INTRODUCTION

Nematocytes (cnidocytes, ‘stinging cells’) are highly specialized cells characteristic of the phylum Cnidaria. The cells harbor a specialized organelle, the nematocyst, which serves various functions such as defense, the capture of prey and locomotion (Weill, 1934; Mariscal, 1974). Nematocysts consist of a capsule with a stiff wall and an internal tubule which is coiled, pleated and barbed with spines. More than 25 morphologically different nematocyst types have been described and all of them are able to ‘discharge’ after appropriate stimulation (Weill, 1934; Mariscal, 1974). During the discharge the long barbed tubule is ejected in one of the fastest known processes in living systems (Tardent and Holstein, 1982). High-speed cinematography has revealed that the initial steps in the eversion of the tubule take less than 10 microseconds and that the spine-bearing part of the tube is accelerated by up to 40,000 \( g \) (Holstein and Tardent, 1984). Spines serve different functions depending on the nematocyst type but all seem to have a high mechanical strength. In particular, stylets, the large spines of stenoteles, have to withstand extreme mechanical stress, since they form the tip which punches a hole in the prey’s integument. Nematocyst discharge is driven by the very high osmotic pressure (15 MPa; Weber, 1989) in the resting capsule, which results from the high concentration of poly-\( \gamma \)-glutamic acid and the corresponding cations in the matrix (Weber, 1990, 1991). The high osmotic pressure in capsules and the extraordinary speed of the evagination process suggest that the capsule wall must have a high tensile strength. Although the structure of the wall is not known in detail, recent work has demonstrated the presence of unusual, small, collagen-like proteins in the wall (Kurz et al., 1991; Engel, 1997) and high-resolution electron microscopy has indicated that these are probably organized in collagen-like fibrils (Holstein et al., 1994). Nothing is known about the structure of the other parts of the nematocyst such as the tubule, spines or operculum, and no other nematocyst-specific genes have been isolated or characterized yet.

The aim of the present study was to investigate another nematocyst-specific protein, which we have named spinalin. Spinalin is not homologous to any protein in the databases but does have regions with partial homology to loricrins (Mehrel et al., 1990; Steinert et al., 1991) and avian keratins (Whitbread et al., 1991). Both proteins are involved in forming cellular supermolecular structures. Spinalin is terminated by a basic region (6 lysines out of 15 residues) and an acidic region (9 glutamates and 9 aspartates out of 32 residues). Western blot analysis with a polyclonal antibody generated against a recombinant 19 kDa fragment of spinalin showed that spinalin is localized in nematocysts. Following dissociation of the nematocyst’s capsule wall with DTT, spinalin was found in the insoluble fraction containing spines and the operculum. Immunocytochemical analysis of developing nematocysts revealed that spinalin first appears in the matrix but then is transferred through the capsule wall at the end of morphogenesis to form spines on the external surface of the inverted tubule and the operculum.

**SUMMARY**

Here we present the cloning, expression and immunocytochemical localization of a novel 24 kDa protein, designated spinalin, which is present in the spines and operculum of Hydra nematocysts. Spinalin cDNA clones were identified by in situ hybridization to differentiating nematocytes. Sequencing of a full-length clone revealed the presence of an N-terminal signal peptide, suggesting that the mature protein is sorted via the endoplasmic reticulum to the post-Golgi vacuole in which the nematocyst is formed. The N-terminal region of spinalin (154 residues) is very rich in glycines (48 residues) and histidines (33 residues). A central region of 35 residues contains 19 glycines, occurring mainly as pairs. For both regions a polyglycine-like structure is likely and this may be stabilized by hydrogen bond-mediated chain association. Similar sequences found in loricrins, cytokeratins and avian keratins are postulated to participate in formation of supramolecular structures. Spinalin is terminated by a basic region (6 lysines out of 15 residues) and an acidic region (9 glutamates and 9 aspartates out of 32 residues). Western blot analysis with a polyclonal antibody generated against a recombinant 19 kDa fragment of spinalin showed that spinalin is localized in nematocysts. Following dissociation of the nematocyst’s capsule wall with DTT, spinalin was found in the insoluble fraction containing spines and the operculum. Immunocytochemical analysis of developing nematocysts revealed that spinalin first appears in the matrix but then is transferred through the capsule wall at the end of morphogenesis to form spines on the external surface of the inverted tubule and the operculum.

