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A genome-wide analysis of biomineralization-related proteins in the sea urchin *Strongylocentrotus purpuratus*

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Abstract

Biomineralization, the biologically controlled formation of mineral deposits, is of widespread importance in biology, medicine, and engineering. Mineralized structures are found in most metazoan phyla and often have supportive, protective, or feeding functions. Among deuterostomes, only echinoderms and vertebrates produce extensive biomineralized structures. Although skeletons appeared independently in these two groups, ancestors of the vertebrates and echinoderms may have utilized similar components of a shared genetic "toolkit" to carry out biomineralization. The present study had two goals. First, we sought to expand our understanding of the proteins involved in biomineralization in the sea urchin, a powerful model system for analyzing the basic cellular and molecular mechanisms that underlie this process. Second, we sought to shed light on the possible evolutionary relationships between biomineralization in echinoderms and vertebrates. We used several computational methods to survey the genome of the purple sea urchin *Strongylocentrotus purpuratus* for gene products involved in biomineralization. Our analysis has greatly expanded the collection of biomineralization-related proteins. We have found that these proteins are often members of small families encoded by genes that are clustered in the genome. Most of the proteins are sea urchin-specific; that is, they have no apparent homologues in other invertebrate deuterostomes or vertebrates. Similarly, many of the vertebrate proteins that mediate mineral deposition do not have counterparts in the *S. purpuratus* genome. Our findings therefore reveal substantial differences in the primary sequences of proteins that mediate biomineral formation in echinoderms and vertebrates, possibly reflecting loose constraints on the primary structures of the proteins involved. On the other hand, certain cellular and molecular processes associated with earlier events in skeletogenesis appear similar in echinoderms and vertebrates, leaving open the possibility of deeper evolutionary relationships.

Keywords: Biomineralization; Genome; Echinoderm

Introduction

Biomineralization is widespread among prokaryotic and eukaryotic organisms. Among present-day metazoans, biominerals composed of calcium carbonate (usually calcite, magnesian calcite, or aragonite), calcium phosphate (usually apatite), or silica (opal) are found in most phyla and often function as supportive, protective, or feeding structures (Bengtson, 1994). In humans, loss of the normal regulatory control of biomineralization contributes to a variety of pathologies. In addition to their important natural functions, biomineralized structures have unique physical properties that may help guide the design of composite materials. An understanding of the molecular basis of biomineralization is therefore of widespread importance in biology, medicine, and engineering.

Sea urchins and other echinoderms have an extensive endoskeleton composed of magnesian calcite (a form of calcium carbonate that contains small amounts of magnesium carbonate) and occluded matrix proteins. All adult echinoderms form

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endoskeletal elements, although these are more elaborate in some groups of echinoderms than others. Adult sea urchins have several calcified structures, including the test, teeth, and spines. In addition, the larvae of some echinoderms (sea urchins, sand dollars [Class Echinoidea], and brittle stars [Class Ophiuroidea]) have extensive, calcitic endoskeletons. In these organisms, biomineral formation begins at early embryonic stages.

The development of the skeleton of the sea urchin embryo has been studied extensively (see reviews by Ettensohn et al., 1997; Wilt, 2002, 2005 Wilt et al., 2003). The skeleton is produced by a specialized population of embryonic cells, the primary mesenchyme cells (PMCs). PMCs are the descendants of the large micromeres, four cells that form at the vegetal pole of the embryo at the 32-cell stage. At the beginning of gastrulation, PMCs undergo an epithelial–mesenchymal transition and ingress from the wall of the blastula into the blastocoel cavity. After ingression, the cells migrate in the blastocoel and fuse with one another via filopodial protrusions, forming a syncytial network. As they migrate, the PMCs gradually accumulate in a characteristic ring-like pattern along the blastocoel wall. Skeletogenesis begins at the mid-gastrula stage with the formation of two triradiate spicule rudiments on the oral side of the embryo. The biomineral is deposited within a privileged extracellular space enshrouded by the fused filopodial processes of the PMCs. During later embryogenesis, the two spicule rudiments elongate and branch in a stereotypical fashion, forming the two spicules of the young pluteus larva. The spicules influence the shape, orientation, and swimming behavior of the larva (Pennington and Strathmann, 1990). Additional skeletal elements arise later in larval development. The cells that produce these elements express molecular markers that are restricted to PMCs at embryonic stages, but it is not known whether the skeletogenic cells found at later stages of larval development are derived from PMCs or from a different lineage of embryonic cells.

