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Micron 37 (2006) 107–120

micron

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Review

Siliceous spicules in marine demosponges (example *Suberites domuncula*)

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Received 2 August 2005; revised 4 September 2005; accepted 5 September 2005

Abstract

All metazoan animals comprise a body plan of different complexity. Since—especially based on molecular and cell biological data—it is well established that all metazoan phyla, including the Porifera (sponges), evolved from a common ancestor the search for common, basic principles of pattern formation (body plan) in all phyla began. Common to all metazoan body plans is the formation of at least one axis that runs from the apical to the basal region; examples for this type of organization are the Porifera and the Cnidaria (diploblastic animals). It seems conceivable that the basis for the formation of the Bauplan in sponges is the construction of their skeleton by spicules. In Demospongiae (we use the model species *Suberites domuncula*) and Hexactinellida, the spicules consist of silica. The formation of the spicules as the building blocks of the skeleton, starts with the expression of an enzyme which was termed silicatein. Spicule growth begins intracellularly around an axial filament composed of silicatein. When the first layer of silica is made, the spicules are extruded from the cells and completed extracellularly to reach their the final form and size. While the first steps of spicule formation within the cells are becoming increasingly clear, it remains to be studied how the extracellularly present silicatein strings are formed. The understanding of especially this morphogenetic process will allow an insight into the construction of the amazingly diverse skeleton of the siliceous sponges; animals which evolved between two periods of glaciations, the Sturtian glaciation (710–680 MYA) and the Varanger-Marinoan ice ages (605–585 MYA). Sponges are—as living fossils—witnesses of evolutionary trends which remained unique in the metazoan kingdom.
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Keywords: Sponges; Porifera; Suberites domuncula; Spicules; Biosilica; Silica formation; Silicatein; Evolution

Contents

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doi:10.1016/j.micron.2005.09.003

1. Introduction

Since Aristotle [384–322 BC] (cited in Camus, 1783) sponges have occupied a distinguished position among the animals because of their biomedical potential (see Arndt, 1937), their beauty and also their enigmatic evolutionary origin. Lord G. Campbell, (1876) a sub-lieutenant on board the 'Challenger' wrote that "sponges are…the most characteristic inhabitants of the great depths all over the world…some of which rival in beauty". Difficulties in their systematic positioning and their relationship to other multicellular organisms have resulted in their designation as 'Zoophytes' or 'Plant–animals', a taxon placed between plants and animals (Spix, 1811), until finally they were recognized as genuine metazoans, which evolved first from the animal ancestor, the urmetazoan (Müller, 2001). Based on intense molecular biological/cell biological studies it became overt that sponges are not 'simple blobs' but contain and express a variety of metazoan-like transcription factors and in turn form sophisticated tissue assemblies (commented by: Pilcher, 2005). The sponges have been grouped into siliceous sponges and calcareous sponges (Haeckel, 1872), and after the discovery/appreciation of the glass sponges (Schulze, 1887) divided into three classes: Demospongiae [mostly sponges with a skeleton, composed of siliceous spicules], Hexactinellida [always siliceous skeleton] and Calcarea [always calcareous skeleton] (see Hooper, 1997). Sponges were united to the phylum Porifera, based on the existence of characteristic and distinct pores on the surface of the animals (Grant, 1835; Lieberkühn, 1859).

Sponges were also termed 'living fossils' (Müller, 1998) since they represent the evolutionary oldest, still extant taxon which testifies the developmental level of animals living in the Neo-Proterozoic Eon (1000–520 million years ago [MYA]). This is important to note since two major 'snowball earth events' occurred during this period of time, the Sturtian glaciation (710–680 MYA) and the Varanger-Marinoan ice ages (605–585 MYA), which very likely resulted in the covering of the earth by a continuous ice layer and supposedly caused extinction of most organisms on earth at that time (Hoffman et al., 1998).

The primordial earth surface comprised initially insoluble silicates, carbonates, and also phosphates. During the cycle of silicate weathering and carbonate precipitation, prior or simultaneously with the glaciations, a dissolution of surface rocks composed of insoluble silicates [$CaSiO_3$] resulted in the formation of soluble calcium carbonate [$CaCO_3$] and soluble silica [SiO_2], under consumption of atmospheric CO_2 (Walker, 2003). These soluble minerals leached subsequently out to the oceans, rivers and lakes and there again led to a re-precipitation of the dissolved minerals to new compositions as part of the sedimentary rocks. Such processes are dependent upon temperature, pH and atmospheric carbon dioxide; passively, the minerals are transformed diagenetically to secondary structures. An example is given from the Proterozoic/Cambrian period (Fig. 1A), the Wilkawillina Limestone at Finders Ranges (Australia). There, through secondary re-precipitation of dissolved minerals, a passive diagenesis of Ca-carbonate into 50 μm large deposits occurred. The resulting spicule-like structures are seen in thin sections and are reminiscent of sterrasters found in sponges from the family of Geodiidae (siliceous sponges); microanalysis, however, revealed that these spicule-like structures do not at all contain silicon (Fig. 1A-c), but calcium (Fig. 1A-b).

In contrast to passive re-precipitation, biogenic deposition of minerals by metazoans is first seen in sponges. The oldest sponge fossils (Hexactinellida) have been described from Mongolia and were assessed to have lived coeval with the diverse Ediacara fauna of Namibia more than 540 MYA (Brasier et al., 1997). Hence, the Hexactinellida are the oldest group of sponges as documented there and later in fossil records of the Sansha section in Hunan (Early Cambrian; China; Steiner et al., 1993), where more or less completely preserved sponge fossils, e.g. *Solactiniella plumata* (Fig. 1B-a), have been found. This fossil is noteworthy since it shows, besides the unusual body preservation also very intact spicules; the approximately 40 mm large specimen comprises 0.5–5 mm long spicules with a diameter of 0.1 mm (Fig. 1B-b). Some of them are broken and present the open axial canals (Fig. 1B-c). The oil-shales of the Messel pit, near Darmstadt (Germany), are very rich in fossil freshwater sponges; among them is *Spongilla gutenbergiana* from the Middle Eocene (Lutetian), approximately 50 MYA (Fig. 1C) (Müller et al., 1982). Sometimes spicule assemblies are found (Fig. 1C-a), which are very reminiscent of complete animals; most spicules found in such nests are 160–230 μm long oxeas (Fig. 1C-b). Many