**Key words:** Hydra, Nematocyst, Nematocyte, Spinalin.

**INTRODUCTION**

Nematocytes (cnidocytes, ‘stinging cells’) are highly specialized cells characteristic of the phylum Cnidaria. The cells harbor a specialized organelle, the nematocyst, which serves various functions such as defense, the capture of prey and locomotion (Weill, 1934; Mariscal, 1974). Nematocysts consist of a capsule with a stiff wall and an internal tubule which is coiled, pleated and barbed with spines. More than 25 morphologically different nematocyst types have been described and all of them are able to ‘discharge’ after appropriate stimulation (Weill, 1934; Mariscal, 1974). During the discharge the long barbed tubule is ejected in one of the fastest known processes in living systems (Tardent and Holstein, 1982). High-speed cinematography has revealed that the initial steps in the eversion of the tubule take less than 10 microseconds and that the spine-bearing part of the tube is accelerated by up to 40,000 \( g \) (Holstein and Tardent, 1984). Spines serve different functions depending on the nematocyst type but all seem to have a high mechanical strength. In particular, stylets, the large spines of stenoteles, have to withstand extreme mechanical stress, since they form the tip which punches a hole in the prey’s integument. Nematocyst discharge is driven by the very high osmotic pressure (15 MPa; Weber, 1989) in the resting capsule, which results from the high concentration of poly-\( \gamma \)-glutamic acid and the corresponding cations in the matrix (Weber, 1990, 1991). The high osmotic pressure in capsules and the extraordinary speed of the evagination process suggest that the capsule wall must have a high tensile strength. Although the structure of the wall is not known in detail, recent work has demonstrated the presence of unusual, small, collagen-like proteins in the wall (Kurz et al., 1991; Engel, 1997) and high-resolution electron microscopy has indicated that these are probably organized in collagen-like fibrils (Holstein et al., 1994). Nothing is known about the structure of the other parts of the nematocyst such as the tubule, spines or operculum, and no other nematocyst-specific genes have been isolated or characterized yet.

The aim of the present study was to investigate another nematocyst-specific protein, which we have named spinalin. Spinalin is not homologous to any protein in the databases but does have regions with partial homology to loricrins (Mehrel et al., 1990; Steinert et al., 1991) and avian keratins (Whitbread et al., 1991). Both proteins are involved in forming cellular
products that tolerate high mechanical stress, i.e. the cornified epidermal cell envelope in the case of loricins, and keratinized epithelial derivatives (claws, nails) in the case of avian keratins. A truncated form of spinalin, named spinalin19K, was recombinantly expressed in *Escherichia coli*. Using a polyclonal antibody generated against spinalin19K we were able to localize the mature protein to the spines and operculum of nematocyst capsules, both structures having high mechanical strength.

**MATERIALS AND METHODS**

**Hydra strains and culture conditions**

*Hydra magnipapillata* strain sf-1 was used for all experiments. This strain contains temperature-sensitive interstitial cells, which can be removed by warming cultures to 28°C (Sugiyama and Fujisawa, 1977). Mass cultures were maintained in M solution at 18°C (Loomis and Lenhoff, 1956) and fed daily with *Artemia nauplii*. Animals were starved for 24 hours before experiments.

**cDNA library and differential screening**

A cDNA library was prepared in lambda gt10 using poly(A)+ RNA isolated from cell fractions enriched for the interstitial cell lineage (Kurz et al., 1991). These cells are smaller than epithelial cells and can be enriched by low-speed sedimentation from suspensions of dissociated *Hydra* cells (Greber et al., 1992). The cDNA library was differentially screened using radioactively labeled cDNA probes prepared from poly(A)+ RNA isolated from temperature-shocked sf-1 (epithelial cell probe) or from an interstitial cell-enriched fraction (interstitial cell probe) as previously described (Kurz et al., 1991). Approximately 13% of clones were interstitial cell-specific; 1% of these clones (6 of 590 isolated clones) represent the spinalin sequence described here.

**Molecular techniques**

Commonly used recombinant techniques such as gel electrophoresis, subcloning, growth of plasmids and bacteriophages, nucleic acid isolation, and DNA and RNA blot analysis were carried out according to standard protocols (Sambrook et al., 1989). DNA sequencing was done with the dideoxy chain termination method (Sanger et al., 1977) to standard protocols suggested by the manufacturer, using 100 μg of the antigen per injection.

