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The primitive metazoan *Hydra* expresses antistasin, a serine protease inhibitor of vertebrate blood coagulation: cDNA cloning, cellular localisation and developmental regulation

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We have isolated and characterized cDNAs from *Hydra* which encode antistasin, a potent inhibitor of factor Xa in the vertebrate blood clotting cascade. *Hydra* antistasin is expressed in gland cells and represents a major class of transcripts from *Hydra*'s head. Sequence analysis revealed that *Hydra* antistasin contains 6 internal repeats of a 25–26 amino acid sequence with a highly conserved pattern of 6 cysteine and 2 glycine residues identical to that in leech antistasin. Conservation of antistasin in a lower metazoan provides a potential link between the vertebrate and invertebrate coagulation systems.

Antistasin; Serine protease inhibitor; Factor Xa; Blood coagulation; *Hydra*

1. INTRODUCTION

Antistasin is a 17 kDa serine protease inhibitor isolated from the salivary glands of the Mexican leech (*Haemateria officinalis*) [1]. This protein (and a similar one from *Haemateria ghilianii*) is a potent anticoagulant by virtue of its ability to inhibit factor Xa in the coagulation cascade [2]. Antistasin also exhibits a strong antimetastatic activity [3–6]. Amino acid sequence analysis revealed that antistasin is a cysteine-rich 119 amino acid peptide with no sequence similarity to other known proteins [7]. A remarkable feature of the primary structure is a 2-fold internal repeat, suggesting that antistasin has evolved by gene duplication [7,8].

We have cloned and sequenced an antistasin-like cDNA from *Hydra*, one of the most primitive metazoans. The complete sequence revealed that *Hydra* antistasin contains a highly conserved 6-fold internal repeat of 25–26 amino acids with the reactive site for the specific inhibiton of factor Xa in four of the six repeats. The positions of all 6 cysteine and 2 glycine residues are identical in leech and *Hydra* antistasin. The structural organisation of *Hydra* antistasin suggests that antistasin-like serine protease inhibitors may have evolved from small ancestral genes corresponding to the basic repeat unit of *Hydra* antistasin.

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2. EXPERIMENTAL

2.1. *Animals and culture*

Hydra magnipapillata strain sf1 [9] was used in all experiments. Mass cultures were kept in M solution [10] at 18 ± 0.5°C and fed daily with *Artemia* nauplii. Experimental animals were starved for 24 h.

2.2. *cDNA cloning and differential screening of an I-cell library from Hydra*

A cDNA library of interstitial cells (I-cells) was constructed in lambda gt10 and 8,000 clones were differentially screened at high stringency conditions using [^{32}P]dCTP-labeled cDNA probes prepared from poly(A)$^+$ RNA of fractions enriched for interstitial cells or epithelial cells [11]. 600 I-cell-specific clones were re-screened with [^{32}P]dCTP-labeled cDNA probes prepared from poly(A)$^+$ RNA of head or gastric tissue (concentration of the probes was 3×10^6 cpm/ml [12]): 41 clones hybridized more strongly with the head probe than with the gastric probe; 19 of these clones cross-hybridized with *Hydra* antistasin under high stringency conditions.

2.3. *DNA sequence analysis*

Commonly used recombinant techniques, such as gel electrophoresis, subcloning (pUC 19), growth of plasmids and bacteriophage were carried out as described [13]. DNA sequencing was done with the dideoxy chain termination method [14] using sequence-specific primers and a USB sequencing kit. Sequence data were analyzed with the sequence analysis software package, Hibio DnasisTM and ProsisTM (Hitachi Software Engineering Co.).

2.4. *Separation of Hydra cells by counterflow centrifugation elutriation*

Hydra cell types are characterized by considerable size differences and can be separated by counterflow centrifugation elutriation [15]. Polyps (1,000) were dissociated into single cells (10^8) with pronase E and separated into size fractions using a Beckman J6 centrifuge (18°C) and a elutriator (JE 5.0) with a 5 ml separation chamber [18]. Four 100 ml fractions were collected using rotor speeds of 4,000, 2,800, 1,700 and 1,100 rpm at 15 ml/min flow rate and two additional fractions at 35 ml/min and 60 ml/min at 1,100 rpm.

