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Molecular Evolution of the Metazoan Extracellular Matrix: Cloning and Expression of Structural Proteins from the Demosponges *Suberites domuncula* and *Geodia cydonium*

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Abstract. One crucial event during evolution to multicellularity was the development of either direct cell–cell contact or indirect interaction via extracellular matrix (ECM) molecules. The identification of those polypeptides provides conclusive data on the phylogenetic relationship of metazoan phyla and helps us to understand the position of the Metazoa among the other kingdoms. Recently it became evident that the ECM of sponges is amazingly complex; it is composed of fibrous molecules, e.g., collagen, and their corresponding receptors, which are highly similar to those existing in other metazoan phyla. While these data already support the view of monophyly of Metazoa, additional studies are required to understand whether these molecules, which are similar in their primary sequence, also have the same function throughout the metazoan kingdom. In the present study we identified the ligand for one of the autopomorphic characters of Metazoa, the single-transmembrane receptor protein with the receptor tyrosine kinase (RTK) from *G. cydonium,* as an example: the putative mucus-like protein from *G. cydonium.* This protein was upregulated during autograft fusion in the homologous system with kinetics similar to those of the RTK. Additionally, a cDNA was isolated from *S. domuncula* whose deduced polypeptide displays a high sequence similarity to dermatopontin, an ECM molecule found exclusively in Metazoa. Furthermore, it is documented that expression of the fibrous ECM molecule collagen is regulated by the characteristic metazoan morphogens myotrophin and endothelial monocyte-activating polypeptide. These data indicate that the ECM of sponges is not an unstructured ground substance but provides the basis for integrated cell communication.

Key words: Sponges — Porifera — *Geodia cydonium* — *Suberites domuncula* — Extracellular matrix — Dermatopontin — Mucus-like protein — Morphogens

Introduction

Tissue from Metazoa is composed not only of cells but also of a matrix into which cells are embedded. This extracellular matrix (ECM) is composed of an intricate meshwork of macromolecules which comprise besides polysaccharides also different types of proteins. The body of sponges (Porifera), the phylogenetically oldest still extant metazoan phylum (Müller 1998), is surrounded by a pinacoderm, a layer of epithelial cells (reviewed by Simpson 1984). The internal tissue of sponges was termed mesohyl (Borojevic et al. 1967), thus stressing the previous suggestion that it comprises cells which are highly variable in terms of their degree of development as well as a bulky noncellular matrix. The proteinaceous ECM was—until recently—thought to contain

The sequences reported herein are deposited in the EMBL/GenBank database (accession Nos. AJ299721 for the *Geodia cydonium* mucus-like protein, termed MUCL_GC, and AJ299722 for the putative *Suberites domuncula* dermatopontin, DERM_SD).

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free collagen fibrils and an unstructured ground substance (Simpson 1984). This view changed fundamentally since it had been shown that the ECM is not, as suggested (Loomis 1988), an inert scaffold to stabilize the physical structure of the autonomous flagellates, but a dynamic, active, and complex network of molecules which regulate the behavior of the cells (reviewed by Müller 1997). In recent years it has become clear that the ECM of sponges has an amazingly complex molecular composition and controls cell positioning/cell adhesion, cell function, and the immune response. Applying molecular biological techniques, which resulted in the isolation of cDNAs/genes coding for informative proteins, it was demonstrated that the molecules present in the ECM are highly related to those found in other metazoan phyla; this allowed molecular biological resolution of the monophyly of Metazoa (Müller et al. 1994). The bulk of the molecular biological data came from studies with the marine demosponges *Suberites domuncula* and *Geodia cydonium.*

Since the genetic code is evolving and not "frozen" (Jukes and Osawa 1991) and the rate of amino acid (aa) substitutions can be used to estimate the evolutionary changes of proteins (Zuckerkandl and Pauling 1965; Jukes and Cantor 1969), the divergence time of the phylogenetically oldest metazoan phylum could be estimated using nucleotide (nt) and aa sequences from demosponges. The first calculation using a protein deduced from a sponge cDNA was based on the galectin sequences; it revealed that sponges have existed for at least 800 million years (Pfeifer et al. 1993; Hirabayashi and Kasai 1993). This time estimate for the divergence of sponges from a common ancestor was confirmed by analysis of the receptor tyrosine kinases (Schäcke et al. 1994b). The oldest fossil sponges have been identified in rocks from South–Central China which date back ~580 million years (Li et al. 1998).

Sponge Molecules Involved in Cell Adhesion. The first sponge cDNA identified encoding a molecule involved in cell–cell adhesion was a galectin (Pfeifer et al. 1993). This polypeptide isolated from *G. cydonium* was found to be one component of the cell–cell adhesion molecule, the aggregation factor (Conrad et al. 1981; Wagner-Hülsmann et al. 1996). The cell surface-associated molecule, the aggregation receptor, was likewise cloned from *G. cydonium* and found to be a complex composed of scavenger receptor cysteine-rich domains (Blumbach et al. 1998).

Collagen is the dominant component of the ECM of sponges that functions as a cell–matrix adhesion molecule (Garrone 1978; Diehl-Seifert et al. 1985). The corresponding gene was cloned from the freshwater sponge *Ephydatia muelleri* (Exposito and Garrone 1990) as well as from the demosponge *S. domuncula* (Schröder et al. 2000). The sponge collagens have been grouped in the short-chain collagens. In protostomians as well as in deuterostomians collagen binds to the cell surface receptor integrin (Eble 1997). As the first nonbilaterian integrins, the sponge molecules have been identified in *G. cydonium* (Pancer et al. 1997) as well as in *S. domuncula* (Wimmer et al. 1999a, b); their function has been partially characterized. A second ligand for the integrin receptors is fibronectin, from which the FN_3 module has been identified in *G. cydonium* (Pahler et al. 1998b).

Sponge Molecules Involved in Signal Transduction. Cell surface-spanning receptors, in particular, the single-pass (1-TMR) as well as the seven-pass transmembrane receptor proteins (7-TMR), serve as receivers for extracellular signaling molecules. Also, these molecules were identified in sponges. The first 1-TMR was the receptor tyrosine kinase (RTK), which represents an autapomorphic character of Metazoa (reviewed by Müller and Schäcke 1996). Interestingly, the tyrosine dephosphorylating enzymes, the protein–tyrosine phosphatases (PTPase), already exist in yeasts (reviewed by C.I. Müller et al. 2001). As a 7-TMR the metabotropic glutamate/GABA-like receptor was identified in *G. cydonium* (Perovic et al. 1999). The latter receptor was found to respond to extracellular agonists as well as to antagonists known from metabotropic glutamate/GABA-like receptors found on nerve cells from mammals (Kaupmann et al. 1997).

The RTK comprises the extracellular domain (ligand-binding domain), the transmembrane domain, the juxtamembrane domain, and the catalytic domain (Schäcke et al. 1994a; Müller and Schäcke 1996). The extracellular domain is composed of two immunoglobulin (Ig)-like segments, which are polymorphic (Pancer et al. 1996). The expression of this receptor is upregulated during the fusion process of autografts (Wimmer et al. 1999a).

Sponge Molecules Involved in the Immune Response. Besides the cytokines identified from *S. domuncula* and *G. cydonium* which might be released into the extracellular space and act on distantly positioned cells [e.g., allograft inflammatory factor, glutathione peroxidase, or pre-B-cell colony-enhancing factor (reviewed by Müller et al. 1999a)] as well as intracellular elements of the interferon pathway (Wiens et al. 1999), receptors showing a high sequence similarity to vertebrate receptors involved in the immune response have been found. The prominent receptors of this class are the polymorphic sponge adhesion molecules, which occur in *G. cydonium* in a short and a long form (Blumbach et al. 1999). Also, in their extracellular part these receptors comprise two Ig-like domains which are grouped in the V-related set of Ig-like sequences (Blumbach et al. 1999).

Morphogens. In the extracellular space of sponges, morphogens have been identified, e.g., endothelial monocyte-activating polypeptide (EMAP) (Pahler et al.

1998a) and myotrophin (Schröder et al. 2000), which are both very likely involved in the organization/differentiation of cells within the sponge body.