Considerable progress has recently been made in elucidating the molecular basis of PMC specification. Many components of a gene regulatory network that operates in the large micromere–PMC lineage have been identified. Upstream components of this network include several maternal proteins as well as early zygotic transcription factors (Kurokawa et al., 1999; Logan et al., 1999; Fuchikami et al., 2002; Oliveri et al., 2002; Amore et al., 2003; Ettensohn et al., 2003; Weitzel et al., 2004). Downstream of these maternal factors and transcriptional regulators are gene products that directly control the morphogenetic behaviors of PMCs; viz., the ingression, migration, and fusion of the cells and their deposition of the biomineralized endoskeleton.

Among the proteins involved in biomineralization are the spicule matrix proteins, secreted proteins contained within the spicule and closely associated with the mineral. Killian and Wilt (1996) identified nearly four dozen proteins comprising the integral spicule matrix using two-dimensional gel electrophoresis. Seven genes have been cloned that encode spicule matrix proteins: *SM50* (Benson et al., 1987; Katoh-Fukui et al., 1991; Killian and Wilt, 1996), *SM30α* (George et al., 1991; Akasaka et al., 1994; Killian and Wilt, 1996), *PM27* (Harkey et al., 1995), *SM37* (Lee et al., 1999), *SM29*, *SM32*, and *SpC-lectin* (Illies et al., 2002). These proteins contain a single C-type lectin (CTL) domain and often proline/glycine-rich repeats (see Illies et al., 2002). During embryogenesis, spicule matrix proteins are expressed only by PMCs. Antisense knockdown experiments have shown that SM50 is essential for normal skeletal rod elongation (Peled-Kamar et al., 2002). PMCs also express a family of cell surface proteins consisting of MSP130 and at least two MSP130-related proteins (MSP130-related-1 and -2), that may function in biomineralization (Leaf et al., 1987; Farach-Carson et al., 1989; Illies et al., 2002). In addition, a novel, PMC-specific transmembrane protein, P16, is essential for skeletal rod elongation (Cheers and Ettensohn, 2005).

Sea urchins and other echinoderms are deuterostomes, a multiphyletic clade that also includes the vertebrates, urochordates (tunicates), hemichordates, and cephalochordates. Among deuterostomes, only the vertebrates and echinoderms form extensive biomineralized structures. Possible relationships between the genetic programs of biomineralization in these two groups have not been systematically explored. It is widely accepted that the earliest vertebrates lacked biomineralized tissues and, therefore, that the skeletons of vertebrates and echinoderms are not homologous in the strictest sense (Donoghue and Sansom, 2002). Although biomineralized structures probably appeared independently in the echinoderms and vertebrates, certain embryological and molecular programs already established in the ancestral deuterostome may have been exploited in similar ways in these two groups to carry out biomineralization. Biomineralization in echinoderms and vertebrates may therefore be related through the deployment of common elements of a pre-existing cellular and genetic toolkit.

In the present study, we used several methods to survey the genome of the purple sea urchin *Strongylocentrotus purpuratus* for proteins involved in biomineralization. Our analysis has significantly expanded the collection of biomineralization-related proteins. We also systematically searched the *S. purpuratus* genome for counterparts of gene products that play a role in biomineralization in vertebrates. Our findings reveal substantial differences in the proteins that mediate biomineral formation in echinoderms and vertebrates. There are, however, certain similarities in molecular and cellular features of biomineralization in the two groups that leave open the possibility of deeper evolutionary relationships. As a whole, our findings advance our understanding of the molecular and developmental pathways that underlie skeletogenesis in echinoderms and shed light on the evolution of biomineralization within the deuterostomes.

Materials and methods

Computational approaches

Identification of biomineralization-related proteins in S. purpuratus

We surveyed the *S. purpuratus* whole genome shotgun (WGS) assembly (07/18/2005) for proteins involved in biomineralization using the following computational methods:

(1) We queried the complete collection of predicted *S. purpuratus* proteins with each of the known sea urchin biomineralization-related proteins

using BLAST (Altschul et al., 1997) to identify new, closely related proteins. In most cases, we also queried the set of gene predictions and the WGS assembly at the nucleotide level. BLAST searches were carried out remotely using the public site created by the Human Genome Sequencing Center at the Baylor College of Medicine (http://www.hgsc.bcm.tmc.edu/blast/blast.cgi?organism=Spurpuratus).