2.5. *Northern analysis*

Total RNA was extracted using the guanidinium isothiocynate

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method [16], electrophoresed in 1% agarose gels containing 2.22 M formaldehyde, and transferred to a nylon membrane (Biodyne B from Pall). The filters were washed and hybridized at high stringency conditions.

2.6. *In situ hybridization*

For cryosections, polyps were anesthesized in 2% urethane, fixed with Lavdowsky's fixative (ethanol/formalin/acetic acid/water, 50/10/4/40) for at least 1 h, washed in PBS, transferred to 10, 20 and 30% sucrose (cryoprotectant) in PBS, and frozen using the quick-freeze device of a Reichert cryomicrotome; cryosections (15 μm) were prepared at 20°C, dried on gelatine-coated slides at room temperature and stored at −80°C. Macerated cell preparations on gelatine-coated slides were prepared as described [11,17]. Probes were labeled with digoxygenin-dUTP using the random primer procedure according to the Boeringer (Mannheim, Germany) labeling kit [11,18]; hybridization was carried out in 100 mM DTT, 5 μg/ml heparin, 4× SSC and 0.5–2 μg denatured probe in a humidified chamber at 58°C for 12 h; slides were washed 3×20 min in 2× SSC at 58°C, and the hybridized probe was detected using anti-digoxygenin AP (2 h) and NBT/X-phosphate substrate (23 h); preparations were mounted using PBS/glycerol (9:1) and viewed with Nomarski interference contrast optics.

3. RESULTS AND DISCUSSION

We identified an antistasin-like sequence in *Hydra* in the course of experiments to isolate cell type and position-specific marker genes from a cDNA library of the interstitial cell lineage. The library was prepared from cells of the interstitial cell lineage (stem cells, nematocytes, nerve cells, gland cells) [11] and differentially screened with interstitial and epithelial cell cDNA to identify interstitial cell specific clones. 19 clones (see section 2) were identified which cross-hybridized to each other and turned out by sequence analysis to encode an antistasin-like peptide (see below).

The cell-type specificity of antistasin expression was determined by Northern blot analysis using RNA from cell-type specific fractions of elutriated cells (see section 2). The expression pattern indicates that *Hydra* antistasin is strongly expressed in gland cells (Fig. 1). In situ hybridization on macerated cells and on cryosections of fixed polyps using digoxygenin-labeled DNA and anti-

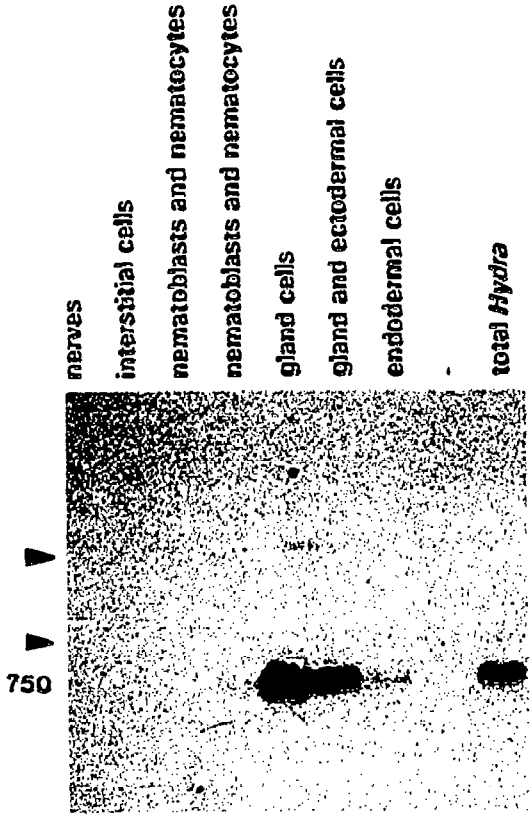

Fig. 1. Hybridization of 10 μg total RNA from elutriated *Hydra* cells (lanes 1–7) and whole animals (lane 9) with ^{32}P-labeled *Hydra* antistasin cDNA; cell size increases from the nerve cell fraction (lane 1) to the endodermal cell fraction (lane 7) [18]; arrowheads indicate the position of 28 S and 18 S ribosomal RNA.