Mineral Deposits. Within the metazoan kingdom the two sponge subphyla, the Demospongiae and the Hexactinellida, contain siliceous deposits, the spicules. The silicon forming the spicules is synthesized enzymatically by silicatein; the expression of silicatein is controlled by the extracellular level of silicate (Krasko et al. 2000). Collagen gene expression, which is very likely under the control of myotrophin (Schröder et al. 2000), is apparently involved in the determination of the form of the spicules (Krasko et al. 2000).

With the exceptions of galectin and the receptor PTPase, all other sponge receptors mentioned are characteristic for Metazoa. Therefore, we proposed that all metazoan phyla including the Porifera evolved from a common ancestor (Müller et al. 1994; Müller 1995), the hypothetical Urmetazoa (Müller and Müller 2000; Müller 2001).

In the present study the answer to a further crucial question about the molecules which guide the organization of the extracellular matrix proteins is approached. One of the polypeptides involved in the assembly of collagen into fibrils is dermatopontin (MacBeath et al. 1993). The cDNA of dermatopontin was isolated from *S. domuncula* and the recombinant protein was prepared. It is demonstrated that the sequence shows a high similarity to the related molecules found in other metazoan phyla. In addition, the recombinant dermatopontin from *S. domuncula* comprises the same function as related molecules, e.g., agglutination of cells by the 18-kDa aggregation factor from *Limulus* (Fujii et al. 1992).

In addition, the extracellular ligand for the receptor tyrosine kinase from *G. cydonium* was identified using the technique of the two-hybrid system. The experiments revealed that a mucus-like protein is the probable RTK ligand. It has been described earlier that sponges produce mucous material, e.g., in *Cyamon neon* (Smith 1968) or in *Eunapius fragilis* (Harrison and Cowden 1975). However, the chemical nature of this substance remained undetermined. The cDNA encoding the putative mucus-like protein from *G. cydonium* comprises the characteristic polythreonine stretches known from other eukaryotic organisms.

Until recently, it was generally suggested that most cells in sponges are functionally independent, a reason most of the species are thought to form amorphous, asymmetrical creatures (Pechenik 2000). Therefore, it is pressing to demonstrate that sponges synthesize factors which determine the growth form by effecting the expression of structural proteins. In the present study we demonstrate that the expression of collagen is modulated by the morphogens myotrophin and EMAP, resulting also in a change in the form of primmorphs. Primmorphs are a special form of aggregates, which reassociate from single cells after transferring them into medium composed of seawater; they contain proliferating cells (Müller et al. 1999b).

Materials and Methods

Materials. Restriction endonucleases and other enzymes for recombinant DNA techniques and vectors were obtained as described earlier (Kruse et al. 1997; Wimmer et al. 1999b). The HybriZAP two-hybrid system was obtained from Stratagene (La Jolla, CA, USA); the CheckMate mammalian two-hybrid system, from Promega (Madison, WI, USA); and DIG (digoxigenin), the PCR-DIG Probe Synthesis Kit, DIG-11-dUTP, anti-DIG AP Fab fragments, and CDP-Star [disodium 2-chloro-5-(4-methoxyspiro{1,2-dioxetane-3,2′-(5′-chloro)-tricyclo-[3.3.1.1^{3,7}]decan}-4-yl)phenyl phosphate], from Roche Mannheim (Mannheim, Germany).

Sponges. Specimens of the marine sponges *Geodia cydonium* (Porifera, Demospongiae, Geodiidae) and *Suberites domuncula* (Porifera, Demospongiae, Hadromerida) were collected in the northern Adriatic near Rovinj (Croatia) and then kept in aquaria in Mainz (Germany) at a temperature of 17°C.

Autografting Procedure. For the autograft experiments the "insertion technique" was used as described previously (Pancer et al. 1996). In brief, tissue pieces from the same specimen of *G. cydonium* were removed with a cork drill (diameter, 1 cm; approximate length, 4 cm) and inserted into other holes in the same individual. The sponges were kept for up to 10 days to be subsequently analyzed. Tissue samples from the fusion zones were taken, approximately 1.5 mm thick, and frozen in liquid nitrogen until use for Northern blotting experiments.

Isolation of the Mucus-like Protein from G. cydonium *in the Yeast Two-Hybrid System.* The interaction of the extracellular part of the *G. cydonium* receptor tyrosine kinase (RTK), comprising the two immunoglobulin (Ig)-like domains, with the putative ligand was studied in the yeast two-hybrid system. The HybriZAP two-hybrid system was applied according to the Instruction Manual (HybriZAP two-hybrid predigested vector kit; Stratagene). The complete extracellular part of the RTK, spanning nt_{391} to nt_{1089} and encoding the deduced polypeptide region aa_{131} to aa_{363} [accession number X98340 (Pancer et al. 1996)], was obtained from the cDNA library (Pfeifer et al. 1993) by polymerase chain reaction (PCR). The forward primer with the adapter sequence for *Eco*RI and the reverse primer with a *Sal*I site spanning the two Ig-like domains were inserted into the *Eco*RI–*Sal*I sites of the yeast *pBD-GAL4* Cam phagemid vector (Stratagene), encoding the bait. The cDNA library (≈1.5×10^9 pfu) of *G. cydonium* was inserted into the HybriZAP (Stratagene) vector to generate the primary λ library. The amplified library was converted into a *pAD-GAL4* (Stratagene) target library by in vivo excision, according to the *Strategene Manual.* The target and bait plasmids were transformed and coexpressed in the yeast host, strain YRG-2. Colonies that contain the target protein, which interacts with the bait protein, have been identified by detection of the β-galactosidase activity, as described (Zhu and Kahn 1997), in the presence of both negative controls (coexpression of *pLamin C* and *pSV40*) and positive controls (*p53* and *pSV40*). The total DNA from the yeast colonies that contain DNA encoding the target protein, which interacts with the bait protein, was isolated (Hoffman and Winston 1987). To isolate the plasmid, containing the gene for the target protein, the isolated DNA was transferred into the *Escherichia coli* strain Sure (Stratagene) by electroporation. Subsequently, the plasmid was isolated from the clone and sequenced with an automatic DNA sequenator (Li-Cor 4200). The resulting insert obtained had a size of 1716 nt and was termed *GCMUCL.*

Verification of protein (target)–protein (bait) interaction was performed with the isolated target plasmid in the presence of the plasmid encoding the bait protein in the yeast YRG-2 system. In addition, the CheckMate mammalian two-hybrid system (Promega) was used to prove the specificity of the protein–protein interaction.

PCR Cloning of the Putative Sponge Dermatopontin. A cDNA fragment, encoding the putative dermatopontin, was obtained from a *S. domuncula* cDNA library (Kruse et al. 1997). This cDNA was completed by PCR using the reverse primer 5′-CATCTTCCAAATCCTGTCCTCAT-3′ (nt_{507} to nt_{529} of the final sequence) in conjunction with the ZAPII vector-specific primer. The PCR was carried out using a GeneAmp 9600 thermal cycler (Perkin Elmer), with initial denaturation at 95°C for 3 min, then 35 amplification cycles each at 95°C for 30 s, 56°C for 30 s, and 72°C for 1.5 min, and a final extension step at 72°C for 10 min as described (Pancer et al. 1997). The plasmid DNAs, *SDDERM,* were sequenced.

Sequence Comparisons. The sequences were analyzed using the computer programs BLAST (1997) and FASTA (1997). Multiple alignments were performed with CLUSTAL W Version 1.6 (Thompson et al. 1994). Phylogenetic trees were constructed on the basis of aa sequence alignments by neighbour-joining, as implemented in the "Neighbor" program from the PHYLIP package (Felsenstein 1993). The distance matrices were calculated using the Dayhoff PAM matrix model as described (Dayhoff et al. 1978). The degree of support for internal branches was further assessed by bootstrapping (Felsenstein 1993). The graphic presentations were prepared with GeneDoc (Nicholas and Nicholas 1997). Hydropathicity analysis (window size, 15 aa), based on the method of Kyte and Doolittle (1982), was performed using the PC/GENE (1995; Soap).