(2) Because sea urchin biomineralization genes are often physically clustered (see Results), we examined all DNA scaffolds that contained known biomineralization genes to identify closely linked genes that might represent new members of this class. Candidate proteins were identified through predicted sequence similarity to known biomineralization proteins and/or evidence that the corresponding mRNAs were abundant and PMC-specific, as reflected by chip hybridization data and the spectrum of matching ESTs (see 4 below).

(3) In a search specifically for new spicule matrix proteins, we identified and analyzed all predicted proteins that contained a signal sequence and a single CTL domain but not other protein motifs. Domain searches were performed locally on the Beowulf cluster of the Center for Computational Regulatory Genomics, Beckman Institute at California Institute of Technology. The IPRSCAN software Version 4.2 from EBI (InterPro Consortium, 2001; ftp://ftp.ebi.ac.uk/pub/databases/interpro/iprscan) and a variety of Hidden Markov Model databases were used. These included the two most commonly utilized sets, PFAM (Bateman et al., 2004) and SMART (Letunic et al., 2006). In order to be backward compatible with searches of previous genomic sequence releases, the predictions were queried using HMMER, version 2.3.2 (Eddy, 1998) and the latest PFAM database, version 18. SignalP 3.0 (http://www.cbs.dtu.dk/services/SignalP/) was used to identify predicted signal sequences.

(4) Using a robotically arrayed cDNA library generated from gastrula-stage PMCs (Zhu et al., 2001), a collection of 51,000 EST sequences was produced (Genbank entries BG780044–BG789446, DN560823–DN586179, and DN781759–DN810571). These ESTs were mapped to the collection of gene models to create a comprehensive list of genes expressed by PMCs at a stage when the cells are engaged in biomineralization. The genes in this list were ordered based on the numbers of matching ESTs. Because the PMC cDNA library was not normalized, the number of ESTs that matched a particular gene model provided a reliable estimate of the abundance of the transcript (see Zhu et al., 2001). In addition, for any candidate gene, a substantial enrichment in matching ESTs from the PMC collection relative to other EST sources was found to be a reliable predictor of PMC-restricted expression as assayed by whole mount in situ hybridization. The other two major sources of ESTs from *S. purpuratus* are a non-redundant catalogue of 20,000 EST clusters derived from several developmental stages (Poustka et al., 2003) and a set of 36,522 ESTs derived from a normalized mesenchyme blastula stage library (contributed to Genbank by J. Coffman and collaborators at Washington University). A more complete analysis of the PMC EST collection and gene list will be published separately (Ettensohn et al., in preparation).

(5) Based upon literature searches, we compiled a comprehensive list of proteins that have been implicated in biomineralization in vertebrates, including transcription factors, extracellular matrix (ECM) proteins, and ECM-modifying enzymes. We queried the *S. purpuratus* gene models with these protein sequences using BLAST to identify candidate sea urchin homologues. Putatively homologous sequences were used in reciprocal BLASTs against the NCBI sequence databases to confirm the similarity.

Other computational approaches

We searched for homologues of sea urchin biomineralization genes in closely related deuterostomes by matching *S. purpuratus* biomineralization-related genes against EST collections from the cephalochordate *Branchiostoma floridae* (amphioxus) and the enteropneust hemichordate *Saccoglossus kowalewski*. Panopoulou and coworkers (2003) have generated a non-redundant catalogue of 14,000 EST clusters from gastrula and neurula stages of *B. floridae*. Sea urchin gene models or documented cDNA sequences were blasted against both the clustered EST sequences (http://www.molgen.mpg.de/~amphioxus/) and the individual ESTs (http://www.ncbi.nih.gov). For searches of the S. *kowalewski* genome, 104,451 ESTs from the trace repository at NCBI were downloaded and collected into a blast database. BLAST analysis was carried out locally using the stand-alone BLAST software BLASTALL version 2.2.6 (Altschul et al., 1997).