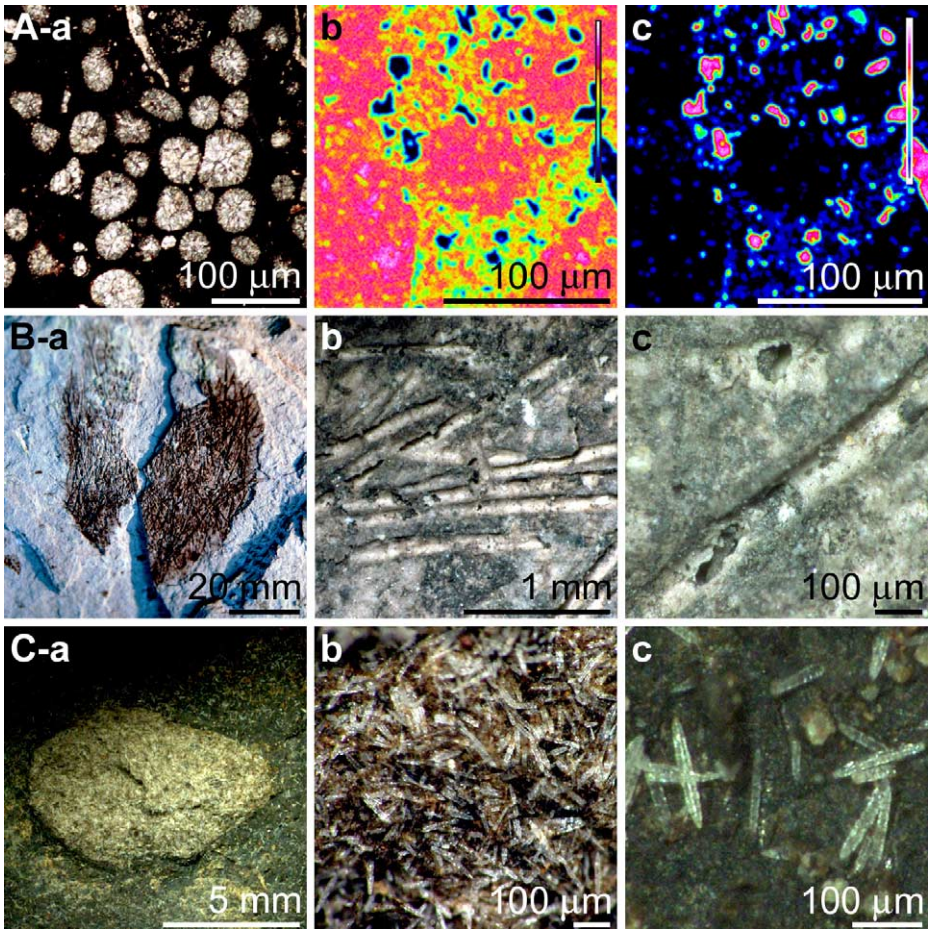

Fig. 1. Proterozoic/Cambrian secondary re-precipitation of dissolved minerals into new forms. (A) Passive diagenesis of Ca-carbonate into 50 µm large deposits. Spicule-like structures are seen in thin sections through the Wilkawillina Limestone at Finders Ranges (Australia); they are reminiscent of sterrasters found in sponges from the family of Geodiidae. (A-a) Polished section. (A-b) Analysis of the calcium level within the section; scanning was performed with a JXA-Joel 890—EPMA electron probe microanalyzer. The bright red areas correspond to the spicule-like structures. (A-c) Scanning for silicon showed that the corresponding areas to the high level of calcium in A-b are dark, thus indicating the absence this element. (B) Earliest sponges in body preservation are known from the Lowermost Cambrian Sansha section (Hunan, China); *Solactiniella plumata* (Hexactinellida) (B-a). This fossil is composed mainly of intact spicules (B-b). (B-c) Some spicules are broken and show the internal axial canals. (C) The fossil freshwater sponge *Spongilla gutenbergiana* from the Middle Eocene (Lutetian) near Messel (Darmstadt, Germany). (C-a) Spicule assembly, reminiscent of a complete animal. (C-b) Oxeas in these nests. (C-c) Oxeas with the flashing centers, representing the axial canals. Size bars are given.

of them show in the center a brightly lit axial canal (Fig. 1C-c). Calcarea are documented from the Flinders Ranges Archaeocyath mounds; isolated spicules as well as the more or less complete rigid skeletons of small calcarean sponges sitting on Archaeocyaths have been identified (Reitner, 1992).

Based on sequence data of informative genes, which code for structural and functional proteins, it had been calculated that the sponges diverged from the common metazoan ancestor approximately 650 MYA (Schäcke et al., 1994). This calculation is in close accordance with fossil records and implies that the sponges evolved between the two glaciations, Sturtian and Varanger-Marinoan. The existence of a large genetic repertoire in the Porifera, the basis for the establishment of complex metabolic and morphogenetic pathways, may have contributed to the rapid evolution of sponges occurred between the snowball periods 710–680 and 605–585 MYA. At present it cannot be ruled out that other animal phyla evolved simultaneously with the Porifera, but became extinct during the last ice age.

2. Historical aspect

First descriptions of sponge spicules were given by Donati (1753). He isolated them from a species belonging to the genus *Geodia* (*Alcyonium*) (Fig. 2A). Distinct cytological studies on the spicule formation were first published in 1856 (Lieberkühn, 1856) with the freshwater sponge *S. fluviatilis* (Demospongiae) as a model. This work gave even detailed analyses on the development and differentiation of fertilized eggs, together with the differentiation stages of somatic sponge cells from the 'Schwärmsporen' to the spicule-forming sclerocytes (Fig. 2B). Spicule formation starts intracellularly mainly within the 'Schwärmsporen' but to a smaller extent also in other cell types (Fig. 2C). It is interesting to note that spicule-forming

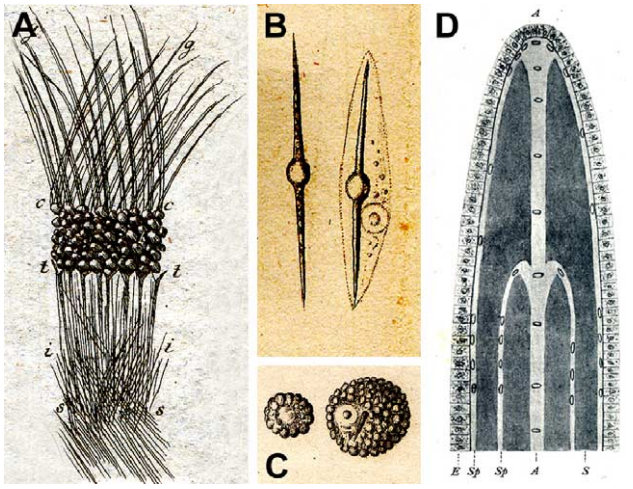

Fig. 2. Historical aspect of spicules. (A) First description of spicules from a species belonging to genus *Geodia* (Donati, 1753). Highlighted are the spheres (asters [between t and c]) and also the spines (protriaenes and oxeas [between c and g]). (B) First description of siliceous spicule formation in sponges, here of the freshwater sponge *S. fluviatilis* (Demospongiae), by Lieberkühn (1856). The spicule formation starts intracellularly within the 'Schwärmsporen'. (C) Also other cells have been suggested to form spicules; especially those which have a large nucleus and nucleolus (Lieberkühn, 1856). (D) Schematic outline of the spicule formation, based on studies with the hexactinellids *Hyalonema* and *Monorhaphis* (Schulze, 1904). The center of the spicules is formed by the axial filament (A), around which the first layer of siphons (silica) is formed. Besides this layer and the next one towards the surface of the spicules, organic layers, termed 'spiculin lamellae' (Sp), exist. The spicule-forming sclerocytes, which he named 'epithelium like cells' (E), surround the spicule.

cells have a large nucleus and nucleolus (Fig. 2C). A few years later, DeLage (1892) observed likewise precisely that the spicules are formed intracellularly. Very notably he mentioned that the spicule-containing cell 'est de même nature que les amoeboïdes'. This remark can also be taken as an indication that he had been aware of the new stem cells concept that originates from this time (Weismann, 1892).

A precise description of the structure and also the growth of spicules of the glass sponges *Hyalonema* and *Monorhaphis* was given by Schulze (1904). The spicules of *Monorhaphis* can reach lengths of 70 cm and may be up to 8 mm thick. Schulze (1904) outlined that the growth starts from axial organic filaments, which appears alveolar to hyaline. He proposed, based on his observations, that thickening of the spicules proceeds by fairly regular apposition of lamellar silica layers under formation of centric rings (siphons). Between the concentric lamellae he described the presence of organic layers (Fig. 2D). In a later phase the spicules are surrounded by sclerocytes, the spicule-forming cells.

The inorganic material which builds the spicules is silica; it had been postulated already by Bowerbank (1864) that in addition to silica also organic material exists. Detailed analyses of the siliceous spicules came from later studies (Sollas, 1888; Bütschli, 1901) which revealed that silicic acid contributes to more than 92% to the mass of a spicule. Sollas (1888) presented comprehensive results on the physical and chemical properties of the siliceous spicules. As an example, he reported that they have a refractive index of 1.5 and that colloidal silica contains about 10% of water and has the general formula of $(SiO_2)_{2-5} \cdot H_2O$.

3. Structural features of the sponge Bauplan

Sponges, as the oldest still extant metazoan phylum, are characterized by a simple Bauplan (reviewed in Müller et al., 2004; Müller, (2005) [these reviews give more detailed literature reference]). They are filter-feeding organisms that are sessile. Their body is composed of an epithelial layer which surrounds a mesogleal compartment, the mesohyl; this is reticulated in a highly organized manner by a canal system. The major structural and functional novelties, which evolved during the major transitions to the Porifera and Cnidaria are summarized in Fig. 3.

3.1. Molecules Involved in cell–cell interaction

Sponges have become a classical model for basic studies to understand metazoan cell–cell adhesion. It was Wilson (1907) who introduced this system to experimental biology which then became a traditional model to study both cell–cell- and cell–matrix adhesion. With the isolation of a galectin sequence as the first cell–cell adhesion molecule, and the integrin sequence as the first cell–matrix adhesion receptor from sponges—predominantly the model species *Suberites domuncula* and/or *Geodia cydonium* had been used—it became overt that sponges contain molecules highly related to those known to promote adhesion also in Protostomia and Deuterostomia.

3.2. Molecules involved in cell–substrate interaction

The dominant molecule present in the extracellular matrix of sponges which functions as a cell–matrix adhesion molecule is collagen. The corresponding gene was cloned from the freshwater sponge *Ephydatia muelleri* as well as from the demosponge *S. domuncula*. Cell surface-spanning receptors, in particular the single—as well as the seven—pass transmembrane receptor proteins, serve as receivers for extracellular signaling molecules. Also these molecules could be identified in sponges. The first, sponge 1-transmembrane receptor (1-TMR) which represents an autapomorphic character for Metazoa, was the receptor tyrosine kinase. As a 7-transmembrane receptor (7-TMR) the metabotropic glutamate/GABA-like receptor was characterized in *G. cydonium*; it was found to respond to extracellular agonists as well as antagonists known from metabotropic glutamate/GABA-like receptors present on nerve cells from mammals.