Northern Blotting. RNA was extracted from liquid nitrogen-pulverized sponge tissue with TRIzol reagent. Five micrograms of total RNA was electrophoresed through 1% formaldehyde/agarose gel and blotted onto a Hybond N^+ membrane following the manufacturer's instructions (Amersham, Little Chalfont, Buckinghamshire, UK) (Wiens et al. 1998). Hybridization was performed with two probes. To detect *GCMUCL* transcripts an ≈ 700-bp PCR product from the clone *GCMUCL* was used. For expression studies in primmorphs the *S. domuncula* collagen *SUBDOCOL1* cDNA [accession number AJ252241 (Schröder et al. 2000)] was applied. Hybridization was performed under high-stringency conditions overnight at 50°C. Washes were performed twice in 2 × SSC (300 m*M* NaCl, 30 m*M* Na_3–citrate, pH 7.0), 0.1% $NaDodSO_4$ at room temperature, then twice in 0.1 × SSC/0.1% $NaDodSO_4$ at 68°C. The probes were labeled with DIG-11-dUTP using the PCR-DIG Probe Synthesis Kit. After washing, DIG-labeled nucleic acid was detected with anti-DIG Fab fragments (conjugated to alkaline phosphatase) and visualized by the chemiluminescence technique using CDP-Star.

As a control for the expression studies of *G. cydonium* mucus-like protein, the complete sequence (1.5 kb) of *G. cydonium* β-tubulin [*GCBTUB*; accession number Y17002 (Schütze et al. 1999)] was used as a probe for the Northern blot experiments. For semiquantitative analysis of the level of expression the bands on the film were scanned with a GS-525 Molecular Imager (Bio-Rad, Hercules, CA, USA).

Dermatopontin cDNA Expression. The insert from *SDDERM* was used for expression in *E. coli*. The cDNA was inserted into the bacterial oligohistidine expression vector pQE-30 (Quiagen) as described before (Schröder et al. 2000). After transformation of *E. coli* strain XL1-blue, expression of fusion protein was induced for 5 h with 1 m*M* isopropyl 1-thio-β-D-galactopyranoside (IPTG). Bacteria were collected and extracted with phosphate-buffered saline (PBS)/8 *M* urea; from the supernatant, termed the "bacterial crude extract," the recombinant dermatopontin (rDERM) was isolated using metal-chelate affinity chromatography, with Ni–NTA–agarose resin (Quiagen), as described by Hochuli et al. (1987). After dialysis and concentration to 0.2 mg protein/ml, rDERM was used for experiments. The purity of the material was checked with 12% polyacrylamide gels containing 0.1% $NaDodSO_4$ (PAGE) according to Laemmli (1970).

EMAP and Myotrophin Expression. The expression of EMAP was performed in *E. coli* as described above, using the complete open reading frame (ORF) of the cDNA *GCEMAPR1* obtained from *G. cydonium* [accession number Y14947 (Pahler et al. 1998a)]. The oligohistidine fusion polypeptide, rEMAP, was purified by Ni–NTA–agarose affinity chromatography and analyzed by PAGE.

A description of the preparation of the recombinant myotrophin, rMYO, was given earlier (Schröder et al. 2000).

Cell Reaggregation Assay. Single cells from *S. domuncula* were obtained by dissociation in Ca^{2+}- and Mg^{2+}-free artificial seawater as described (Müller et al. 1999b). For the study of the effect of dermatopontin on cell–cell adhesion, a suspension of 25×10^6 cells/ml of seawater was incubated in a standard reaggregation assay of 3 ml (Müller and Zahn 1973) and rolled in glass tubes at 35 rpm and 20°C. Where indicated rDERM was added at a concentration of 5 μg/ml and incubation proceeded for 30 min. The size of the aggregates formed was determined optically and is given as micrometers (Müller et al. 1979).

Incubation of Primmorphs with Morphogens. Primmorphs, the special form of aggregates, are formed from single cells after transferring them from Ca^{2+}- and Mg^{2+}-free artificial seawater, used to dissociate the cells, into natural seawater. The procedure was applied as described (Müller et al. 1999b). Five days after starting primmorph formation the aggregates were used for experiments.

Results and Discussion

Cloning of G. cydonium *Mucus-Like Protein*

The cDNA encoding the *G. cydonium* mucus-like protein was identified by the yeast two-hybrid system and termed *GCMUCL.* The two Ig-like domains were fused with the *pBD-GAL4* CAM–bait system, while the cDNA library was inserted into *pAD-GAL4.* After coexpression in yeast the cDNA encoding the interacting target protein was isolated using the *E. coli* strain Sure. The resulting 1716-nt-long sequence comprises one ORF ranging from nt_3 to nt_{1616}, encoding a 538-aa-long polypeptide sequence, named MUCL_GC (Fig. 1). The calculated size of the translation product (M_r) is 58,792 and the estimated isoelectric point (p*I*) is 6.0 (PC/GENE 1995; Physchem). Northern blot analysis performed with the sponge *GCMUCL* clone as a probe yielded one prominent band of approximately 1.9 kb, confirming that a full-length cDNA was isolated (see below). The instability index of 50.5 classifies this protein as an unstable one.

The databank search revealed that the deduced *G. cydonium* polypeptide shows the highest sequence similarity to mucus(-like) proteins found in eukaryotes, especially to the human mucin-2 protein [M74027 (Toribara et al. 1991)], the mucin-like proteins from *Caenorhabditis elegans* (T22808) and *Trypanosoma cruzi*