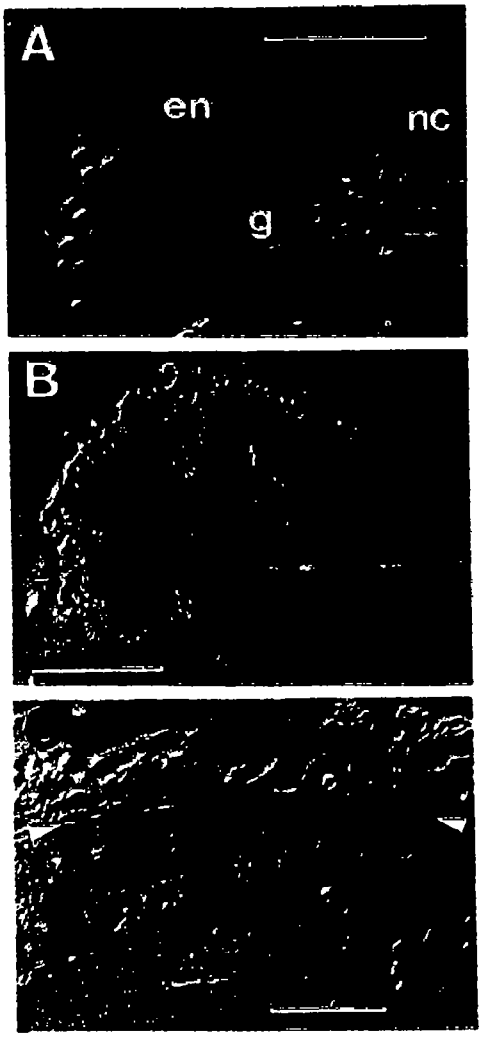

Fig. 2. Expression of *Hydra* antistasin in macerated cells (A) and in cryosections (B,C). (A) Gland cells (g) are labeled; ectodermal and endodermal epithelial cells (en), differentiating nematocytes (nc) and all other cells types are unlabeled; bar = 50 μm. (B) Cross-section through the head region; all gland/mucous cells are heavily labeled; bar = 300 μm. (C) cross-section through the gastric region; arrowheads indicate mesoglea separating ectoderm and endoderm; scattered gland cells in endoderm are labeled; bar = 150 μm.

digoxygenin alkaline phosphatase confirm this result. Fig. 2A shows macerated cells hybridized with *Hydra* antistasin. Only gland and mucous cells are heavily stained; all other cell types are unstained. Quantitative analysis of the preparations revealed that 95% of all gland/mucous cells in the head region were labeled and 47% in the gastric region. Cryosections demonstrate this spatial pattern: only gland and mucous cells in the endoderm are stained and the density of stained cells in the head region (Fig. 2B) is very high compared to the gastric region (Fig. 2C).

Northern blot analysis confirms that *Hydra* antistasin is more strongly expressed in head than in gastric tissue (Fig. 3A, lanes 1 and 5). Indeed, it constitutes one of the major transcripts from the interstitial cell lineage in head tissue (see section 2). To follow the expression of antistasin transcripts during head differentiation, regenerating head tissue was assayed for antistasin transcripts. Fig. 3A shows that the level of antistasin RNA increases continuously during the 2-day period of head differentiation indicating that expression of *Hydra* antistasin is stimulated early during head formation.