3.3. Molecules involved in morphogenesis

During development of animals, a set of genes, most of them transcription factors, that are responsible for cell fate and pattern determination, is expressed. Among them, T-box, Forkhead and Homeobox genes families have been found to be extremely conserved, in their DNA sequence and their function. Among the T-box family, *Brachyury* is involved

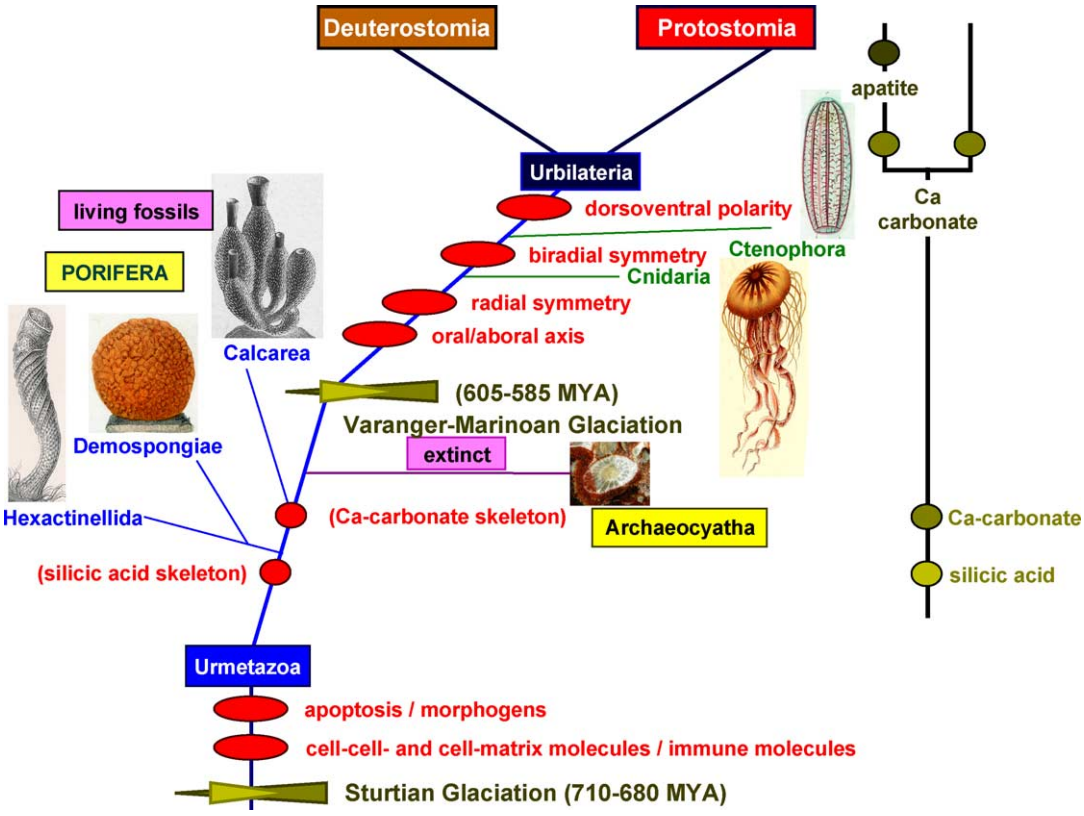

Fig. 3. Phylogenetic position of the Porifera between the Urmetazoa and the Urbilateria. The major evolutionary novelties which have to be attributed to the Urmetazoa are those molecules which mediate apoptosis and control morphogenesis, the immune molecules and primarily the cell adhesion molecules. The siliceous sponges with the two classes Hexactinellida and Demospongiae emerged first and finally the Calcarea, which possess a calcareous skeleton, appeared. These three classes of Porifera are living fossils that provide a reservoir for molecular biological studies. The Archaeocyatha, sponge related animals with a calcareous skeleton, became extinct. The Calcarea are very likely a sister group of the Cnidaria. From the latter phylum the Ctenophora evolved which comprise not only an oral/aboral polarity but also a biradial symmetry. Finally the Urbilateria emerged from which the Protostomia and the Deuterostomia originated. Very likely the Urmetazoa emerged between the two major 'snowball earth events', the Sturtian glaciation (710–680 MYA) and the Varanger-Marinoan ice ages (605–585 MYA). In the two poriferan classes Hexactinellida and Demospongiae the skeleton is composed of amorphous and hydrated silica, while the spicules of Calcarea are composed of Ca-carbonate. The latter biomineral is also prevalent in Protostomia and also in Deuterostomia. In vertebrates the bones are composed of Ca-phosphate [apatite].

in the formation and differentiation of the third germ layer, the mesoderm, in tripoblastic animals. Recently, two T-box genes have been isolated from the sponge *S. domuncula*; a *Brachyury* gene and a homologue of the *Tbx2-3-4-5* genes from chordates, which there are interestingly involved in the formation of the limbs. Functional studies demonstrated that the expression of the *Brachyury* gene is upregulated in differentiating sponge cells during formation of canal-like structures. Based on this observation we postulate that already in sponges a primordial axis formation is genetically fixed. This assumption was subsequently confirmed by the isolation and phylogenetic characterization of five members of the forkhead gene family from the sponge *S. domuncula.*

3.4. Transcription factors: homeodomain molecules

The developmental processes resulting in the formation of a body axis require a head center; e.g. in bilaterians, the Spemann's organizer. The genes which are involved in the establishment of the head organizer during embryogenesis have been grouped into three classes of homeobox genes, the Paired-class, the Antennapedia-class and the Lim-class genes. Also in this area a strong progress was made in sponges in the last few years. A paired-class (*Pax-2/5/8*)-gene had been isolated from the freshwater sponge *E. fluviatilis*, which encodes a complete although substantially degenerated homeodomain. In *S. domuncula* a cDNA encoding a LIM/homeobox protein has been isolated which comprises high sequence similarity to the related LIM homeodomain proteins in other animals.

3.5. Genes in S. domuncula indicative for Wnt signaling

The Wnt signaling pathway is a cell communication system which regulates cell-fate decisions, tissue polarity and morphogenesis. The Frizzled protein is the membrane receptor for the Wnt secreted glycoproteins. Through the canonical Wnt signaling pathway, the activated Frizzled binds to Dishevelled (Dsh), which leads to the stabilization and accumulation of ß-catenin in the nucleus, where it activates the TCF/LEF transcription factor. Very recently we isolated the gene encoding the Frizzled receptor from *S. domuncula*, suggesting that a Wnt-pathway involved in cell-fate determination and morphogenesis was already established in sponges.