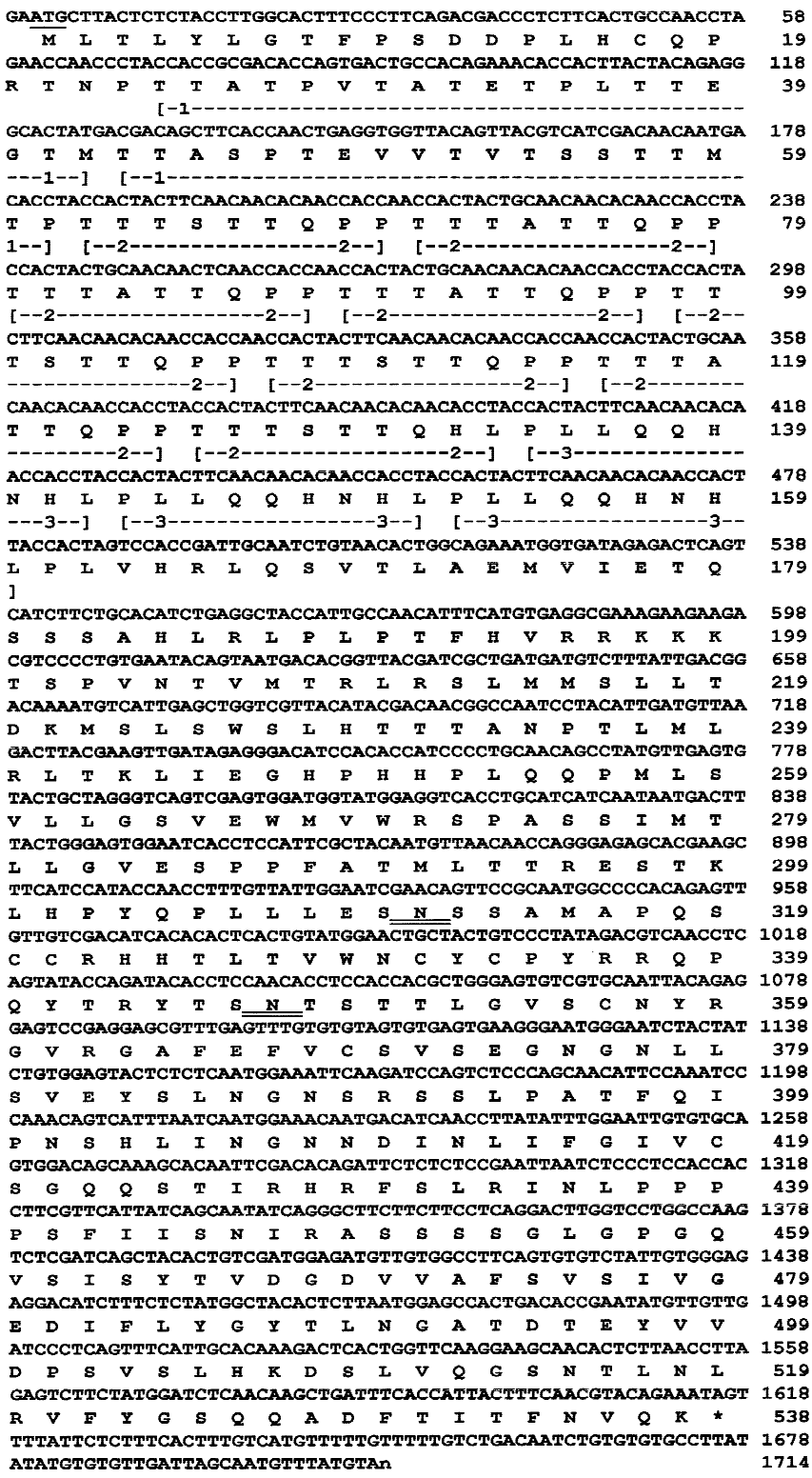

Fig. 1. Nucleotide (nt) sequence of the mucus-like protein cDNA, *GCMUCL*, and the deduced aa sequence MUCL_GC. The nt's are numbered in the 5′-to-3′ direction, starting with the nt triplet encoding the start methionine; the putative start methionine is *underlined* and the stop codon is marked with an *asterisk.* The locations of the N-glycosylation sites (*double underlined*) are indicated. The first repeat, with a consensus of T–T–A–x–P–x–x–x–x–x–x–x–x–T–x–x–T–x, is found at aa_{24} to aa_{42} and aa_{43} to aa_{61}; the second repeat (T–T–T–x–T–T–Q–x–x) is found eight times (within the segment aa_{62} to aa_{133}); and the third repeat (P–L–L–Q–Q–H–N–H–L) is found three times. All three are indicated in *brackets.* Further details are described under Results and Discussion.

[U32447.1 (Di Noia et al. 1995)], and the promastigote surface antigen-2 from *Leishmania major* [C41710 (Murray and Spithill 1991)]. The high sequence relationship to the mucus-like protein is due to the presence of polythreonine stretches in all sequences. In the *G. cydonium* polypeptide threonine accounts for 17% of the total aa, followed by 11% of leucine and 7% of serine. Mucins are cell surface glycoproteins (Shimizu and Shaw 1993). The *G. cydonium* mucus-like protein has two potential N-glycosylation sites (aa_{311} and aa_{347}).

As in other mucus(-like) proteins from eukaryotic organisms, the unicellular euglenozoa *Trypanosoma cruzi* (mucin-like protein; U32447), the yeast *Saccharomyces cerevisiae* (membrane protein YJR151c; S57180), the

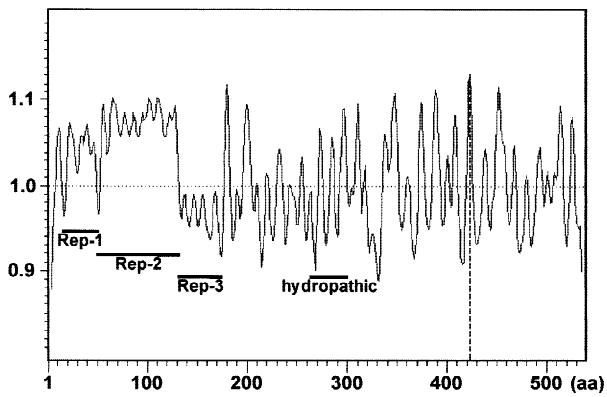

Fig. 2. Prediction of flexibility of the mucus-like protein from *G. cydonium*. The prediction pattern shows that the first and second repeats (Rep-1 and Rep-1) span the region of high flexibility, while the third repeat (Rep-3) is located in the region of low flexibility; in addition, the hydropathic region determined according to the method of Rao and Argos (1986) is indicated. The vertical axis shows units of flexibility (with an average flexibility of 1.0; the *dashed line* indicates the region of maximal flexibility). Further details are given under Results and Discussion.

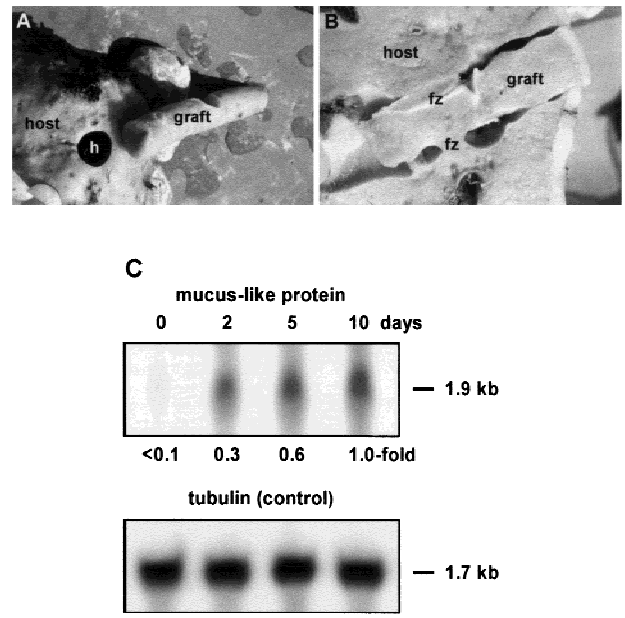

Fig. 3. Autograft fusion of tissue from *G. cydonium* (A and B). **A** A graft was removed from one specimen and transplanted into a hole (h) from the same specimen (the host). **B** During a period of 3 to 10 days the transplant fused with the host; here a fusion of the graft with the host 7 days after grafting is shown. The fusion zone (fz) is indicated. **C** Increased expression of the genes encoding the mucus-like protein in the fusion zone of autografts. The analysis was performed immediately after removal of the tissue (time 0) and from tissue taken from the fusion zone 2 to 10 days after grafting. The intensities of the transcripts for *GCMUCL* were semiquantitatively determined as described under Materials and Methods; the expression of β-tubulin was determined in parallel. (A, B) Original magnifications, × 0.6.

protostomian *Drosophila melanogaster* (hemomucin; U42014), and the deuterostomian human mucin-2 protein (M74027), polythreonine stretches are also present in the *G. cydonium* polypeptide. It is interesting that three clusters of related repeats are found in MUCL_GC. The first repeat, with a consensus of T–T–A–x–P–x–x–x–x–x–x–x–x–x–T–x–x–T–x, is found twice, at aa_{24} to aa_{42} and aa_{43} to aa_{61}; the second repeat (T–T–T–x–T–T–Q–x–x) is found eight times (within the segment aa_{62} to aa_{133}); and the third (P–L–L–Q–Q–H–N–H–L) is found three times consecutively, between aa_{134} and aa_{160} (Fig. 1). Similar repeats have been identified in the larval glue protein Lgp-1 from *Drosophila virilis* [A60095 (Swida et al. 1990)]; there the repeats read T–T–T–T–R–T–T–T–T–P. The mucus-like protein of *G. cydonium* comprises no transmembrane region (PC/GENE 1995; Soap).

The mentioned repeats of the mucus-like protein of *G. cydonium* render a characteristic flexibility pattern (Fig. 2) (PC/GENE 1995; Flexpro). The prediction of flexibility of the mucus-like protein shows that the first two repeats are characterized by a high flexibility, while the third repeat is a region of low flexibility. The segment of highest hydropathicity, presumably the site of interaction of the mucus-like protein with the *G. cydonium* RTK, is found between aa_{275} and aa_{300} (Fig. 2).

Expression of Mucus-like Protein During Autograft Fusion

As applied earlier (Pancer et al. 1996; Blumbach et al. 1999) autografting experiments have been performed to determine the level of expression of ECM molecules, here of *GCMUCL*. As shown in Figs. 3A and B tissue pieces (grafts) were removed and inserted into holes from the same species (host). After a period of approximately 2 to 3 days the grafts fused with the host (Müller et al. 1999a). At time 0 (analysis of RNA from grafts isolated immediately after grafting the sample), the level of *GCMUCL* expression was very low (Fig. 3C); no bands could be detected by Northern blot analysis. However, 2 days later the expression of *GCMUCL* in the fusion zone increased drastically (detection of a 1.9-kb band), reaching a maximum after 10 days (Fig. 3C). In a control series of experiments the expression of tubulin was analyzed to assure that the same amount of RNA was loaded onto the gel (Fig. 3C).