Multiple sequence alignments were performed using ClustalW or ClustalX (1.8) and tree construction was carried out using TreeView (Win32) version 1.6.6, NJPlot, MEGA, or PAUP 4.0 (neighbor-joining method).

Chip hybridization data

As one means of assessing the embryonic expression of sea urchin genes, we used the chip hybridization data of Samanta et al. (2006). Briefly, Samanta and coworkers designed ~11 million 50-mer oligonucleotides that covered the entire sequenced *S. purpuratus* genome with a gap of 10 nt between consecutive oligos. The oligonucleotides were synthesized on glass slides (~400,000 oligos/slide) and hybridized with probe generated from a mixed pool of poly-A RNA from egg, early blastula, gastrula, and prism stage *S. purpuratus* embryos. Samanta et al. (2006) quantified the amount of probe that hybridized to each of the oligonucleotides. These data provide a global picture of genomic sequences that are transcribed into mRNA during embryogenesis.

RT-PCR analysis

Total RNA was isolated from *S. purpuratus* embryos and adult tissues as described by George et al. (1991) and Richardson et al. (1989). RT-PCR analysis was carried out using the One-Step RT-PCR kit (Qiagen). cDNA was generated from 0.1 μg of total RNA. PCR was performed using the following primers: *SpSM30A*—(5′-CGGTGGTGGTGATCCCTTTTAC-3′) and (5′-GTAGCTGGAGTATCCGTAGTTGG-3′); *SpSM30B*—(5′-CGTATTGGCTTTGGCCTCTTTC-3′) and (5′-GGTAGGTGGTGTGATTGGG-3′); *SpSM30C*—(5′-CGTATTGGCTTTGGCCTCTTTC-3′) and (5′-GGGTCGATGGTGTGACTAGA-3′); *SpSM30D*—(5′-GACTGGACAACCGCGTATGCAAC-3′) and (5′-CATGGTAGCTCCTGGCTGCGTTG-3′); *SpSM30E*—(5′-GTCGTATTGATCCCGTAGTTCACG-3′) and (5′-CATCCTGGTGACAGGCATCCTAG-3′); *SpSM30F*—(5′-GAACCACCCCATCACAGCTGGTC-3′) and (5′-GGCAGTTTAGTTGTCCCATAACCGTTG-3′); SPU_012518—(5′-GACGTTGTTGTACCAAGAATGTTTAG-3′) and (5′-TCCGTTGCCGTTCCACCAAC-3′); SPU_027906—(5′-GATGGACGTACTCCCTCAAG-3′) and (5′-ACTTCGCATGGGGCCAATG-3′); *SpUbiquitin* (SPU_015276—(5′-GACAGCATCGAGAATGTCAAG-3′) and (5′-CAGGTCATTGGAGTGTCC). Thirty two thermal cycles were performed for each primer set. The annealing temperature for each PCR reaction was optimized.

In situ hybridization

Whole mount in situ hybridizations were performed according to Zhu et al. (2001).

Results

Analysis of known classes of biomineralization-related proteins in S. purpuratus

Spicule matrix proteins

Seven proteins were previously identified as likely spicule matrix proteins (Benson et al., 1987; George et al., 1991; Katoh-Fukui et al., 1991; Harkey et al., 1995; Killian and Wilt, 1996; Lee et al., 1999; Illies et al., 2002). Five of these proteins (PM27, SM29, SM32, SM37, and SM50) have similar structures and were previously suggested to constitute a subfamily (Illies et al., 2002). These proteins have a basic p*I* and contain a signal sequence, one CTL domain, and a region that contains a variable number of proline-rich repeats. The remaining two spicule matrix proteins, SM30α and C-lectin,

also contain a signal sequence and CTL domain but have an acidic p*I*. A portion of another SM30 protein, designated SM30β, was also cloned (Akasaka et al., 1994).