For western blot analysis nematocyst proteins or total *Hydra* proteins were dissolved in SDS-sample buffer, separated by Tricine SDS-PAGE (Schägger and von Jagow, 1987), transferred to nitrocellulose membranes and incubated with spinalin19K antibody (1/1000 dilution) and a secondary goat anti-rabbit antibody coupled to horseradish peroxidase (Bio-Rad, Hercules, CA 94547). The filters were developed employing the ECL chemoluminescence system (Amersham, Arlington Heights, IL 60005).

**Cell type separation**

Intact *Hydra* were enzymatically dissociated into single cells and various cell types were separated into size fractions using the counterflow centrifugation elutriation with a Beckman J6 centrifuge and the Beckman JE 5.0 elutriator (Greber et al., 1992). The following size fractions were collected: 4000, 3000, 2500, 2200, 1800, 1300 and 1100 rpm at 15 ml flow rate (fractions 1-7) and 1100 rpm at 30 ml flow rate (fraction 8). Cells were eluted with 150 ml in each fraction, and poly(A)+ RNA was isolated using a poly(A)+ isolation kit (Qiagen, 5911 KJ Venlo, Netherlands).

**Construction of spinalin expression vectors**

Spinalin cDNA cloned in a pUC19 vector (Stratagene, La Jolla, CA 92037) was used as a template for PCR amplification of spinalin fragments. Different fragments were cloned into the expression vectors pET-15b (Novagen, Madison, WI 53711) and pGEX (Pharmacia, 751 82 Uppsala, Sweden), but only the spinalin19K fragment was purified and further characterized (see Results). Plasmid stability tests were carried out according to the manufacturer’s suggestions (pET system manual, Novagen, Madison, WI 53711).

To prepare spinalin19K, a DNA sequence coding for residues 18-207 was generated by the use of a 5’ primer introducing a *NdeI* site (5’ end: CCC CAT ATG AGG CCA TGG GGA TCC and a 3’ primer introducing a stop codon and a *BamHI* site (3’ end: CCC GGA TCC TTA ATA AAC ATG TAG ACC ACC AAC TC). The amplified product was cloned into the *NdeI/BamHI* site of the pET-15b expression vector, which contains an N-terminal histidine tag followed by a thrombin recognition site (Novagen, Madison, WI 53711).

**Recombinant expression and purification**

The pET-15b vector encoding spinalin19K was transformed into *E. coli* BL21(DE3) cells (Novagen, Madison, WI 53711) and expression of spinalin19K was induced by IPTG. Cells were lysed by sonication and the debris removed by centrifugation (13,000 g for 20 minutes). To purify histidine-tagged spinalin19K crude extract was chromatographed on a nickel sepharose column under denaturing conditions according to the manufacturer’s instructions (Novagen, Madison, WI 53711). Protein samples used for antibody generation were dialyzed against TBS buffer (20 mM Tris, 150 mM NaCl, pH 7.4), containing 2 M urea. Ultrafiltration was performed with Ultrafree-15 centrifugal filters (Millipore, Bedford, MA 01730-2271) having a nominal molecular mass limit of 30 kDa. Spinalin19K was recovered in the flowthrough, as judged by SDS-PAGE.

To remove the histidine tag, thrombin digestion was carried out in cleavage buffer (20 mM Tris-HCl, pH 8.4, 150 mM NaCl, 2.5 mM CaCl₂, containing 2 M urea) at room temperature for 4 hours using 5 i.u. thrombin per mg protein. Cleavage was monitored by SDS-PAGE as described by Laemmli (1970) using 12x13 cm slab gels. Protein bands were visualized by Coomassie Blue staining and molecular masses were estimated by comparing bands with molecular mass standards (Pharmacia, range 94-14.4 kDa).

**Antibodies and immunoblotting**

A polyclonal antibody directed against spinalin19K was generated in rabbits by Eurogentec Bel S.A. (4102 Ougrée, Belgium). Purified spinalin19K, dissolved in 20 mM Tris-HCl, pH 7.4, containing 2 M urea, was directly injected into rabbits. Immunization was carried out according to the manufacturer's suggestions (pET system manual, Novagen, Madison, WI 53711). The sequence data are available from EMBL/GenBank/DDBJ under accession number AF043907.