Cloning of S. domuncula *Dermatopontin*

The complete cDNA, *SDDERM*, encoding the putative *S. domuncula* dermatopontin protein, termed DERM_SD, was isolated as described under Materials and Methods. The cDNA is 723 nt long; the ORF spans nt_{20} to nt_{572}. From the 184 aa of the polypeptide a M_r of 21,698 and a p*I* of 8.5 were computed (PC/GENE 1995; Physchem) (Fig. 4A). As for the *G. cydonium* mucus-like protein, an instability index of 48.8 was calculated, which also classifies the sponge dermatopontin as an unstable protein.

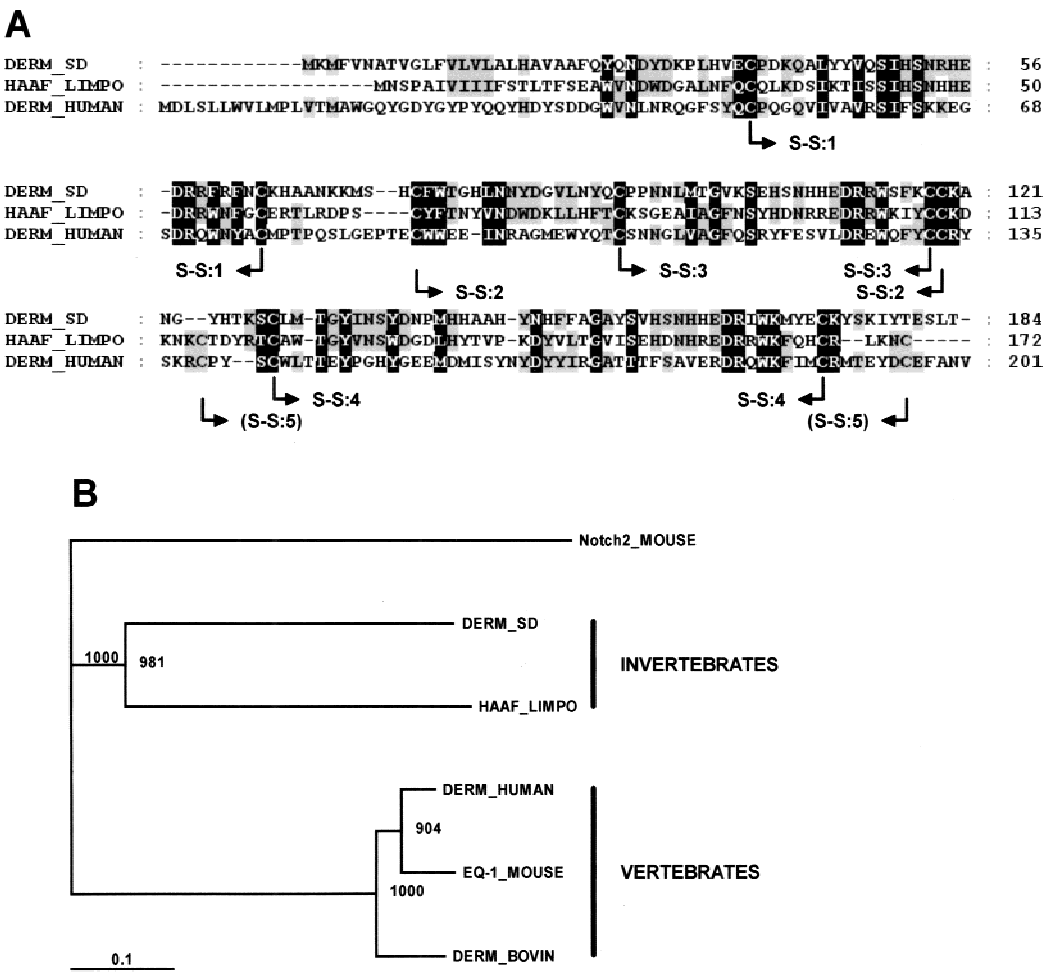

Fig. 4. *S. domuncula* dermatopontin. **A** The deduced aa sequence from the *S. domuncula* cDNA, *SDDERM*, termed DERM_SD, is aligned with related sequences from the amebocyte aggregation factor precursor of *Limulus polyphemus* [HAAF_LIMPO; accession number Q01528 (Fujii et al. 1992)] and human dermatopontin precursor [DERM_HUMAN; Q07507 (Superti-Furga et al. 1993)]. Residues conserved (similar or related with respect to their similar physicochemical properties) in all sequences are shown in *white on black*, and those in at least two sequences in *black on gray*. The four characteristic disulfide bridges (S–S:1 to S–S:4) present in the *Limulus*, human, and sponge molecules are indicated; in addition, the borders of the fifth bridge (S–S:5), present only in human, is given in *parentheses*. **B** The phylogenetic relationship of the sponge dermatopontin sequence from *S. domuncula* (DERM_SD) with the aggregation factor of *Limulus polyphemus* [HAAF_LIMPO; accession number Q01528 (Fujii et al. 1992)] and human dermatopontin precursor (DERM_HUMAN; Q07507; identical to AL049798) given in A together with the early quiescence protein-1 from *Mus musculus* (EQ-1_MOUSE; AF143374) and the bovine dermatopontin [DERM_BOVIN; P19427 and A32851 (Neame et al. 1989)] was performed after alignment. The rooted tree was computed by neighbor-joining and distance matrix determinations as described under Materials and Methods. The scale bar indicates an evolutionary distance of 0.1 aa substitution per position in the sequence. The cell surface protein Notch2 from *Mus musculus* [Notch2_MOUSE; D32210 (Lardelli and Lendahl 1993)] was used as outgroup.

Using the method of Von Heijne (1986) to predict the N-terminal secretory signal (PC/GENE 1995; Psignal), the potential cleavage site was localized to between aa_{23} and aa_{24}. This position is identical to the end of the potential transmembrane N terminus of the sponge dermatopontin as determined according to Rao and Argos (1986). Using the same approach the potential cleavage site for human dermatopontin was identified at position aa_{18}, which is identical to that found experimentally for the native protein (Neame et al. 1989), suggesting that the sponge molecule is also secreted into the extracellular space. In the *Limulus* aggregation factor (Fujii et al. 1992) the potential cleavage site has been predicted at aa_{17} (Fig. 5A).

Five disulfide bonds have been described for the bovine dermatopontin, which are marked in Fig. 4A (Neame et al. 1989). In the sponge molecule four bonds can be predicted (Fig. 4A), indicating that the sponge molecule is also formed in a loop-like manner.

