicated by the N. Asterisks indicate groups that are significantly different from control groups. Thio-oligonucleotides were designed from the cDNA sequence of Hcol-I and represent portions of the 5'UTR, initiation site, coding region and 3'UTR. Controls are shown with mismatch (randomised sequence) oligonucleotides and sense oligonucleotides to the 3'UTR region. In the upper panels are shown the effects of antisense and sense thio-oligonucleotides on the deposition of Hcol-I at the apical border of hydra 24 hours post-decapitation. The arrowheads indicate the apical border of the ectoderm and the arrow in the sense panel indicates the position of the ECM. Scale bar: 100  $\mu$ m.

the monomeric  $\alpha$ -chains were most prominent even under nonreducing conditions. Vertebrate fibrillar collagens contain specific crosslinking sites with essential lysine residues in the N-terminal and C-terminal parts of the molecule. One major site is located in the non-collagenous domain at the border of the N-propeptide and the major triple helix. The corresponding sequence in Hcol-I (position 84-92; second amino acid sequence shown in *italic* in Fig. 2) does not contain any lysine residues, so that at this site at least, the classical crosslinking via lysine-lysine aldehydes cannot occur. It seems quite reasonable to assume that extensive crosslinking is not essential for hydra because of its short replication time.

Taken in total, the low proline content, the inability to form fibres and the absence of important lysine residues that normally form crosslinks in collagens are all properties of Hcol-I that contribute to the formation of an ECM that can be structurally supportive to the epithelial bilayer, but at the same time can be flexible enough to allow the organism to perpetually contract and extend as it does in nature. Previous ultrastructural studies by Davis and Haynes (1968) reported

that the thin fibrils of hydra ECM become irregular in their orientation when the organism was contracted as compared with the normal parallel alignment of fibrils along the longitudinal axis when hydra were extended. Davis and Haynes (1968) suggested that hydra collagens had the ability to fold on themselves and that this folding property facilitated flexibility in hydra ECM during contraction of the organism. Our structural and biochemical data support the interpretations presented by Davis and Haynes (1968).

The localisation of Hcol-I to the central fibrous zone also supports the proposal that this region equates to an interstitial-like matrix in contrast to the subepithelial zones that equate to basal lamina. Our biochemical analysis together with the immunolocalisation studies further demonstrates that this interstitial matrix has a rather simple composition. In our ECM preparations (EDTA extract), we found only two major components, the fibrillar collagen, Hcol-I, and the typical basement membrane collagen type IV, thus making hydra an attractive simple model for studying the structure and biogenesis of ECM. These studies indicate that the overall organisation of hydra ECM and specifically the unique structural characteristics of Hcol-I are fundamental to the basic morphology of this simple epithelial metazoan. The organisation and molecular composition of hydra ECM also has profound implications as to developmental processes in this organism, as will be discussed next.

### Fibrillar collagen is essential to morphogenesis in hydra

Previous studies have established that the ECM of hydra is important to processes related to cell migration, morphogenesis and cell differentiation in this simple metazoan (Sarras et al., 1991b, 1993, 1994; Zhang and Sarras, 1994; Zhang et al., 1994; Agbas and Sarras, 1994; Yan et al., 1995, 2000a,b; Leontovich et al., 2000). These studies have established that an underlying principle of hydra biology is that morphogenesis does not proceed in this organism until an ECM has formed. This is reflected by studies with hydra cell aggregates (Sarras et al., 1993), as well as studies related to head or foot regeneration (paper in preparation). The fundamental importance of cell/ECM interactions during development is reflected by the fact that such interactions have also been shown to extend to other groups within the Cnidarian phylum (Schmid et al., 1999), as well as to the more ancient phylum, Porifera (Wimmer et al., 1999).

As stated above, the ECM of hydra is actually composed of two basal lamina layers (subepithelial zones) and a central fibrous zone that can now be classified as a true interstitial-like matrix based on the studies presented in the current study. From a functional standpoint, it is not difficult to understand the importance of the basal lamina to cell-ECM interactions in hydra since these matrix zones are adjacent to the epithelial layers (Sarras et al., 1991a) and contain molecules that have been shown to interact with the basal plasma membrane of the ectoderm and endoderm (Sarras et al., 1994; Agbas and Sarras, 1994; Zhang and Sarras, 1994). Incorporation of basal lamina components into a newly forming ECM is crucial to morphogenesis in hydra (Sarras et al., 1994), but the precise nature of the cell/ECM interactions are not clearly understood. In this regard, it is not certain to what degree the basal lamina imparts structural integrity to the developing epithelium of



hydra or provides differentiation signals through endogenous amino acid sequences within the matrix molecules themselves (Zhang et al., 1994; Sarras et al., 1994; Agbas and Sarras, 1994). Such endogenous signalling from matrix molecules has been clearly established in vertebrate systems (Giannelli et al., 1997; Pilcher et al., 1997; Zhao et al., 1999). The strongest support for the role of endogenous signals from components of the hydra basal lamina comes from competition studies using either analogous peptides or antibodies to matrix sequences or integrins (Sarras et al., 1993, 1994; Agbas and Sarras, 1994; Zhang and Sarras, 1994; Zhang et al., 1994). In contrast, less is known about the importance of the central fibrous zone of hydra ECM. The current studies clearly establish that Hcol-I is localized to the central fibrous zone of hydra ECM and that it is upregulated during head regeneration. The finding that it is expressed in the ectoderm while basal lamina components such as laminin are expressed in the endoderm (Leontovich et al., 2000) indicate that some type of signalling must occur between the bilayer during biogenesis of the ECM in hydra. The ability to knockout precisely the translation of Hcol-I and to block head morphogenesis reversibly in hydra shows that the central fibrous zone of hydra ECM is also crucial to normal development in this organism. Given that Hcol-I is separated from the epithelium by the two intervening subepithelial zones or basal lamina, it is assumed that the importance of this fibrillar collagen relates to the structural integrity of the hydra ECM. It should be noted, however, that cellular processes from the ectoderm and endoderm extend through the ECM to form cell-cell contracts (Wood, 1983) and therefore the basal plasma membrane of these cells is in direct contact with Hcol-I as well as laminin. Additional studies related to cell-ECM signalling and to the normal biogenesis of hydra ECM are required to clarify this point.

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