**Whole mount preparations**

Animals were relaxed in 2% urethane in M solution for 1 minute and fixed in Ladvovsky’s fixative for at least 24 hours (Techman and Holstein, 1995). After washing extensively CCA TGG GGA CCT GGA T¢ and a 3’ "¢ end: CCC GGA TCC TTA ATA AAC ATG TAG ACC ACC AAC TC. The amplified product was cloned into the *NdeI/BamHI* site of the pET-15b expression vector, which contains an N-terminal histidine tag followed by a thrombin recognition site (Novagen, Madison, WI 53711).
Preparations of isolated capsules
Nematocysts were isolated as described (Kurz et al., 1991; Weber, 1989). Nematocyst suspensions were induced to discharge on gelatin-coated slides by using 0.1 N NaOH (30 minutes), then treated for 1 hour with 65 μM DTT (in 100 mM Tris-HCl, pH 9.5) in order to partly solubilize the spines, and were finally fixed in 4% formaldehyde. After washing extensively in PBS (pH 7.2), slides were incubated overnight in spinalin19K antibody (1:1000 in PBS/1% BSA/0.1% sodium azide), washed and incubated with FITC-conjugated goat anti-rabbit antibody (Boehringer; diluted 1:50 in PBS/1% BSA/0.1% sodium azide).

Microscopy and photography
All preparations were analyzed using a Zeiss Axiovert microscope equipped with epifluorescence, and photography was performed with Kodak Tungsten 320 (1200 ASA). Confocal laser scanning microscopy was performed using a Leica TCS NT confocal microscope.

Electron microscopy
Transmission electron microscopy (TEM) analysis was performed as previously described (Holstein, 1981). For fixation the specimens were chilled to 4°C for 1 hour, then double-fixed with 3.5% glutaraldehyde (2-3 hours) and with 1% osmium tetroxide (1-2 hours); the fixatives were buffered with 0.05 M collidine buffer (pH 7.2). Specimens were embedded in epon-araldite with propylene oxide as infiltration solvent; serial sections (Reichert Ultracut microtome) were stained with uranyl acetate and lead acetate, and examined with a Zeiss EM 9S electron microscope.

Scanning electron microscopy (SEM) was performed as previously described (Tardent and Holstein, 1982). Whole Hydra or isolated nematocysts were fixed in 2.5% glutaraldehyde solution containing 50 μM phosphate buffer, which was also used for the post-fixation in 1-2% OsO₂. Samples were dehydrated through a graded series of acetone followed by critical-point drying. Specimens were sputtered with gold and analyzed with a Cambridge S-4 scanning electron microscope (this analysis was done in the laboratory of Prof. Pierre Tardent, Zoological Institute, University of Zürich).

RESULTS
Isolation of the nematocyte specific cDNA for spinalin
We identified nematocyte specific cDNAs by in situ hybridization of individually picked clones. The clones were derived from a cDNA library prepared from cells enriched for the interstitial cell lineage. Interstitial cell-specific clones were identified by differentially screening the library with interstitial cell and epithelial cell probes. From a set of 590 interstitial cell- and epithelial cell-specific clones, we identified six clones that cross-hybridized with each other and which hybridized in situ to clusters of differentiating nematocytes in the ectoderm throughout the body. In northern blots these clones hybridized to a 1000 bp transcript that was expressed in differentiating nematocytes but not in other cell types (Fig. 1). We have named the protein encoded by these cDNA clones spinalin, based on its localization in the spines of nematocytes (see below).

Sequence analysis and putative domain organization of spinalin
Fig. 2A shows the nucleotide and deduced amino acid sequence of spinalin. There is a single open reading frame of 254 amino acids beginning at nucleotide 126. The start codon ATG is immediately followed by a G residue and preceded at position -3 by an A, which is in agreement with the consensus sequence for eukaryotic translation initiation sites (Kozak, 1987). No homology to other proteins in the database (SWISS-PROT; Bairoch and Apweiler, 1997) was found by amino acid sequence comparisons using the entire spinalin sequence. Based on features of the primary sequence and similarities of parts of the spinalin sequence to known protein motifs we propose the domain organization shown in Fig. 2B.