Phylogenetic Analysis of S. domuncula *Dermatopontin*

The deduced aa sequence of the sponge dermatopontin shows 22% identical aa and 37% similar aa compared to the human sequence; the degree of similarity to the *Limulus* aggregation factor is higher (30% identical aa, 50% similar aa). It is interesting to note that dermatopon-

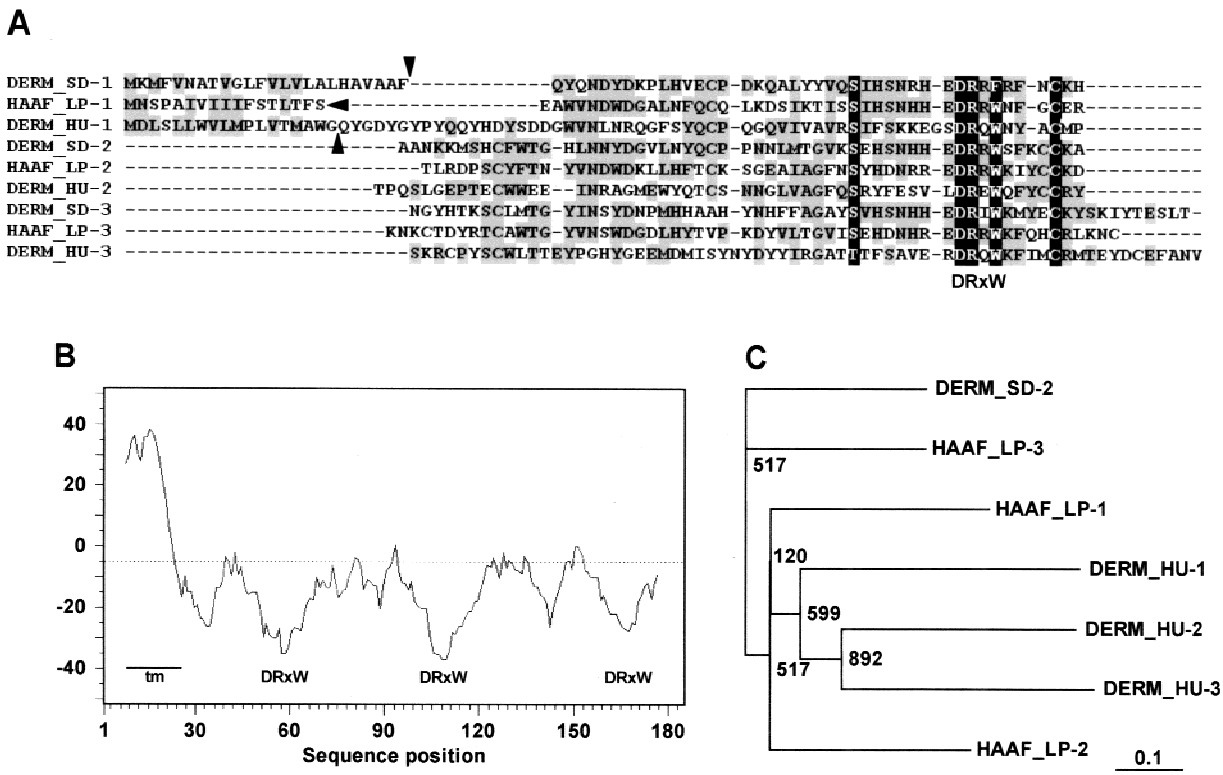

Fig. 5. Three repeating sequences of dermatopontin. **A** The sequences from *S. domuncula* (DERM_SD), *Limulus* (HAAF_LP), and human (DERM_HU) were dissected into three segments and subsequently aligned: segment 1 of *S. domuncula*, from aa_1 to aa_{66} (DERM_SD-1), segment 2, within aa_{67} to aa_{121} (DERM-SD-2), and segment 3, from aa_{122} to aa_{184} (DERM_SD-3); segment 1 of *Limulus*, from aa_1 to aa_{60} (HAAF_LP-1), segment 2, from aa_{61} to aa_{113} (HAAF_LP-2), and segment 3, from aa_{114} to aa_{172} (HAAF_LP-3); and segment 1 of human, aa_1 to aa_{79} (DERM_HU-1), segment 2, from aa_{80} to aa_{135} (DERM_HU-2), and segment 3 from aa_{136} to aa_{201} (DERM_HU-3). Residues conserved (similar or related with respect to their similar physicochemical properties) in all sequences are shown in *white on black*, and those in at least four sequences in *black on gray;* the conserved Asp–Arg–Xxx–Trp (DRxW) signature is indicated. The C termini of the transmembrane regions (*arrowheads*) of the three sequences are shown; they were determined according to Rao and Argos (1986). **B** Hydropathicity plot of the sponge dermatopontin sequence; the calculation was performed according to the method of Kyte and Doolittle (1982). The horizontal axes show the aa numbers along the protein versus the corresponding hydropathicity. The *dotted line* at the −5 value divides hydrophobic regions (above) from hydrophilic regions (below). The transmembrane region (tm) and the DRxW signatures are indicated. **C** Phylogenetic tree built by the three segments of the *Limulus* as well as the human dermatopontin, constructed using segment 2 of the *S. domuncula* sequence as an outgroup.

tin molecules have been described in mammalians (human and bovine) but not in *Drosophila melanogaster* or *Caenorhabditis elegans*. This finding confirms earlier observations which indicate that the sponge genome comprises genes which have been lost during evolution to the Protostomia (Gamulin et al. 2000), e.g., the apoptotic genes *Bcl-x* and the death domain-containing molecules (Wiens et al. 2000a, b). The fact that in *Limulus* a factor is found which comprises such a high sequence conservation to the sponge dermatopontin suggests that a dermatopontin-like molecule(s) was (were) present in the early protostomian taxa.

The bovine dermatopontin contains three conserved stretches with Asp–Arg at the N terminus (Neame et al. 1989); in *S. domuncula* this conserved region reads Asp–Arg–Xxx–Trp (Fig. 5A) and is found in the sequence at the three positions of highest hydrophilicity (Kyte and Doolittle 1982) (PC/GENE 1995; Soap), which are very likely exposed to the surface of the molecule (Fig. 5B).

Earlier studies using the bovine dermatopontin suggested that this polypeptide is composed of three related repeats (Neame et al. 1989). The sponge dermatopontin sequence was dissected into three segments at the borders adjacent to Asp–Arg–Xxx–Trp; the segments were aligned with the corresponding three segments from the human and the *Limulus* sequences (Fig. 5A). The similarity calculation reveals that the sponge segment 2 (DERM_SD-2) shows the highest similarity to the human and *Limulus* segments, with ~35% identical aa and ~50% similar aa (Fig. 5C), while the similarities of segment 1 (DERM_SD-1) and of segment 3 (DERM_SD-3) are considerably lower, with ~25% identical aa and ~35% similar aa and ~30% identical aa and ~40% similar aa, respectively (the tree is not shown). Therefore, a phylogenetic tree was constructed with sponge segment 2 only and the three segments from both human and *Limulus*. The tree, rooted with the sponge segment, shows that the sponge segment 2 builds, together with the *Limulus* segment 3, the basis of the tree from which, first, the branches of the two other *Limulus* segments and, finally,

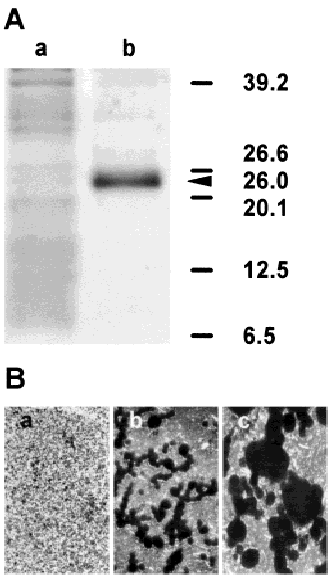

Fig. 6. Effect of recombinant dermatopontin (rDERM) on adhesion of *S. domuncula* cells. **A** Analysis of the recombinant oligohistidine–rDERM fusion protein. *SDDERM* was expressed in *E. coli;* the "bacterial crude extract" (lane a) as well as the recombinant rDERM (lane b) was purified by affinity chromatography and size-separated by 12% PAGE. In lane M the protein size markers are given. **B** Effect of rDERM on cell–cell adhesion. Single cells (a) were transferred into Ca^{2+}- and Mg^{2+}-containing seawater (b and c). In the assay shown in c rDERM was added at a concentration of 5 μg/ml original magnification, ×15.

the three human segments split off (Fig. 5C). This finding also shows that the ancestor of the dermatopontin molecule contained one segment from which the final sequence arose—very likely—by gene duplication. Computer searches with the dermatopontin segments revealed only a distant relationship to the chitinase from *Aedes aegypti* (De la Vega et al. 1998); however, no characteristic Asp–Arg–Xxx–Trp stretch was detected in the chitinase sequence.

Effect of Dermatopontin on Aggregation of S. domuncula *Cells*

Preparation of recombinant dermatopontin, rDERM, was achieved by expression of *SDDERM* in *E. coli* as recombinant oligohistidine–rDERM fusion protein as described under Materials and Methods. The bacterial crude extract was analyzed by PAGE (Materials and Methods) (Fig. 6A). The staining pattern of proteins obtained from induced cultures (Fig. 6A, a), as well as of the purified fusion protein, rDERM, is shown (Fig. 6A, b). The recombinant protein preparation is almost completely pure; a size of 26 kDa was determined. The bacterial crude extract from noninduced cultures did not contain rDERM (not shown).