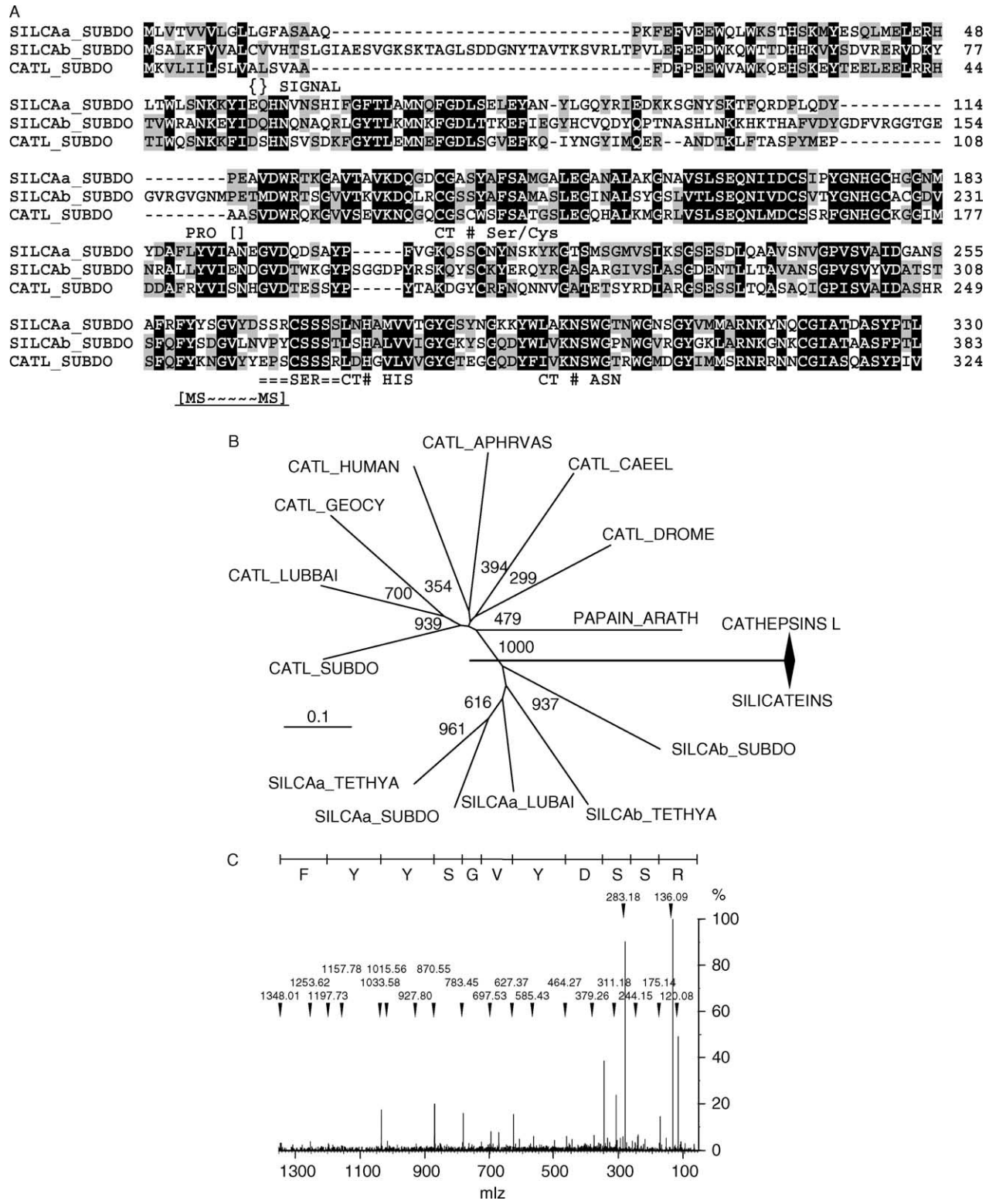

Fig. 4. Sponge silicateins and cathepsins. (A) The *S. domuncula* enzymes, silicatein-α (SILICAa_SUBDO; CAC03737.1) and the β-isoenzyme (SILICAb_SUBDO, AJ547635.1) as well as the cathepsin L sequence (CATL_SUBDO, AJ784224) were aligned. Residues conserved (similar or related with respect to their physico-chemical properties) in all sequences are shown in white on black and those in at least two sequences in black on gray. The characteristic sites in the sequences are marked: the catalytic triad (CT) amino acids, Ser (#) in silicateins and Cys in cathepsin, as well as His (#) and Asn (#), and the processing site for the conversion of the proenzyme to the mature enzyme ([] PRO). The serine cluster ([∼Ser∼]) and the cleavage site of the signal peptide are indicated ({ }). The region of the silicatein-α protein sequence, identified by ESI-MS, is marked (MS∼ ∼ ∼MS). (B) The radial phylogenetic tree was constructed after the alignment of these sequences together with the silicateins-α from *Tethya aurantia* [*T. aurantium*] (SILICAa_TETYA, AAC23951) and *Lubomirskia baicalensis* (SILICAa_LUBAI, AJ872183), silicatein-β from *T. aurantia* (SILICAb_TETYA, AF098670), as well as with the cathepsin L sequences from the sponges *Geodia cydonium* (CATL_GEOCY, Y10527), *L. baicalensis* (CATL_LUBAI, AJ96849) and *Aphrocallistes vastus* (CATL_APHRVAS, AJ968951) as well as from higher Metazoa:

3.6. Molecules present in tight junctions

Our screening for a gene encoding a tight junction scaffold protein from a sponge, here *S. domuncula*, was successful; the scaffold protein membrane-associated guanylate kinase with inverted arrangement (MAGI) had been identified. In addition, the existence of one tetraspan receptor, tetraspanin, in *S. domuncula* has been reported. The tetraspanins belong to a group of hydrophobic proteins, comprising four transmembrane domains with a series of conserved aa residues in the extracellular loops.

Besides these mentioned genomic regulatory systems, primarily those structural and enzymatic molecules which govern the skeletal formation especially in the phylum Porifera are crucial for the establishment of the body plan (Bauplan). Therefore, an understanding of the spicule formation and the association of spicules within the sponge body is crucial for an understanding of the sponge body plan. In the class of Demospongiae, spicule formation is a dynamic process and involves both anabolic and the catabolic enzymes.

4. Anabolic enzyme for the synthesis of silica: silicatein

In the last years, the silicate metabolism in sponges has been elucidated to some extent (reviewed in Weaver and Morse, 2003). The formation of the spicules is a rapid process; in the freshwater sponge *E. fluviatilis* the 100–300 μm long spicules are synthesized under optimal conditions within 40 h (Weissenfels, 1989). The rate of appearance of new spicules is apparently correlated with the growth of the oscular tube, implying that an efficient relocation system must be active to place spicules to their functional destination. In addition, the re- and de-organization processes in sponges, like the bud formation, require an efficient machinery to translocate or replace spicules.

The group of Morse (Shimizu et al., 1998; Cha et al., 1999) discovered that the organic filament in the central canal of the spicules is composed of a cathepsin L-related enzyme, which they termed silicatein. They cloned two of the proposed three isoforms of silicateins, the α- and β-form, from the demosponge *Tethya aurantium* (Cha et al., 1999). In subsequent years these molecules were cloned also from other sponges, among them *S. domuncula* (Krasko et al., 2000, 2002; Schröder et al., 2004a) (Fig. 4A). Phylogenetic analysis was performed with the silicateins from the demosponges, *Lubomirskia baicalensis*, *T. aurantium*, *Aphrocallistes vastus* and *S. domuncula* as well as with the cathepsins L from Metazoa and the papain cysteine peptidase from *Arabidopsis thaliana* (Fig. 4B). The radial tree shows that the cathepsin L sequences form a branch, which is separated from the silicatein sequences.

In a recent study it could be clarified that silicatein occurs in several isoforms in the axial filament. Even though several silicatein cDNAs have been isolated also from *S. domuncula* (Schröder et al., 2004b), a proof of different isoforms on protein level could not be given unequivocally. Two-dimensional gel electrophoresis of the axial filament (= silicatein) revealed that silicatein in the axial filament undergoes stepwise phosphorylation. Five phospho-isoforms with p*I* values of 5.5, 4.8, 4.6, 4.5 and of 4.3 have been identified (Müller et al., 2005). The sizes of these phosphorylated proteins are around 25 kDa, compatible with the predicted mature form of the silicatein. Interesting was the finding that under native conditions, in the absence of urea in the sample buffer, silicatein forms not only monomers but also dimers and trimers.

Surprising was also the finding that if a total extract of *S. domuncula* was subjected to two-dimensional gel electrophoresis the predominant proteins were the silicateins, again present in five isoforms (Fig. 5A). The identification of the five isoforms was performed by Western blotting using polyclonal antibodies against silicatein (Fig. 5B). This finding suggests that silicatein is not only present within the spicules, but should exist to a considerable extent outside of the spicules.

As an additional proof of the silicatein protein identified immunologically by Western blotting (Fig. 5B), electrospray ionization (ESI)-mass spectrometry (MS) was applied as described (Claverol et al., 2003). In brief, SDS (sodium dodecyl sulfate) electrophoresis was performed and the gels were stained with Coomassie brilliant blue in order to localize the spots. After digestion with trypsin, distinct spots were cut out and the dye, SDS and salts were removed from the protein samples. Proteins were then eluted and aliquots were injected into the mass spectrometer (Dionex; Amsterdam). Spray voltage was set at 1.8 kV and capillary temperature to 180 °C. By using this approach the 11 amino acids long peptide (F:Y:Y:S:G:V:Y:D:S:S:R; see in Fig. 4A), has been

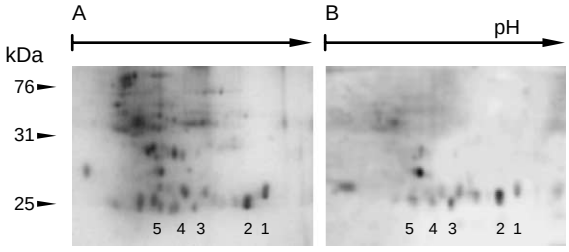

Fig. 5. Analysis of the proteins in total extract of *S. domuncula*. Extract was prepared in 10 mM Tris–HCl buffer (containing 2 mM EDTA, 100 mM NaCl; pH 8.2). An aliquot was analyzed by two-dimensional gel electrophoresis (first isoelectric focusing and then size separation) and stained with Coomassie brilliant blue (A). (B) In a parallel series the proteins were blotted and the membranes were incubated with polyclonal antibodies, raised against silicatein. The five major isoforms are marked.

human (CATL_HUMAN, X12451), *D. melanogaster* (CATL_DROME, S67481) and from *C. elegans* (CATL_CAEEL, NP_507199.1) and the related papain-like cysteine peptidase XBCP3 from *Arabidopsis thaliana* (PAPAIN_ARATH, AAK71314). The numbers at the nodes are an indication of the level of confidence for the branches as determined by bootstrap analysis [1000 bootstrap replicates]. (C) ESI-MS analysis of gel-eluted silicatein isoform; spot 2 in (A). The ordinate gives the relative abundance in percent; the abscissa the *m/z* units. Above the spectrum the identified 11 amino acids long stretch of the silicatein sequence is given.

identified (Fig. 4C). This region in the silicatein protein is especially interesting, since there the serine-rich region starts.