Fig. 4 shows the nucleotide and deduced amino acid sequence (single letter code) of *Hydra* antistasin determined from five cDNA clones. The putative protein is 220 amino acids long and has a clearly defined N-terminal hydrophobic domain (19 amino acids) with the characteristic features of a signal peptide [19,20] indicating that *Hydra* antistasin is synthesized on the endoplasmic reticulum and probably exported from the cell.

The most striking feature of the *Hydra* antistasin sequence is a 6-fold internal repeat of a 25–26 amino acid sequence (Figs. 4 and 5A). The repeats are not identical but are easily recognized due to the presence of six highly conserved cysteine residues, as well as R/KM, GF and DENG motivs at conserved positions (Fig. 5A). Comparison of the *Hydra* antistasin sequence with the EMBL DNA data library indicated, depending on the repeat, a 35–55% sequence identity to antistasin from *Haemateria officinalis* [7,8] and to the corresponding protein from *Haemateria gitilianii* (ghilanten) [21]. The homology is restricted to residues 28–53 and 80–108 of leech antistasin and is based primarily on the conserved spacing of the 6 cysteine and 2 glycine residues. Due to the perfect conservation of these residues, it appears likely that leech and *Hydra* antistasin fold to form very similar tertiary structures. The reactive site for the specific inhibiton of factor Xa is the arginine/methionine sequence at position 34/35 in the leech antistasin [2]. These residues are conserved as Arg/Lys and Met in four of the six repeats in the *Hydra* antistasin sequence (Fig. 5A).

There is an additional consensus motif (PEKK) in the 3' flanking region of the 1st, 2nd, and 4th repeat which may encode an endopeptidase cleavage site and thus lead to fragmentation of *Hydra* antistasin into 4 functional peptides. If this occurs repeats 3/4 and 5/6 would

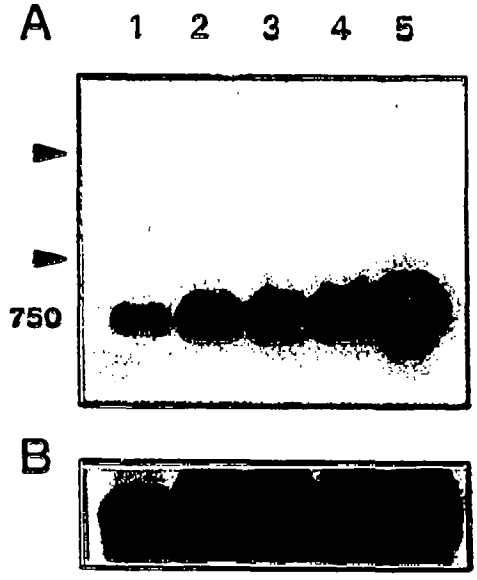

Fig. 3. (A) Hybridization of 10 μg total RNA from gastric tissue (lane 1), from presumptive head tissue of regenerating animals 12, 24, and 36 h after head removal (lanes 2–4), and from head tissue (lane 5); arrowheads indicate the position of 28 S and 18 S ribosomal RNA. (B) The filters were reprobed with ribosomal DNA to show that equal quantities of DNA were loaded.