Dermatopontin causes an increase in the degree of aggregation of single cells obtained by dissociation in Ca^{2+}- and Mg^{2+}-free artificial seawater. If these cells are transferred to Ca^{2+}- and Mg^{2+}-containing seawater, they form aggregates of 190 ± 38 μm after an incubation period of 30 min. If rDERM at a concentration of 5 μg/ml is added to the cells, the size of the aggregates increases to 440 ± 65 μm. The images of the single cells (Fig. 6B, a) as well as of the aggregates formed in the absence or presence of dermatopontin are shown (Fig. 6B, b and c).

Previous functional studies with dermatopontin revealed that in mammalian systems this polypeptide displays a striking tendency to bind to dermatan sulfate proteoglycans (Neame et al. 1989; Okamoto et al. 1996) and promotes cell attachment (Lewandowska et al. 1991). Furthermore, it has been demonstrated that dermatopontin binds to collagen and accelerates the assembly of its fibrils (MacBeath et al. 1993). In *Limulus* the related molecule is likewise an adhesion molecule (Fujii et al. 1992). Based on the finding with the sponge recombinant dermatopontin, it can be proposed that this molecule has conserved not only its sequence but also its function—cell adhesion and ECM molecule—during evolution within the metazoan kingdom.

Regulation of Collagen Expression in S. domuncula *Primmorphs by Morphogens*

It is reasonable to assume that molecules have changed their function during evolution. An example is βγ-crystalline, which has been isolated from *G. cydonium*, where it might function as a protection molecule against dryness (Krasko et al. 1997), while this molecule is the major class of soluble proteins in lenses of mammals (Wistow and Piatigorsky 1988). Therefore, it is necessary for the understanding of the functional molecular evolution of Metazoa also to elucidate—at least on selected examples—whether the function of molecules obtained from distantly related taxa, e.g., Porifera and Mammalia, share the same or closely related roles in the organism.

Recently, two potential morphogens have been identified and cloned from sponges: endothelial monocyte-activating polypeptide (EMAP) from *G. cydonium* (Pahler et al. 1998a) and myotrophin from *S. domuncula* (Schröder et al. 2000). These two molecules received great interest and helped to clarify the etiology of human disease: EMAP as an antitumor cytokine (Schwarz et al. 1999) and cardiac myotrophin, which stimulates protein synthesis in myocytes, suggesting a crucial role in the formation of cardiac hypertrophy (reviewed by Sil et al. 1998). To identify the role of these two morphogens in sponges the polypeptides were prepared recombinantly. The procedure for the preparation of purified recombinant EMAP (rEMAP) is described here, while the preparation of the recombinant myotrophin (rMYO) was described previously (Schröder et al. 2000). These two proteins were used to study their effect on the expression of collagen in primmorphs.

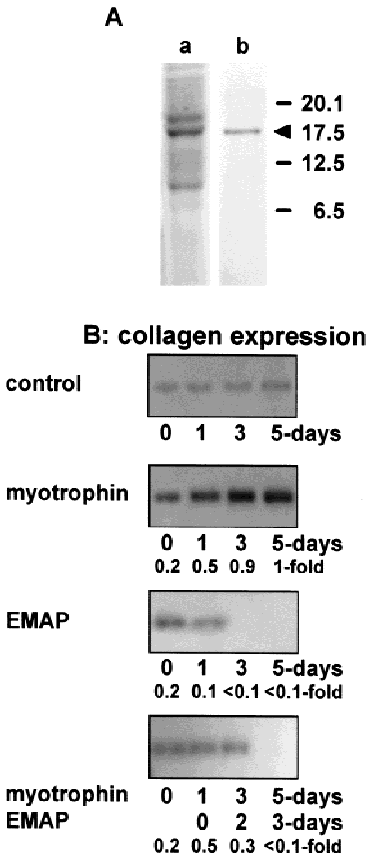

Fig. 7. Effect of recombinant EMAP on the expression of collagen (transcript size: ~1.1 kb) in primmorphs from *S. domuncula.* **A** Preparation of rEMAP. The sponge cDNA was expressed in *E. coli;* subsequently the bacterial cells were lysed and the bacterial crude extract (lane a) and the purified rEMAP (lane b) were analyzed by 12% PAGE. **B** Effect of rEMAP on the myotrophin-induced expression of the collagen gene. The control assays were not supplemented with morphogens. In parallel, rMYO (3 μg/ml) or rEMAP (5 μg/ml) was added to the cultures and incubation proceeded for up to 5 days. In one series rMYO was present in the cultures during the complete 5-day incubation period; at day 2 the cultures were coincubated, in addition, with 5 μg/ml of rEMAP for the final 3 days. RNA was extracted, and the level of collagen expression determined semiquantitatively by Northern blotting. The levels of expression are correlated with the highest value measured at day 5 in the assays with rMYO alone; this value was arbitrarily set at onefold. Five micrograms was applied per slot.

rMYO. In the absence of any morphogen, the expression level of collagen (size of transcript, ~1.1 kb) in primmorphs is low (Fig. 7B; control). The addition of rMYO (3 μg/ml) caused a dramatic increase in the steady-state level of collagen transcripts; this effect could be seen after just 1 day and the maximum was reached after 5 days (Fig. 7B; myotrophin).

rEMAP. The rEMAP was expressed and purified as an oligohistidine fusion polypeptide. The Western blot of oligohistidine fusion polypeptide, present both in the bacterial crude extract (Fig. 7A, a) and after purification (Fig. 7A, b), is shown. The size of the molecule was determined to be 17.5 kDa (including the six-histidine tag), while the M_r of the polypeptide, deduced from the ORF, is 16.5 kDa (Pahler et al. 1998a).

The addition of rEMAP (5 μg/ml) caused an almost-immediate downregulation of collagen gene expression (Fig. 7B; EMAP). The reduction could be measured after just 1 day of incubation. If rEMAP is coincubated with rMYO, rEMAP causes a block of the myotrophin-mediated collagen gene expression (Fig. 7B; myotrophin/EMAP). The addition of rEMAP to the cultures after 1 day of treatment with rMYO suppresses collagen expression almost totally.

Conclusion

The formation of the ECM is a highly complex process. Even though only a few structural molecules have been identified until now in sponges, e.g. collagen, dermatopontin, and mucus-like protein, these examples are sufficient to document that sponge cells are embedded in a matrix which allows reversible stabilization within the organism and provides the basis for integrated cell communication. These fibrous proteins interact with cell surface receptors, e.g., the receptor tyrosine kinase(s), or integrins, cell surface molecules which are restricted to Metazoa. The complex network of secreted proteins that builds the extracellular space between the sponge cells is also a reservoir for morphogens, controlling cell growth and differentiation. Thus sponges are provided with key molecules that allowed the evolution of multicellularity. Even more, since the molecules display a high sequence similarity/homology to related molecules in other metazoan phyla, this also supports the view that sponges have a common evolutionary origin with the more complex metazoan animals. The hypothetical common ancestor for all Metazoa has been termed Urmetazoa (Müller and Müller 2000; Müller 2001). Molecular clock estimates are controversial, but those estimates suggest an evolution to the Urmetazoa of about 800 million years (see introduction) or possibly even of about 1000 million years (Nikoh et al. 1997). The introduction of the term Urmetazoa allows us to avoid the distinction of multicellular animals into Parazoa (“alongside” animals), Mesozoa (“middle” animals), and Eumetazoa (“true” animals), terms which imply qualitative assessments that appear not to be helpful for the systematic classification based on quantitative analyses. In 1884 the Porifera were given the subkingdom status of Parazoa by Sollas.