The first 17 amino acids fulfill the criteria for a signal peptide (Von Heijne, 1986), indicating that spinalin is synthesized on the endoplasmic reticulum and processed through the Golgi. The presence of the leader sequence is consistent with spinalin being a component of nematocysts, which are the major Golgi products of differentiating nematocytes (Slautterback and Fawcett, 1959; Holstein, 1981). Following removal of the signal peptide, the mature protein consists of 237 amino acids

Table 1. Homologies of parts of the spinalin sequence to protein sequences of the SWISS-PROT database*

<table>
<thead>
<tr>
<th>Spinalin region†</th>
<th>Homology to</th>
<th>% identity</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Mouse type II keratin</td>
<td>36</td>
<td>Steinert et al. (1985)</td>
</tr>
<tr>
<td>I</td>
<td>Human cytokeratin 9</td>
<td>36</td>
<td>Langbein et al. (1993)</td>
</tr>
<tr>
<td>I</td>
<td>Mouse loricin</td>
<td>29</td>
<td>Mehrel et al. (1990)</td>
</tr>
<tr>
<td>II</td>
<td>Chick claw keratin</td>
<td>70</td>
<td>Whitbread et al. (1991)</td>
</tr>
<tr>
<td>II</td>
<td>Chick tropoelastin</td>
<td>48</td>
<td>Bressan et al. (1987)</td>
</tr>
</tbody>
</table>

*The FASTA program (Pearson and Lipman, 1988) was used for computer homology searches (Wisconsin Package, Version 8.1, Genetics Computer Group) in the SWISS-PROT database (Bairoch and Apweiler, 1997).
†Annotation according to Fig. 2B.
and has a calculated molecular mass of 23.7 kDa. It can be divided into four distinct regions. The first part of the sequence (region I, 154 residues) is glycine- and histidine-rich (31 and 21 mol%, respectively). For this region, sequence homologies to mouse loricin and the C-terminal regions of different cytokeratins were found (Table 1).

Region I is followed by a sequence of 35 amino acids (region II) in which, with only few exceptions, two of three amino acids are glycines. This sequence could adopt a polyglycine type II (PGII) conformation with three residues (a, b, c) per turn, where large, hydrophobic residues are located in a position of Gly residues in b and c positions (Ramachandran et al., 1966; Traub and Yokoth, 1966; Lotz and Keith, 1971). This region reveals sequence homology to the glycine-rich regions of chick claw keratin and to chick elastin (Table 1).

Region II is succeeded by a stretch of 16 amino acids (region III) with a high content of lysine residues and with weak homology to histone sequences (not shown). The last 32 residues (region IV) represent a region with a high content of negatively charged residues designated as the acid tail.

**Recombinant expression of spinalin fragments**

Full-length spinalin could not be overexpressed in *E. coli*. Although different expression vectors and different conditions were tried, plasmid instability was observed in all cases (see Materials and Methods), which is an indication that the spinalin gene product is toxic for the host cells. Since our aim was to prepare antibodies against spinalin, we also tried to express fragments of the spinalin sequence. Fragments that included the lysine-rich region (region III) could not be expressed in *E. coli*. However, a large fragment comprising regions I and II, designated spinalin19K, could be successfully overexpressed with a histidine tag for affinity purification. The protein was localized in inclusion bodies and could be purified under denaturing conditions using nickel sepharose affinity chromatography (Fig. 3, lane 1). The histidine-tagged spinalin19K was soluble in TBS buffer containing 2 M urea. It was further purified by ultrafiltration and directly used for antibody production (Fig. 3, lane 2). The histidine tag was cleaved by thrombin to prepare spinalin19K (Fig. 3, lane 3). Purified samples used for antibody generation were further purified by ultrafiltration (lane 2). The histidine tag was removed by thrombin digestion to prepare spinalin19K (lane 3).

**Spinalin is present in mature nematocysts**

In western blots of total *Hydra* protein the spinalin19K antibody reacted specifically with a protein of apparent molecular mass 24 kDa, which is the expected size of the mature spinalin polypeptide (Fig. 4A, lane 1). Purified...
staining at this stage was still confined to the matrix. The lumen
sequestered within the capsule (Holstein, 1981). Spinalin
stained.

during the next stages of capsule maturation spinalin staining
disappeared progressively from the matrix, became
concentrated along the inverted tubule, and then appeared to
pass through the tubule wall to fill the lumen of the tubule. This
process was not well visualized in the smaller nematocyst types
but could be clearly seen in developing stenoteles because of
their relatively large size. Fig. 5D shows a stenotele at this stage
of development. The stylets (large spines) have developed
within the lumen of the inverted tubule and can be seen to form
a prominent structure in the center of the capsule. Optical
sections of immunostained preparations, taken with a confocal
microscope, demonstrated that the stylets are strongly stained
by spinalin antibody and that staining has disappeared from the
matrix of the capsule at this stage (Fig. 6). This suggests that
spinalin is ‘transferred’ from the matrix of the capsule to the
lumen of the inverted tubule where stylets develop. Shortly
thereafter spinalin immunostaining disappeared completely.
Spinalin protein was, however, still present, as shown by
western blotting of mature nematocysts (Fig. 4A).