In the present study, we identified unique gene models corresponding to each of these gene products (*SpPM27*=*SPU_028945*, *SpSM29*=*SPU_005990*, *SpSM32*=*SPU_018810*, *SpSM37*=*SPU_018813*, *SpSM50*=*SPU_018811*, *SpSM30α*=*SPU_000826*, *SpSM30β*=*SPU_000827*, and *SpC-lectin*=*SPU_007882*). Previous evidence suggested that SM32, SM37, and SM50 were encoded by genes clustered in the genome (Lee et al., 1999; Illies et al., 2002). The genome assembly confirms that that these genes are clustered on Scaffold77437. Illies et al. (2002) reported that the sequences of *SpSM32* and *SpSM50* mRNAs were identical at their 5′ ends and suggested that they might be products of alternative splicing. This is indeed the case, and we propose that the single gene encoding both proteins should be designated *SpSM50*. *SpSM30α* and *SpSM30β* lie adjacent to one another as reported by Akasaka et al. (1994).

We searched for new spicule matrix genes in the *S. purpuratus* genome by (1) querying the complete set of predicted proteins with each of the known spicule matrix proteins, (2) examining genes that were closely linked to known spicule matrix genes (i.e., located on the same DNA scaffolds), and (3) searching for proteins that contained a predicted signal sequence and a single CTL domain but not other common protein motifs. The third strategy led to the identification of thirty-three proteins with this general domain structure, not including the previously identified spicule matrix proteins. We analyzed these proteins further to identify any that contained proline-rich repeats, were expressed by PMCs as evidenced by matching ESTs from the PMC EST collection, or were encoded by genes that were closely linked to known spicule matrix genes.

Using this combination of approaches, we identified eight new candidate spicule matrix genes. We found that *SpSM30α* and *SpSM30β* are part of a cluster of four closely related, tandemly arranged genes that we designate *SpSM30-A* (*SPU_000825*), *SpSM30-B* (formerly *SpSM30α*) (*SPU_000826*), *SpSM30-C* (formerly *SpSM30β*) (*SPU_000827*), and *SpSM30-D* (*SPU_000828*). We also identified two other *SM30*-like genes, *SpSM30-E* (*SPU_004867*) and *SpSM30-F* (*SPU_004869*). These genes are located on a separate scaffold in the WGS assembly but a more recent, provisional, BAC-based assembly (03/16/2006) suggests that all six *SM30*-like genes are arranged in tandem on a single scaffold. In addition to the four new *SM30*-like genes, we identified three candidate spicule matrix genes (*SPU_005989*, *SPU_005991*, and *SPU_005992*) that immediately flank *SpSM29* on Scaffold253 and another (*SPU_027906*) located ~165 kb distant from *SpPM27* on Scaffold1445.

A diagram of the general domain structures of the eight new putative spicule matrix proteins and the previously identified spicule matrix proteins is shown in Fig. 1. In addition, a ClustalX alignment of the six SM30-like proteins is shown in Fig. 2. Each new putative spicule matrix protein contains a predicted signal sequence and a CTL domain, although in several cases (e.g., SPU_005992, SPU_027906, and SpSM30-E) the CTL domain appears to be incomplete. Most of these proteins contain extensive proline-rich regions, usually consisting of 3–6 amino acid repeats very similar in sequence to those described for other spicule matrix proteins. For example, SPU_005992 contains an extensive region of PNX repeats, with X usually N or Q, that are similar to those observed in SpSM29 and SpSM32. These repeats make up most of the SPU_005992 protein and are organized into larger repeating units. Similarly, SPU_027906 contains a region of PXY repeats (with X usually N, G, or H) and the N-terminal half of SpSM30-E consists of slightly longer proline-rich repeats (5–6 residues).