In the present study major novelties which can be attributed to the Urmetazoa have been summarized in an exemplary way. It is shown that Porifera (sponges), the first phylum which evolved from the hypothetical ancestor, the Urmetazoa, are provided (a) with ECM proteins, such as the mucus-like protein, that interact with cell surface receptors, here with the Ig-like domains of the RTK, and (b) with a variety of ECM molecules includ-

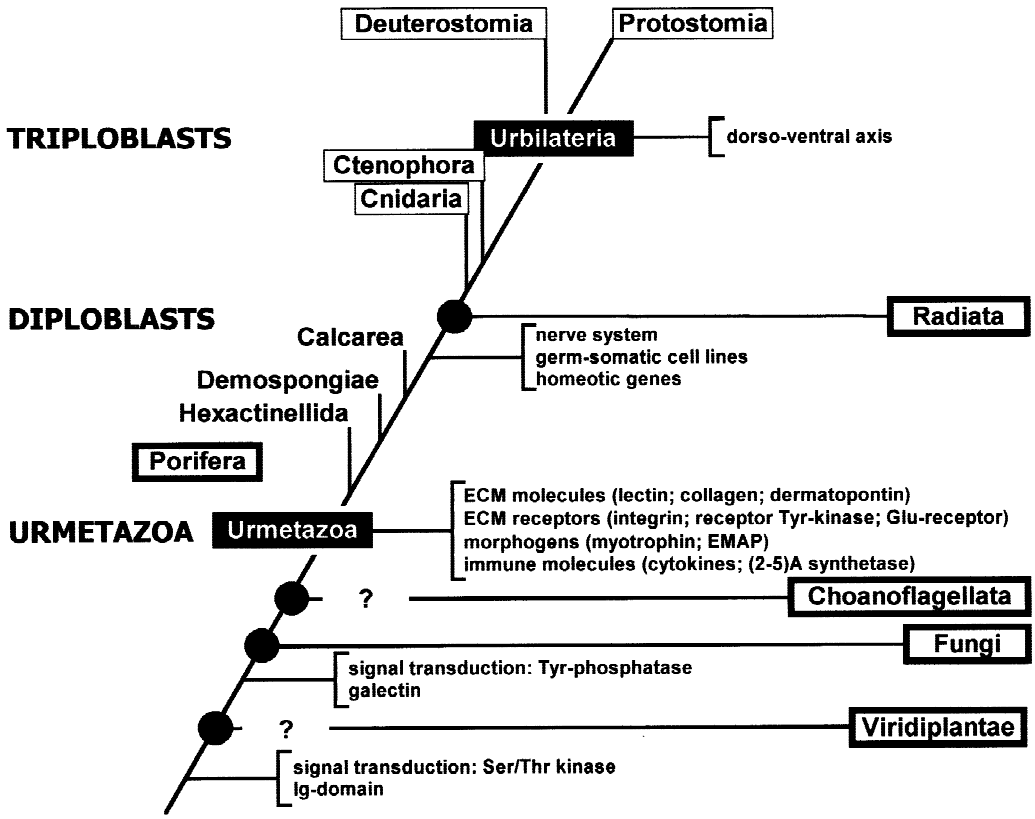

Fig. 8. The proposed relationships of multicellular eukaryotes. The cladogram represents relationships with well-defined monophyletic assemblages. The hypothetical Urmetazoa is provided with the novelties, the newly identified ECM molecules, their receptors, and morphogens which regulate their expression. Molecular analyses indicate that the Hexactinellida branched off first, while the Demospongiae and Calcarea appeared later in evolution. The diploblastic taxon Radiata is defined by the presence of nerve, germ, and somatic cells as well as HOX genes, which constitute the anterior–posterior body axis. The triploblastic Urbilateria appeared later in evolution. It might be hypothesized that the Choanoflagellata diverged from the Urmetazoa. The close relationship between Fungi and Metazoa was established by molecular data. It remains open whether the Viridiplantae form a common lineage with the Fungi. Further details are given in the text.

ing, besides the characteristic collagen, further classes of proteins, e.g., dermatopontin. In addition, it is documented that (c) the ECM contains morphogens, e.g., myotrophin and EMAP, which allow coordinated expression of the fibrous protein, with collagen as an example. The ECM proteins interact with their receptors. In response to these interactions, signal transduction pathways are initiated, which effect the intracellular metabolism. Examples of interactions of extracellular ligands—proteins or low molecular weight molecules—with their corresponding receptors which result in signaling events in the cytoplasm have recently been described in the sponges *G. cydonium* and *S. domuncula*, e.g. interaction of the adhesion motif peptide RGD(S) with integrin (Wimmer et al. 1999a, b), glutamate or quisqualic acid with the metabotropic glutamate/GABA-like receptor (Perovic et al. 1999), and mucus-like protein with the Ig-like domains of the RTK (in this study). The elucidation of further ligand–receptor interactions, e.g., the interaction of EMAP or myotrophin with their unknown receptors, is in progress. In spite of intense screening, cDNA coding for high molecular weight fibronectin or proteins forming the backbone of proteoglycans has not been successful until now. In addition, immune molecules, cytokines, and (2′, 5′)-oligoadenylate synthetase, as a member of the interferon-mediated pathway, already exist in sponges (Fig. 8). Detailed analyses of housekeeping proteins, e.g., heat shock protein (Koziol et al. 1997) and β-tubulin (Schütze et al. 1999), and proteins involved in signal transduction, e.g., Ser/Thr kinase (Kruse et al. 1997 and 1998) and calmodulin (Schütze et al. 1999), revealed that among the three classes of Porifera, the Hexactinellida are the phylogenetically oldest taxon, while Calcarea is the class closest related to higher metazoan phyla (Fig. 8).

Later in evolution the diploblastic Metazoa—phyla Cnidaria and Ctenophora—appeared which are characterized by true epithelial layers (Fig. 8). Besides on expanding systems of signaling proteins and transcription factors, e.g., the *Para*/*Hox* clusters (homeobox genes), which pattern the main body axis, the organization of these animals is based on those building blocks which are already present in Porifera, e.g., cell surface receptors. One further feature of the diploblastic Metazoa is the novel acquisition of developmental characters that distinguish between germ and somatic cells. In cnidar-

ians germ cells arise from interstitial cells (reviewed by Müller 2001).

The basic genetic principles of the morphological and developmental processes which gave rise to the triploblastic Metazoa, to the arthropod (Protostomia) and mammalian (Deuterostomia) lineages, have been elucidated in recent years; a hypothetical ancestral animal has been proposed for both of them, named Urbilateria (DeRobertis and Sakai 1996; Knoll et al. 1999) (Fig. 8). The Urbilateria possess an array of intracellular signaling systems and a divergent complement of transcription factors allowing the evolution of a simple mode of bilaterian development (reviewed by DeRobertis and Sakai 1996).

An important piece of evidence for the proposition that all multicellular eukaryotic organisms evolved from a common ancestor is the fact that in the kingdom Fungi, with *Coprinus cinereus* as an example (Cooper et al. 1997), galectins have already been identified. Galectins diverged into a huge family in Metazoa but are absent in Viridiplantae and Protozoa (Fig. 8). Hence, the galectins can be considered as one autopomorphic character for Fungi and Metazoa. Furthermore, analysis of the receptor tyrosine phosphatase (Tyr-phosphatase) from *G. cydonium* revealed that the two PTPase domains, present in that receptor, are related to the domains already found in yeasts (reviewed by Müller 2001). Until now the PTPases have been identified only in Metazoa and Fungi. Some molecules present in yeast and Metazoa have been identified in Viridiplantae: the Ser/Thr kinases (reviewed by Kruse et al. 1996) and polypeptides, comprising Ig-like domains (reviewed by Müller 2001). These findings might be taken as a first hint that the major kingdoms of multicellular organisms had a common ancestor (Fig. 8).

The phylogenetic position of the Choanoflagellata remains a problem. Unfortunately only rDNA sequences have been presented, which suggest a close relationship to the Metazoa (Wainright et al. 1993). However, an extended statistical analysis could not substantiate this grouping (Kumar and Rzhetsky 1996), and it may be considered not to be as accurate as originally thought (Maley and Marshall 1998). Considering the similarity of the choanocytes, found in Choanoflagellata and Porifera (Cantell et al. 1982), it appears not to be unlikely that the Choanoflagellata branched off from the Fungi–Metazoa lineage (Fig. 8).

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