TEM images of sections through the center of stenoteles
reveal the structure of the stylets in more detail. Stylets appear
to be formed as a series of laminar structures projecting into
the lumen from the wall of the inverted tubule (Fig. 7A). These
lamina are 20-25 nm thick and exhibit a distinct substructure
with an outer electron-dense layer (4-5 nm thick) enclosing an
inner textured core (15-20 nm thick). Transverse sections
through the developing stylet apparatus indicate that the stylets
and spines are formed in three sectors spaced around the inner
circumference of the inverted tubule and that they consist of a
clearly demarcated series of lamina (Fig. 7C). Continued
development, however, leads to a dramatic condensation of the
lamina to form the mature stylet structure shown in cross
section in Fig. 7D and in the DIC image in Fig. 7B. This
extreme condensation is consistent with the observation that
mature stylets could be pelleted from suspensions of DTT-
dissociated capsules (see above).
Operculum formation

The operculum of nematocysts is formed after inversion of the tubule by a process which appears to be very similar to spine formation (Holstein, 1981). In particular, TEM images indicate the presence of laminar structures in the developing operculum similar to those observed in developing spines (Fig 7A,C,D). At later stages, these structures are no longer visible, probably due to condensation similar to that observed during spine formation. Fig. 6 shows a reconstruction of serial optical sections through two stenoteles at the stage of operculum formation. A ring of spinalin staining is present at the site of operculum formation (Fig. 6B), suggesting that spinalin was transferred from the capsule matrix through the wall of the inverted tubule to the site of operculum formation at the apical end of the capsule. This process would initially form a ring, which would fill up to become a cap as a result of further extrusion of spinalin. The cap is brightly stained at this stage but loses immunoreactivity as the capsules mature.

Localization of spinalin in stylets/spines of discharged nematocysts

Stylets and spines are most clearly visualized in SEM images of discharged nematocysts. Fig. 8 shows such images for desmonemes, stenoteles and isorhizas. The spines of desmonemes are long thin structures which become oriented down the central axis of the tight coil formed by the tubule following discharge of the nematocyst (Fig. 8A,B). Stenoteles have three large stylets and a series of smaller spines or lamellae located at the base of the everted tubule (Fig. 8C); the tubule appears to be free of spines (not shown). By comparison, in atrichous isorhizas the entire length of the tubule is covered with the small barbs or spines (Fig. 8D).

Western blotting (Fig. 4A) indicated the presence of spinalin in mature nematocysts despite the lack of spinalin immunoreactivity. This suggests that spinalin epitopes are
masked during formation of spines. To test this idea we searched for a method to restore immunoreactivity to mature capsules and thus directly demonstrate the presence of spinalin in spines. We used discharged nematocysts for these experiments because they permit good visualization of spines. To reduce the extent of condensation of spinalin-containing structures we treated capsules with 0.1 M NaOH and 65 μM DTT (see Materials and Methods). Both treatments were insufficient to dissolve capsules but they did restore spinalin immunoreactivity. The stylets and spines of stenoteles were clearly stained; the capsule and tubule were not stained (Fig. 9A,A'). The tightly coiled tubule of discharged desmonemes was strongly stained by spinalin antibody; the capsule wall was unstained (Fig. 9B,B'). In isorhizas the entire length of the everted tubule was stained whereas the capsule wall was unstained (Fig. 9B,B'). Although the barbs were difficult to resolve by light microscopy, the pattern of immunoreactivity along the tubule of the discharged isorhiza suggests that only the barbs were stained.

DISCUSSION

Structure of nematocysts
The cnidarian nematocyst is one of the most complex secretory products produced by an animal cell (Holstein, 1981; Hessinger and Lenhoff, 1988). Despite a wide diversity of morphological types, the basic structure of all nematocysts is the same: an inner and outer capsule wall, an inverted tubule armed with spines, and an operculum or opercular flaps.
the nematocyst discharges, the inverted tubule everts and the spines are exposed on the outer surface.