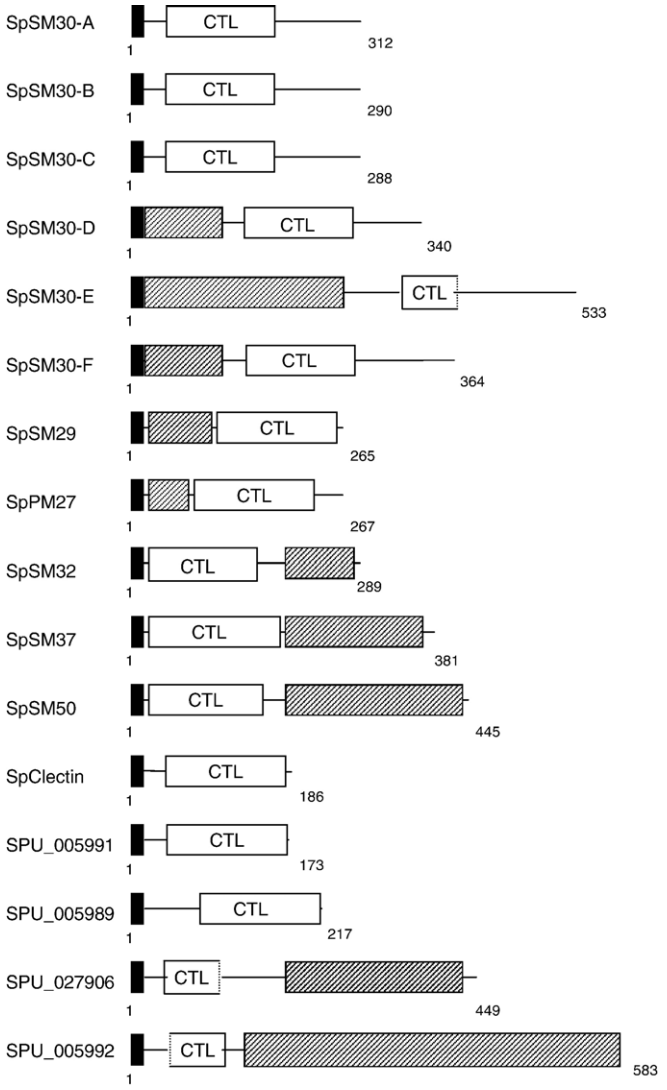

Fig. 1. Domain structures of spicule matrix family proteins. Black box=signal sequence, hatched box=proline-rich repeat domain, CTL=C-type lectin domain.

Several of the newly identified genes are transcribed during embryogenesis. Of the four new *SM30*-like genes, *SpSM30-A* and *SpSM30-E* are expressed at relatively high levels as determined by EST counts, chip hybridization data, and RT-PCR analysis, while *SpSM30-D* and *SpSM30-F* show relatively low levels of embryonic expression. The numbers of PMC ESTs that match *SpSM30-A* and *SpSM30-E* (30 and 14, respectively) are similar to those that match the previously identified genes *SpSM30-B* and *SpSM30-C* (28 and 29, respectively). *SpSM30-*

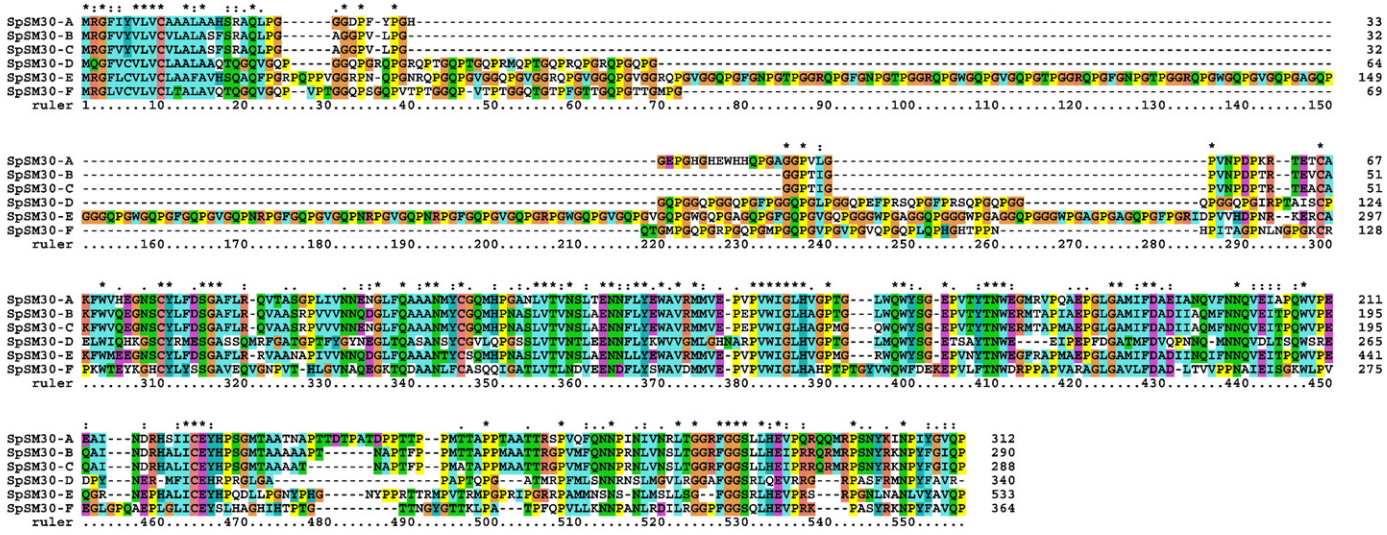

Fig. 2. ClustalX alignment of the six proteins of the SpSM30 subfamily.