5. Catabolic enzyme: silicase

In the course to further elucidate the metabolism of siliceous spicules in Demospongiae another enzyme, silicase, was identified from the marine sponge *S. domuncula*; silicase is able to depolymerize amorphous silica. The cDNA was isolated and the deduced polypeptide identified as an enzyme similar to carbonic anhydrases. Recombinant silicase displays besides a carbonic anhydrase activity the ability to dissolve amorphous silica under formation of free silicic acid (Schröder et al., 2003).

6. Morphology of spicules in Suberites domuncula

The marine demosponge *S. domuncula* was collected in the Northern Adriatic near Rovinj (Croatia). From this species tissue samples, primmorphs and spicules were obtained (summarized in Müller et al., 2005). Primmorphs, a special type of 3D-cell aggregates, containing proliferating and differentiating cells, allow during incubation in medium supplemented with silicic acid to study the differentiation of archaeocytes to sclerocytes (see Müller et al., 2004). Spicules and their filaments were prepared from tissue samples that were treated first with sulfuric acid/nitric acid and then with n-butanol/water/SDS. For immunofluorescence studies the spicules were obtained from the tissue by treatment with EDTA (ethylenediaminetetraacetic acid). The axial filaments were isolated from the spicules by dissolution of the silica material with hydrofluoric acid. The axial filaments were collected, dialyzed against distilled water and collected by centrifugation. The in vitro primmorph cell system was used (Adell et al., 2003) 5 days after formation of these 3D-cell aggregates. The electron microscopical procedures, e.g. scanning electron microscopy (SEM) analysis of spicules, transmission microscopical analysis (TEM) and electron immunogold labeling was performed as described (Müller et al., 2005).

The skeleton of *S. domuncula* is composed of only two types of megascleres, monactinal tylostyles and a smaller fraction of diactinal oxeas. No detailed studies on microscleres are available. The spicules reach up to 450 μm in length and diameters of 5–7 μm (Fig. 6A–C); they grow through apposition of lamellar silica layers. While the two ends of the oxeas are pointed, the tylostyles have one pointed end and one swollen knob. All spicules have an axial canal of 0.3–1.6 μm in width in the center. As shown in SEM images (Fig. 6A–C) the central canal is surrounded by lamellated layers approximately 0.3–1 μm thick. To monitor the location and distribution of silicatein in and around the spicules, polyclonal antibodies (PoAb) were raised against purified filaments from spicules in rabbits (Müller et al., 2005). Immunofluorescence studies with cryosections through tissue revealed that the surfaces of the spicules in the tissue (Fig. 6D) and of isolated spicules (Fig. 6E and F) are surrounded by silicatein.

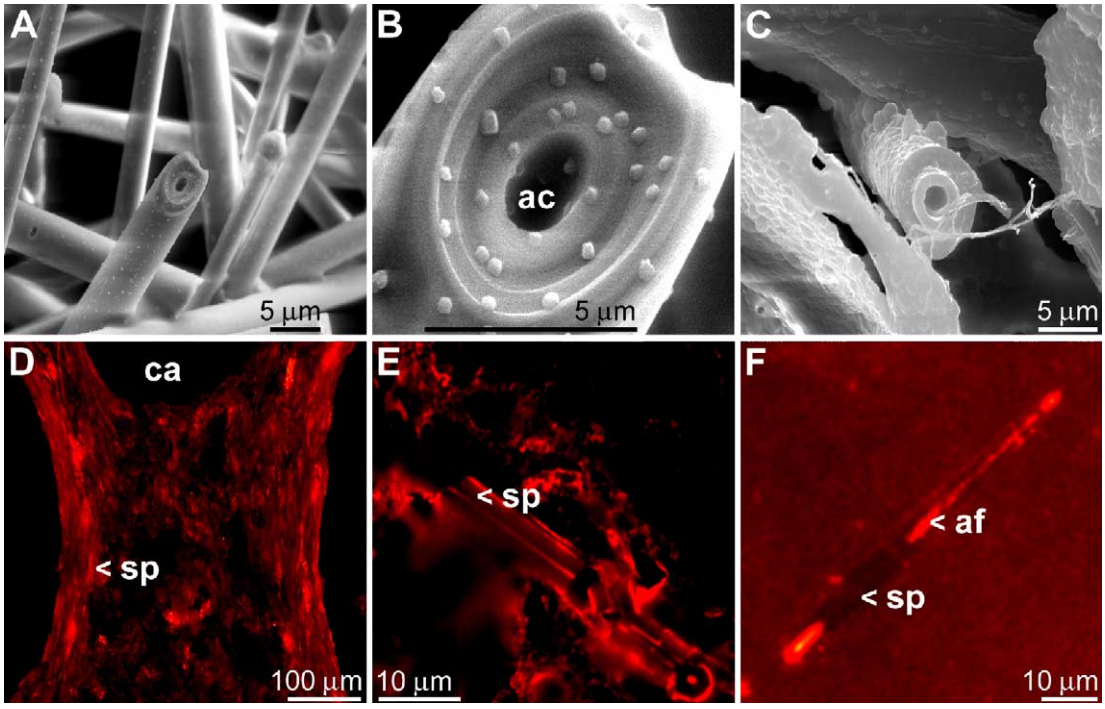

Fig. 6. Spicules of *S. domuncula*. (A–C) SEM analysis. The cross sections show the circular axial canal (ac) and the lamellated siliceous deposits which surround the canal. Immunofluorescence analysis: (D) Immunostaining of cryosections through tissue of *S. domuncula*. 8-μm thick frozen sections were reacted with polyclonal silicatein antibodies. Spicules (sp) and one canal of the aquiferous system (ca) are marked. (E and F) Reaction of antibodies against silicatein with partially digested (hydrofluoric acid) spicules. The preparations were placed onto glass slides and treated with the antibodies. (F) The immuno-stained specimen showed that both the surface of the spicules (sp) and also the axial filaments (af) were brightly stained.

From these studies we conclude that silicatein is not only present in the axial filament of the spicules, but is also located on their surface.

7. Development of spicules in primmorphs

In *S. domuncula*, the formation of spicules is a rapid process and surely proceeds more frequently in embryos or in primmorphs, than in adult specimens. Since all studies on the formation of spicules had hitherto been performed with sections through tissue samples from adult animals the conclusion was published that the formation of the megascleres proceeds extracellularly in the bulky mesohyl of the animals (Uriz et al., 2000; Weaver and Morse, 2003). We applied the primmorph system and could establish unequivocally that the initial steps of spicule formation occur intracellularly in the sclerocytes (Müller et al., 2005).

Sclerocytes in primmorphs produce after an cultivation period of 5 days readily spicules, provided the medium contains silicic acid. As shown in Fig. 7A, the 15 μm large sclerocyte produces one spicules of a length of 6 μm; the lengths of the spicules in those cells vary between 0.7 and 8 μm with diameters of up to 0.9 μm. After the spicules reach a size of > 5 μm, the sclerocytes contract and expand to surround the entire spicule. At higher magnification it can be observed that the blunt end of one spicule, within a sclerocyte, is tightly associated with fibrils, which are very likely involved in the extrusion of the spicules out of the cells (Perović-Ottstadt et al., 2005). It is apparent that especially in the beginning spicule formation starts within vesicles of the cells (Fig. 7A).