SDS-PAGE analysis indicates that Hydra nematocysts consist of a complex mixture of proteins varying from 12 to 200 kDa in size (Kurz et al., 1991). Most of the proteins larger than 40 kDa can be removed from nematocysts by treatment with Pronase or dilute DTT. Capsules remain intact and are still refractile in phase contrast under these conditions, although the outer wall is removed by these treatments (Kurz et al., 1991). Atomic force microscopy has demonstrated that the inner wall consists of bundles of fibrils (Holstein et al., 1994) and it has been proposed that these fibrils are polymers of small 12-15 kDa collagen-like proteins (mini-collagens), which are major components of the capsule wall (Kurz et al., 1991; see also Blanquet and Lenhoff, 1966). Mini-collagens are encoded by a family of nematocyte-specific genes which were first identified in Hydra but have more recently also been found in a reef-building coral, another member of the Cnidaria (Wang et al., 1995).

The present experiments have identified a novel 24 kDa protein, spinalin, as an additional component of nematocysts and have shown that it is a constituent of stylets, spines and opercula. The open reading frame includes a signal peptide at the N terminus, indicating that spinalin is synthesized on the endoplasmic reticulum, processed through the Golgi and sorted to the developing nematocyst capsule. Immunocytochemistry with a polyclonal antibody prepared against a 19 kDa fragment of spinalin confirmed these conclusions. The antibody recognized a single protein of 24 kDa in extracts of whole Hydra and in extracts of purified nematocysts. When purified capsules were dissociated with DTT and separated by centrifugation into soluble and insoluble fractions, spinalin was only found in the insoluble fraction, which contained stylets.

Fig. 8. SEM images of spines in discharged nematocysts. (A,B) Discharged desmonemes. Spines are long thin structures protruding from the center of the coiled tubule. The two desmonemes shown in (A) are wrapped around a bristle. (C) Discharged stenotele showing the apical end of the capsule and the base of the everted tubule. Three stylets project from the base of the tubule; distal to the stylets are lamellae or small spines. (D) Discharged holotrichous isorhiza. Small barbs or spines decorate the surface of the everted tubule; the capsule is located toward the bottom of the micrograph. Bars, 2 µm (A); 1.5 µm (B); 2 µm (C); 0.5 µm (D).

Fig. 9. Immunolocalization of spinalin in discharged nematocysts. Isolated nematocysts were treated with 0.1 N NaOH and 65 µM DTT to induce nematocyst discharge and partial denaturation of the spines. Samples were dried on slides and immunostained with spinalin antibody. Phase contrast micrographs (A,B) and immunofluorescence micrographs (A’,B’) show staining of the spines in a discharged stenotele (A,A’) and in two desmonemes and a holotrichous isorhiza (B,B’). No staining was observed with undischarged, isolated capsules (data not shown). Bar, 20 µm.
Spinalin, a new *Hydra* nematocyst protein

Structure and function of spinalin

Spinalin shows no overall similarity to other proteins in the databases. Nevertheless, parts of the sequence have features characteristic of particular secondary structures. The N-terminal half of spinalin shows sequence similarity to loricins (Mehrel et al., 1990; Steinert et al., 1991) and to the glycine-rich C-terminal parts of cytokeratins (Steinert et al., 1985, 1991). Loricins are involved in shaping the cornified cell envelope of epidermal keratinocytes; the terminal parts of cytokeratins are important for bundling keratin filaments by non-covalent supramolecular interactions and by disulfide cross-linking (Steinert et al., 1991). Spinalin, loricins and the bundling regions of cytokeratins share a high glycine content and other sequence similarities. For loricins and the terminal parts of cytokeratins ‘glycine loop’ structures were postulated (Steinert et al., 1991). ‘Glycine loops’ are defined by the form X(Y)n, where X is an aromatic or long-chain aliphatic residue and Y is usually glycine, although polar residues like serine, asparagine, arginine and cysteine may also occur. The value of n can range from 1 to 35 and ‘glycine loop’ regions are thought to be formed when several such X(Y)n quasi repeats are tandemly arranged (Steinert et al., 1991). Region I of spinalin fulfills these defining characteristics for ‘glycine loop’ domains, although the high content of histidine in this region of spinalin is unusual. Very recently it has been proposed that related histidine- and glycine-rich regions may be involved in Zn2+ and pH-dependent supramolecular self-association (Coyne et al., 1997).

Region II has an even higher glycine content (54%) and contains a large number of Gly-Gly-X triplets, where X stands for long chain aliphatic or aromatic residues. Such sequences can adopt a polyglycine II conformation, which is a left-handed helix with three residues per turn (Rich and Crick, 1961; Lotz and Keith, 1971; Navarro et al., 1995). Such Gly-Gly-X triplets occur in avian keratins, which are highly abundant in claws and scales (Whitbread et al., 1991). Both ‘glycine loop’ domains and polyglycine II helices have been proposed to associate by hydrogen bond formation and to play a role in organizing higher-order structures (Steinert et al., 1991; Traub and Yonath, 1966). In the case of spinalin, the polyglycine-like structure of regions I and II might be also stabilized by disulfide formation between the eight cysteines in these regions.