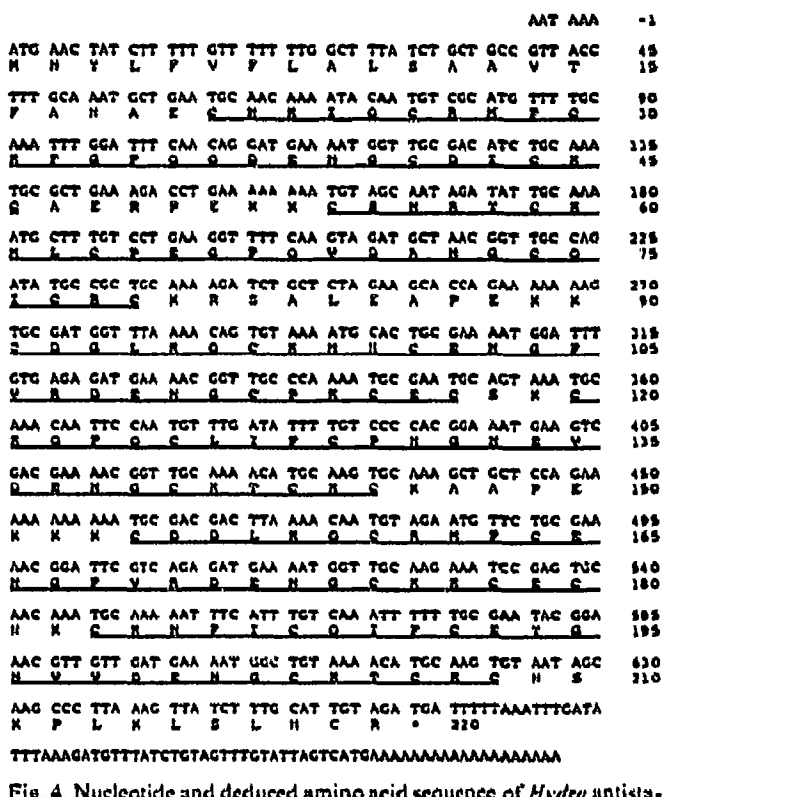

Fig. 4. Nucleotide and deduced amino acid sequence of *Hydra* antistasin. Nucleotide 1 is the first nucleotide of the ATG translation start codon; the termination is indicated by an asterix; six internal repeats are underlined and are in bold letters.