The formation of—at least—the first silica layer around the axial filament starts within the sclerocyte. It was reported that the central canal of the spicules is filled only with the axial filament, which is composed of silicatein (Uriz et al., 2000). Also in the primmorph system (Müller et al., 2005) we could show that in the primordial stage the spicule growth starts around the axial filament. There clods with highly electron dense material become visible, which represent the first

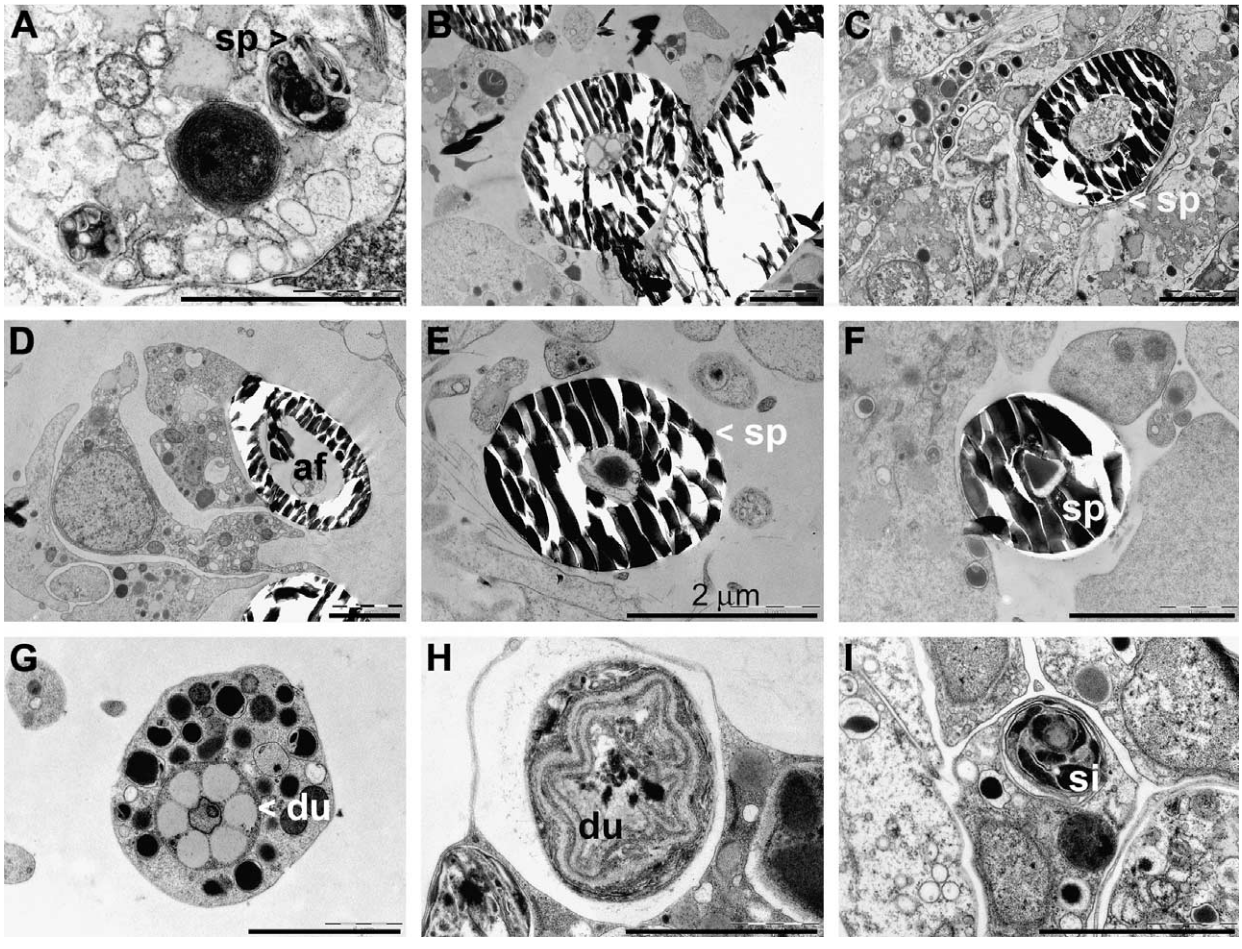

Fig. 7. Maturation stages of the axial filament; TEM analysis of the different phases of spicule formation in primmorphs. (A) In sclerocytes the spicule (sp) formation starts within vesicles of sclerocyte. (B) The axial canal of newly growing spicule comprises hollow fibrillar structures of three (shown here) to six tube-like ducts. (C) Subsequently, the axial filament shows membrane structures, which in a later stage (D) comprises besides those structures also a thin, electron dense axial filament. (E) The electron dense axial filament in the canal increases and displaces the membrane structures. (F) Finally, the axial canal changes from nearly circular to triangular and comprises the electron dense axial filament. (G) The cross section of one hollow fibrillar structure, in the initial phase of spicule formation in the sclerocyte, is shown. Six ducts (du) are seen. (H) All ducts fuse together. Both in the center and around the hollow fibrillar structures, very dense silica deposits (de) are see. (I) Finally, the hollow ducts disappear and larger silica (si) deposits are seen in the primordial spicule. Scale bars: 2 μm.

deposits of spicules. During progression of growth in the extracellular space, the diameter of the axial filament decreases from 1.5 to 0.8 μm while simultaneously the size of the spicule increases up to 450 μm and a diameter of 5 μm. In this phase the spicules are found extracellularly (Fig. 7B). In the next growth stage, two types of structures can be distinguished within the axial canal. First, the 1.6 μm thick axial canal is filled primarily with membrane structures (Fig. 7B) or a comparably thin axial filament is seen, besides the membrane structures (Fig. 7C–E). The 0.8–0.2 μm axial filament has the same electron density as in the initial stage of spicule formation. Among the cellular structures are many 10–15 nm round fibrils of not yet defined nature. The axial filament in the canal increases again in size and becomes more electron dense (Fig. 7E). In the final stage the axial canal changes from nearly circular to triangular (Fig. 7F). Interesting are the hollow fibrillar structures which are seen in sections through spicules in the transition phase (Fig. 7B) from those which comprise complete membrane structures in the axial canal (Fig. 7C) and spicules which show in the canal a more compact axial filament (Fig. 7F). In the transition phase (Fig. 7B), the hollow fibrillar structure fills almost the complete space in the canal.

These hollow fibrillar structures, newly described here, show six tube-like ducts, which are initially regularly arranged around a central filled canal of spicules inside the cells (Fig. 7G). The diameter of those hollow fibrillar structures measures approximately 1.3 μm. In the following stage the regular arrangement of this structure changes and the hollow ducts fuse which each other and also with the central filled canal (Fig. 7H). Now first silica depositions are seen within the structure and also outside of it. Finally the fused hollow ducts disappear and larger silica deposits are observed (Fig. 7I); in this phase the growing spicule is still located within the cells. At present we assume that the function of the hollow fibrillar structure is to provide the guidance for the formation of the silicatein axial filament and to allow also the deposition of the first silica clods. Until now, no solid evidence is available to indicate that in the axial filaments of the spicules entire cells occur.

8. Distribution of silicatein within primmorphs

To obtain results on the distribution of silicatein within the 3D-cell aggregates, the primmorphs, immunogold labeling studies with antibodies against silicatein were performed. Pre-immune serum gave only a low (if at all) labeling signal. More detailed information was given in a recent study (Müller et al., 2005). If, however, the immune serum was used, a dense accumulation of gold granules became visible in the sclerocytes (Fig. 8A). Fine structure analysis revealed that at