The 19 kDa fragment of spinalin comprising regions I and II was found to be insoluble under physiological condition. This behavior is similar to observations made on loricins and cytokeratins and is consistent with the high association potential predicted from the spinalin19K sequence. At 1-2 M urea spinalin19K becomes soluble, although circular dichroism measurements (A. W. Koch, unpublished data) suggest that it is still partly folded under these conditions. Association of such partially folded structures could be the mechanism by which spinalin molecules form higher order structures. More conformational data, however, are needed to unravel the details of spinalin association.

Electron micrographs (Fig. 7) suggest that spines are very densely packed structures. The observation that spinalin immunoreactivity disappears during spine formation also supports this conclusion. Tight packing and network formation are probably important for the function of spines. Stylets, the large spines in stenoteles, are needed to puncture the cuticle of prey organisms when capsules discharge (Tardent and Holstein, 1982), while spines in desmonemes and isorhizas appear to function as barbs binding the discharged nematocyst to prey or to the substrate. These results, together with observations on the structure and function of loricins, cytokeratins and avian keratins (see above), suggest that polyglycine-like structures may be particularly well adapted to formation of organelles having a high mechanical strength.

Morphogenesis of nematocyst capsules and formation of styllets

Immunocytochemical staining of *Hydra* polyps demonstrated that spinalin first appears in developing nematocysts in the body column at an intermediate stage of differentiation. Spinalin is homogeneously distributed in the matrix at this stage and also extends into the tubule when it forms on the apical end of the capsule (Fig. 5B,C). Following inversion of the tubule into the capsule, spinalin becomes concentrated in the lumen of the inverted tubule, which corresponds topologically to the external surface of the everted tubule. Spines develop on this external surface (Holstein, 1981). At the apical end of the capsule spinalin also appears to be transferred from the matrix to the outside of the tubule in order to form the operculum.

Upon further maturation, spinalin staining ceases in both the operculum and the spines, although western blot analysis demonstrated that spinalin continues to be present in these structures (Fig. 4A). We interpret this loss of immunoreactivity in the mature operculum and spines as an indication of extreme condensation of spinalin in these structures. This suggestion is supported by the observation that spinalin immunoreactivity could be restored by mildly denaturing conditions (Fig. 9).

The mechanism by which spinalin is transferred through the tubule wall is not known. However, the wall at this stage is still ‘soft’, since it can be deformed during the process of fixation. At a slightly later stage the wall becomes rigid and is no longer deformed by fixation. At this stage the capsule wall also becomes impermeable to polypeptides since poly-$\gamma$-glutamate polymers are retained inside capsules (Weber, 1990). Even at this stage, however, the wall can and does stretch with the increase in osmotic pressure which results from the synthesis of poly-$\gamma$-glutamate in the matrix (Weber, 1990, T. W. Holstein and C. N. David, unpublished results).

It is interesting to note that a similar pattern of staining has been observed for the H22 antigen during capsule formation. The monoclonal antibody H22 recognizes a 100 kDa protein in the outer capsule wall (Kurz et al., 1991). Like spinalin, the H22 antigen first appears in the matrix and then moves through the inner capsule wall to form the outer wall (T. W. Holstein, unpublished results). Some of the H22 antigen also appears to move through the wall of the tubule and to become localized in spines.

The relationship between spinalin structure and spine morphogenesis requires further investigation. Although the spines of stenoteles, desmonemes and isorhizas are quite different in shape (Fig. 8), they all contain spinalin (Fig. 9). Whether other proteins are also present in spines and contribute to formation of these different shapes is not clear from the
present experiments. However, an attractive alternative hypothesis is that differences in the folding of the inverted tubule in different nematocyst types affect the transfer of spinalin across the tubule wall and hence the morphology of spines. Which molecular features of spinalin are important for spine morphogenesis is also unclear. At present we can only speculate that the highly charged regions III and IV might be involved in solubilization of the protein when transported to or located in the matrix while the glycine- and histidine-rich region I might be involved in pH- or Zn$^{2+}$-dependent assembly.

REFERENCES