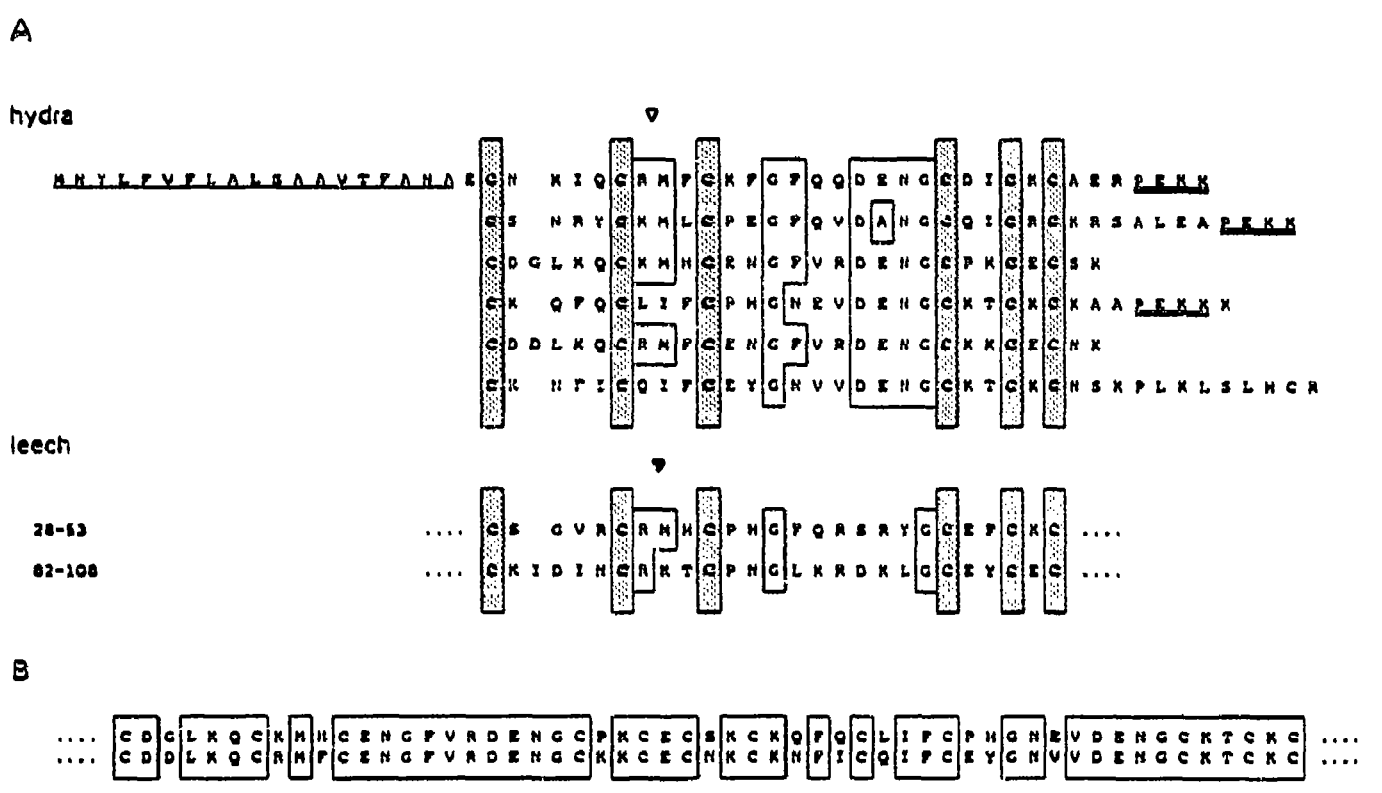

Fig. 5. (A) Amino acid sequence of *Hydra* antistasin, showing the 6-fold internal repeat and the structural homology to leech antistasin [7,8]. Amino acid homologies are boxed, cysteines are indicated by shadowing, the putative leader region is underlined, and possible endopeptidase cleavage sites are indicated by double lines. The filled arrowhead indicates the scissile peptide bond in leech antistasin [2]; the open arrowhead indicates the putative scissile peptide bond in *Hydra* antistasin. (B) Internal homology between repeats 3/4 and 5/6 of *Hydra* antistasin; amino acid homologies are boxed.

form independent peptides with greater than 80% sequence identity (Fig. 5B).

Antistasin is a new member of the large family of serine protease inhibitors [2,7,8]. The mechanistic features shared by most of these inhibitors include binding and cleavage at the reactive site of the inhibitor, which is often presented to the target protease in a disulfide-bonded loop structure; the P1 residue in the reactive site of the inhibitor determines the specifity for distinct serine proteases [22,23].

The function of serine protease inhibitors in coelenterates is unknown. *Hydra* feeds on small crustaceans; however, some coelenterates, e.g. *Physalia* and *Cironex*, use their highly poisonous nematocysts to attack and feed on vertebrates such as fish [24]. Digestion of such tissue might be facilitated by secretion of anticoagulant. Alternatively the secretion of antistasin by mucous cells in the gastric cavity of *Hydra* suggests that this inhibitor may function to protect gastric tissue from its own digestive enzymes, similar to the protease inhibitors in various mucosal secretions in mammals [25–27].

A striking characteristic of many protease inhibitors is the presence of multiple homologous reactive site domains in the same protein, which presumably have arisen by repeated gene duplication events [22,23]. The structural organisation of *Hydra* antistasin suggests that antistasin-like serine protease inhibitors may have evolved from small ancestral genes corresponding to the basic repeat unit of *Hydra* antistasin.

A cascade of serine protease zymogens leading to coagulation of hemolymph has been described for invertebrates [28,29]. In the horseshoe crab (*Limulus*) this cascade is based on three serine proteases (factor C, factor B, and the proclotting enzyme) and one clottable protein (coagulogen). This coagulation system may be phylogenetically related to the vertebrate blood clotting system [28–31]. Supporting this hypothesis are recent experiments showing that the *Limulus* proclotting enzyme has a substrate specifity similar to mammalian factor Xa, and 34–35% homology on the amino acid level [30,31]. The existence of a highly specific factor Xa/proclotting enzyme inhibitor, antistasin, in a coelenterate is the first evidence that the two coagulation systems may have had a common precursor in lower metazoans.

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