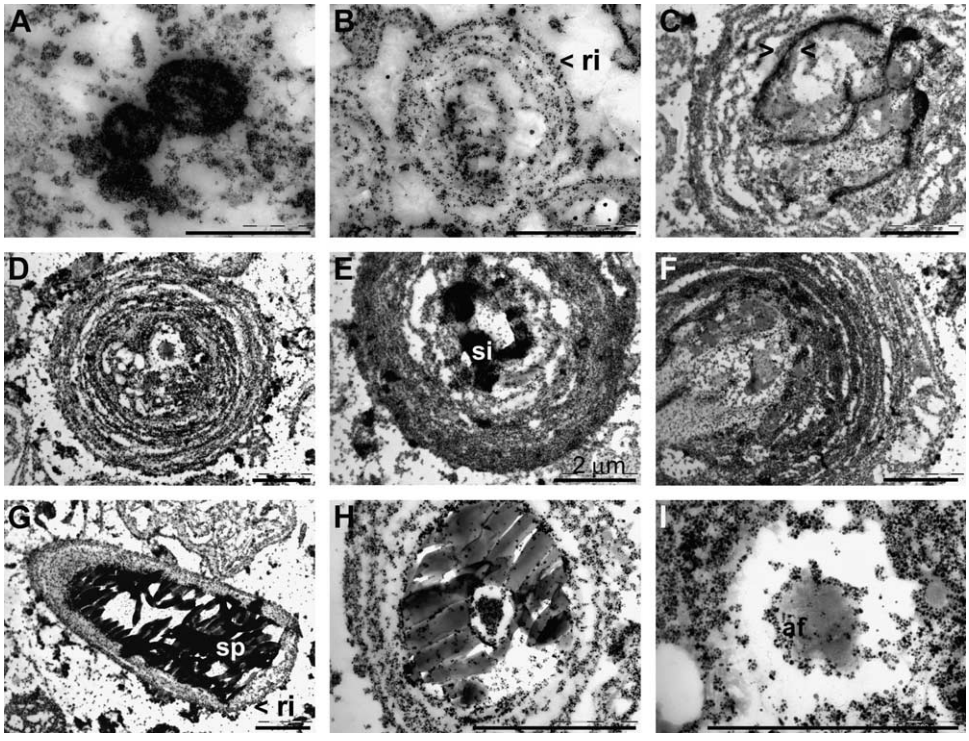

Fig. 8. Immunogold electron microscopy of cross sections through growing spicules in primmorphs; the immune complexes between the polyclonal anti-silicatein antibodies had been visualized with nanogold anti-rabbit IgG. (A) Sclerocytes with a strong accumulation of gold granules, reflecting the condensation of silicatein molecules. (B) At a higher magnification concentric rings (ri) are seen that surround a center with granules of a higher density. (C) In a later stage first electron dense linear clods (> <) become visible around which the rings are arranged. (D) The inner rings fuse and electron dense linear clods are formed. (E) The electron dense material (silica [Si]) accumulates in the center of the multi-lamellar silicatein-reacting material. (F) Higher magnification of (E). (G) The first layer of silica at a growing spicule (sp) is formed around which the concentric ring(s) (ri) remain present. (H) Finally, the silica layer grows and the number of rings increase. (I) The silicatein-antibodies react with the axial filament (af) and the deposits of the silica rings, leaving space for non-reacting material. Scale bars: 2 μm.

first concentric rings are seen around the forming spicules which are 0.2–0.5 μm apart (Fig. 8B). Subsequently, the inner rings fuse and electron dense linear clods become visible (Fig. 8C). Later, during maturation, the number of the concentric rings increases from two to three rings with a diameter of 2–3 μm (Fig. 8B) to 10 rings and a total diameter 4–6 μm (Fig. 8D). At higher magnification the clods appear round-shaped (Fig. 8E and F). Finally, the first layer of silica is formed, showing again around these silica structures concentric rings of gold particles, reflecting silicatein antibodies (Fig. 8G). The number of gold/antibody rings around the primordial spicules, composed of a first silica layer, is larger than three (Fig. 8H). Under the experimental conditions used here, also the axial filament (= silicatein) reacts with the antibodies (Fig. 8H and I). It should be mentioned that around the axial filament and the first layer of the silica deposition, a space filled with non-reacting material is seen (Fig. 8I). Probably there the substrate for the silicatein is stored.

9. Formation of the morphology of the spicules

As recently highlighted, biomineralization is the process by which Metazoa form hard minerals for support, defense, and feeding (see Wilt, 2005). In sponges, the major structural elements which contribute to morphogenesis are the spicules. Until now, no experimental data on the underlying cellular mechanism(s), or even developmental genes, that are involved in the formation of the filigree architecture of the spicules and the arrangement of spicules within the sponge body exist. However, understanding of the morphology of spicules is one of the keys towards an unraveling of the Bauplan of sponges. It is obvious that the size/length of the spicules (megascleres) exceeds that of a cell. Several mechanisms might be postulated for the intracellular and subsequent extracellular growth of the spicules, giving rise to a complex morphology. Sollas (1888) assumed that cells migrate along the spicules to allow growth along their axis. Until now, no kinematical analysis has been presented. With the TEM technique we analyzed sections through adult animals (Fig. 9). By that approach we almost exclusively identified cells only around the knob, almost never at the tip of the tylostyles (Fig. 9A and B). Around the knob the cells are very tightly associated with the spicule; there clusters of cells are arranged (Fig. 9C and D). It might be functionally important that in this region the cells have a ramified morphology and their protrusions appear to surround—and perhaps—to govern the formation of the shape of the knobs. At a higher magnification it becomes overt that between those cellular protrusions and the spicules, frequently vesicles are seen which comprise an almost homogenous content (Fig. 9E). Even small filamentous structures can be identified in those vesicles (Fig. 9F) and occasionally even rod-like structures exist which might be silica deposits (Fig. 9G).

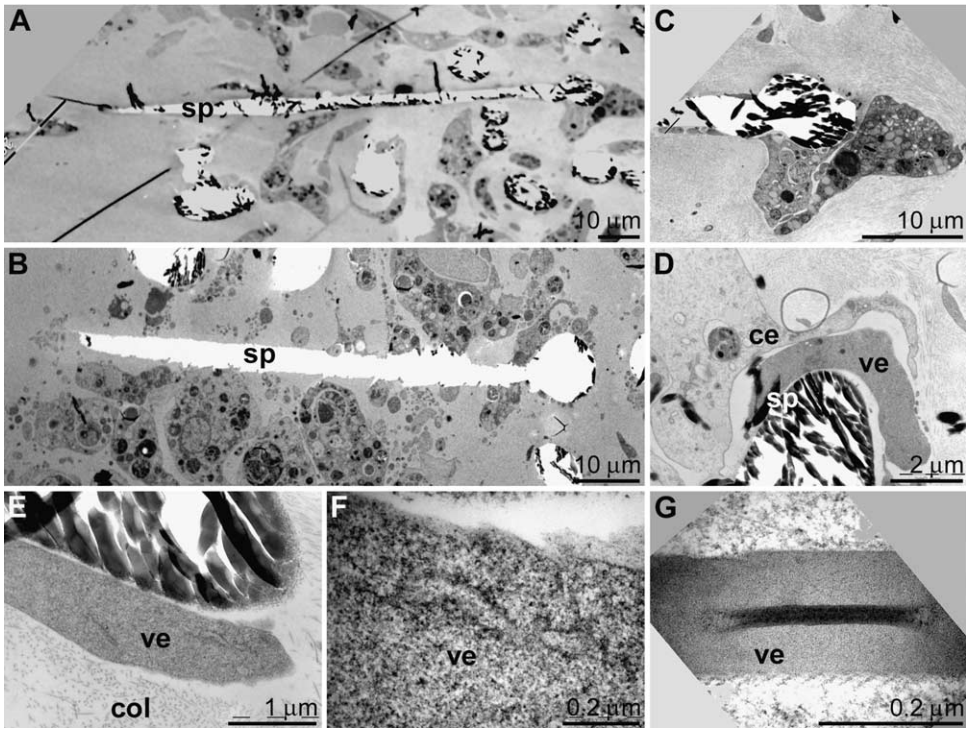

Fig. 9. Formation of the spicules in tissue; TEM analysis. (A and B) A tangential section though an area harboring a tylostyle (sp). The spicule itself had been removed during cutting of the section. Notable are the cells which are located around the knobs of the spicules. (C) The cluster of cells surrounding the knob are closely associated with the spicule. (D) Between the cellular protrusions (ce) and the spicule (sp), vesicles (ve) are seen which comprise a homogenous content. (E) The vesicles are surrounded by a net of collagen (col) fibers. (F) In rare cases the vesicles comprise structures which increase in size (G), allowing the formation of rod-like structures.

10. Extracellular arrangement of silicatein in tissue

The silicatein-specific antibodies were applied to examine if also in tissue from adult specimens silicatein exists in the bulky extracellular space. The immunogold electron microscopical analysis was chosen. Sections show that in the mesohyl compartment strings, which are decorated with nanogold, can be readily identified (Fig. 10). Especially dense are the strings around the area of spicule formation (Fig. 10A). At a higher power it becomes obvious that the strings are regularly arranged and follow the surface/shape of the spicule (Fig. 10B and E). Especially at places where the growth of two or more spicules takes place it is obvious that the direction of the silicatein/immunogold strings is organized. Especially dense are the strings at the ends of the spicules, where the longitudinal growth of the spicules proceeds (Fig. 10F). It remains to be substantiated if extracellular collagen might be involved in the organization of the strings consisting mainly of silicatein.

11. Phases of silica deposition during spicule formation

Based on the electron microscopical studies, and especially using the primmorph system, it can be deduced that the process of spicule formation can be divided into two phases; the intracellular initial steps and the extracellular final and shaping phase.

11.1. Intracellular phase

An outline of the first steps in spicule formation has been recently documented (Müller et al., 2005) (Fig. 11). Silicatein is synthesized as a pro-enzyme (signal peptide–propeptide–mature enzyme: 36.3 kDa) and processed via the 34.7 kDa form (propeptide–mature enzyme) to the 23 kDa mature enzyme. Very likely during the transport through the endoplasmic reticulum and the Golgi complex, silicatein undergoes phosphorylation and is transported into vesicles where it forms rods, the axial filaments. Our studies indicate that the association of the silicatein monomers to the axial filaments involves steps of posttranslational modifications. After assembly to filaments the first layer(s) of silica is (are) formed. Silica deposition occurs in two directions; first from the axial canal to the surface and second from the mesohyl to the surface of the spicule. Finally the spicules are released into the extracellular space where they grow in length and in diameter by appositional growth.

11.2. Extracellular phase

Silicatein is present also in the extracellular space, as documented earlier (Müller et al., 2005). However, it came surprising that also there the silicatein molecules are organized to larger entities. The immunogold electron microscopical analysis showed that the silicatein molecules are arranged along strings, which are organized in parallel to the surfaces of the spicules. It is a task for the future to identify whether those strings are formed solely from silicatein, or if this arrangement is ‘guided’ by an associated protein. It is assumed that these silicatein strings mediate the stepwise and lamellar apposition of the silica layers around the longitudinal axis of the spicules. This view is schematically summarized in Fig. 12.

12. Final remark

Skeletal elements are formed in all metazoan phyla to stabilize the body and to allow growth. In triploblasts, two different strategies have been followed. While in Protostomia an exo-skeleton (integument) which is made of organic molecules (chitin), especially well developed in Arthropoda/Antennata, is formed, Deuterostomia form their the skeletal structures (bones) from the somatic mesoderm. In diploblasts, in coelenterates, the basic inorganic biomineral is calcium carbonate.

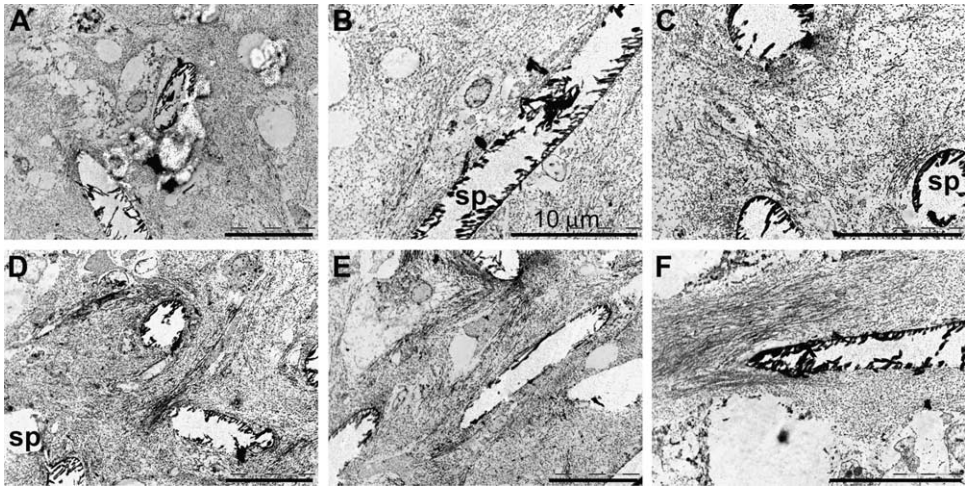

Fig. 10. Arrangement of the silicatein/immunogold strings in the bulky extracellular space (mesohyl) in tissue from adult specimens; the analysis is performed by immunogold electron microscopical analysis. (A) The silicatein/immunogold strings are highly abundant in those areas of high spicule (sp) synthesis. (B–D) The band-like structures are regularly arranged around the surface of the spicules. (E and F) Especially dense are the string-structures at the ends of the spicules. Scale bars: 10 µm.

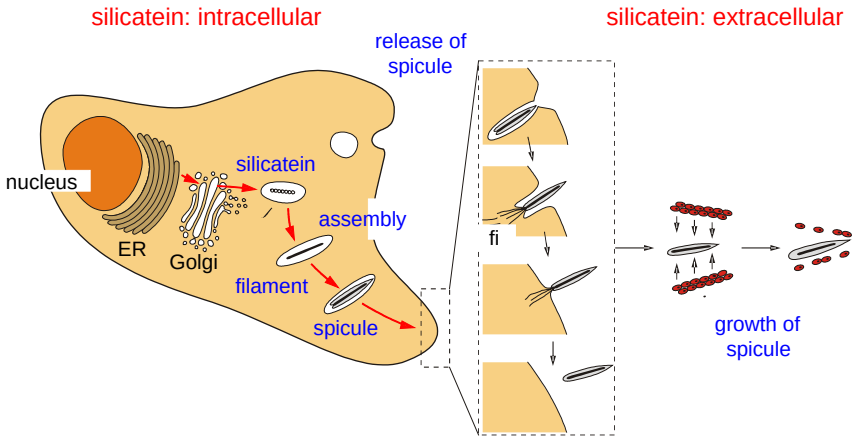

Fig. 11. Schematic outline of spicule formation in *S. domuncula*: intracellular phase. The initial steps of spicule synthesis occur intracellularly where the silicatein is processed to the mature form. During this processing the silicatein undergoes phosphorylation. Very likely with the help of other proteins the silicatein molecules assemble to a rod, the axial filaments. Around this filament the first deposition if silica proceeds. Small spicules are released/extruded from the cells perhaps facilitated by forces coming from associated filaments (fi). These structures have been identified in the axial canal of the spicules. In the extracellular space the spicules growth top their final size. Until now only the 34.7 kDa pro-silicatein has been identified in the extracellular space, suggesting that only small amounts of the enzyme undergoes maturation in this compartment.

Silica is the major constituent of sponge spicules in the classes Demospongiae and Hexactinellida. The spicules of these sponges are composed of hydrated, amorphous, noncrystalline silica. The initial secretion of spicules occurs in Demospongiae in specialized cells, the sclerocytes; there silica is deposited around an organic filament. The synthesis of spicules is a rapid process. Inhibition studies revealed that skeletogenesis of siliceous spicules is enzyme-mediated, more particularly, by an Fe^{++}-dependent enzyme.

The formation of siliceous spicules in sponges is certainly genetically controlled; this process initiates the morphogenesis phase. A series of genes, coding for structural proteins (silicatein and mannose-binding lectin) as well as for a regulatory protein (mago nashi), that are assumed to be involved in the control of spicule synthesis and Bauplan formation in demosponges have recently been identified (to be published). In addition, data demonstrated that at suitable concentrations, silicate induces genes, e.g. those encoding collagen, silicatein and myotrophin (Krasko et al., 2000). A major step forward to elucidate the formation of the siliceous spicules on molecular level was the finding that the ‘axial organic filament’ of siliceous spicules is in reality an enzyme, silicatein, which mediates the apposition of amorphous silica and hence the formation of spicules. The skeletal framework of the sponges is highly ordered. Both in demosponges and in hexactinellids, it can be seen that the growth of the sponges proceeds in a radiate accretive manner, meaning that growth zones which are highly ordered are delimited by growth lines.

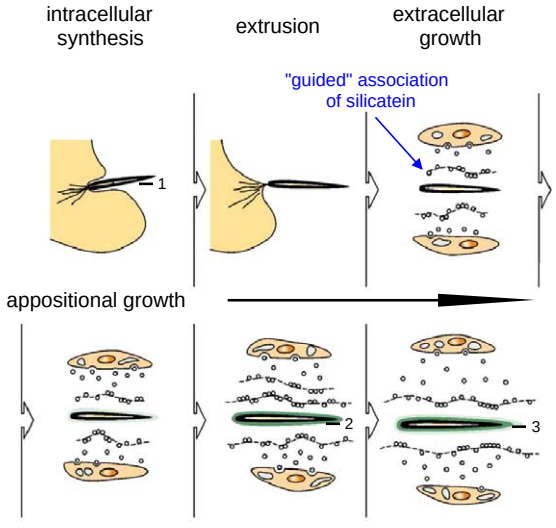

Fig. 12. Scheme of the process of the appositional growth of the spicules in the extracellular space (mesohyl). There, the silicatein molecules are arranged in strings which follow the shape and orientation of the spicules. It is assumed that the silicatein strings mediate laterally the formation of the different lamellar layers around the spicules (appositional growth). It is not yet known if the formation of the strings is based on silicatein–silicatein interactions alone, or if it is structured by a associated protein (‘guided’ association). In this scheme the appositional growth of the spicules by three layers are highlighted.

In conclusion, this survey provides an attempt towards an understanding of spicule formation. Future studies should provide experimental data to solve the question how biosilica, the product of the enzyme silicateins, is formed to give the spicules with their complex morphology. It can be assumed that soluble morphogens which might interact with their corresponding receptors, activate transcription factors that are involved in morphogenesis of the spicules.

Acknowledgements

We thank Dr M. Steiner (TU Berlin) for providing us with fossils and Ms E. Sehn (Zoological Institute; University of Mainz [Germany]) for the valuable technical assistance. Furthermore we are thankful to Ms U. Schloßmacher for valuable information and help to analyze spicule formation. This work was supported by grants from the European Commission (SILIBIOTEC), the Deutsche Forschungsgemeinschaft, the Bundesministerium für Bildung und Forschung Germany [project: Center of Excellence *BIOTEC*marin] and the International Human Frontier Science Program.

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