A and *SpSM30-E* also show strong chip hybridization signal. In contrast, *SpSM30-D* and *SpSM30-F* show little or no chip hybridization signal and do not match any ESTs from the three major EST collections. RT-PCR studies demonstrate that *SpSM30-A* and *SpSM30-E* are expressed zygotically by the prism stage when skeletogenesis is underway and confirm that *SpSM30-D* and *SpSM30-F* are expressed only at very low levels at this stage (Fig. 3). *SpSM30-D* and *SpSM30-F* are expressed at very high levels selectively in adult spines, however, and appear to be the most abundant of the *SM30*-like transcripts in this mineralized tissue (Fig. 3). These findings are in accord with those of Killian and Wilt (1996), who performed immunoblots by probing adult spine matrix proteins with an anti-SM30 antibody and observed a protein doublet that had a different apparent molecular weight from that observed in embryonic spicule matrix proteins. From these results, Killian and Wilt (1996) suggested that there might be an adult form of SM30.

Of the four remaining putative spicule matrix genes, *SPU_027906* exhibits strong chip hybridization signal and matches a large number of PMC EST sequences (32 ESTs). This gene is expressed in prism stage embryos and in adult spine based on RT-PCR analysis (Fig. 3). *SPU_005989* and *SPU_005991* have lower numbers of PMC EST matches (4 and 13, respectively) and lower chip hybridization signal. *SPU_005992* exhibits strong chip hybridization signal and many EST matches. Almost all of these ESTs are from the collection derived from whole mesenchyme blastula stage embryos, however, while none is from the PMC EST collection. This suggests that the protein encoded by *SPU_005992*, which shows every sequence hallmark of a spicule matrix protein, might be expressed by cell types other than PMCs during embryogenesis.

Proteins of the MSP130 family

MSP130 was the first PMC-specific protein to be identified (Leaf et al., 1987). More recently, two closely related gene products, MSP130-related-1 and MSP130-related-2, were identified and shown to be expressed only by PMCs (Illies et al., 2002).

To search for new members of the MSP130 family, we used the sequences of the three known proteins to query the collection of gene models by protein–protein BLAST. This approach identified four new MSP130-related proteins, which we named MSP130-related-3, -4, -5, and -6 (encoded by *SPU_013823*, *SPU_014496*, *SPU_015763*, and *SPU_014492*, respectively). Additional gene models were identified that were nearly identical to MSP130 and MSP130-related-2, -3, and -6, but because it seemed likely that some or all of these models

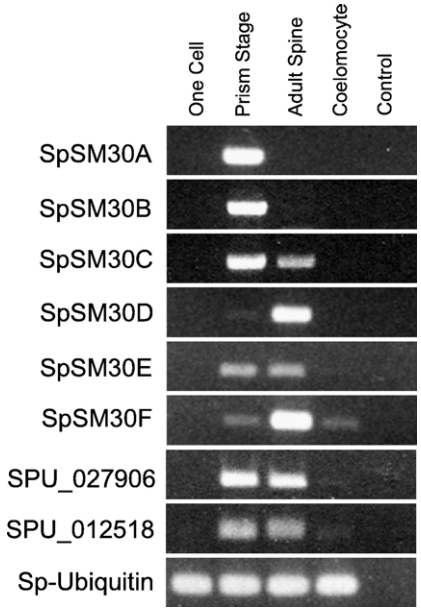

Fig. 3. Semi-quantitative RT-PCR analysis of select biomineralization-related genes. Total RNA was isolated from one-cell stage embryos, prism stage embryos (a stage when overt skeletogenesis is underway), adult spine (a mineralized tissue), and adult coelomocytes (a non-mineralized tissue). As a negative control, RNA was omitted from the reaction.

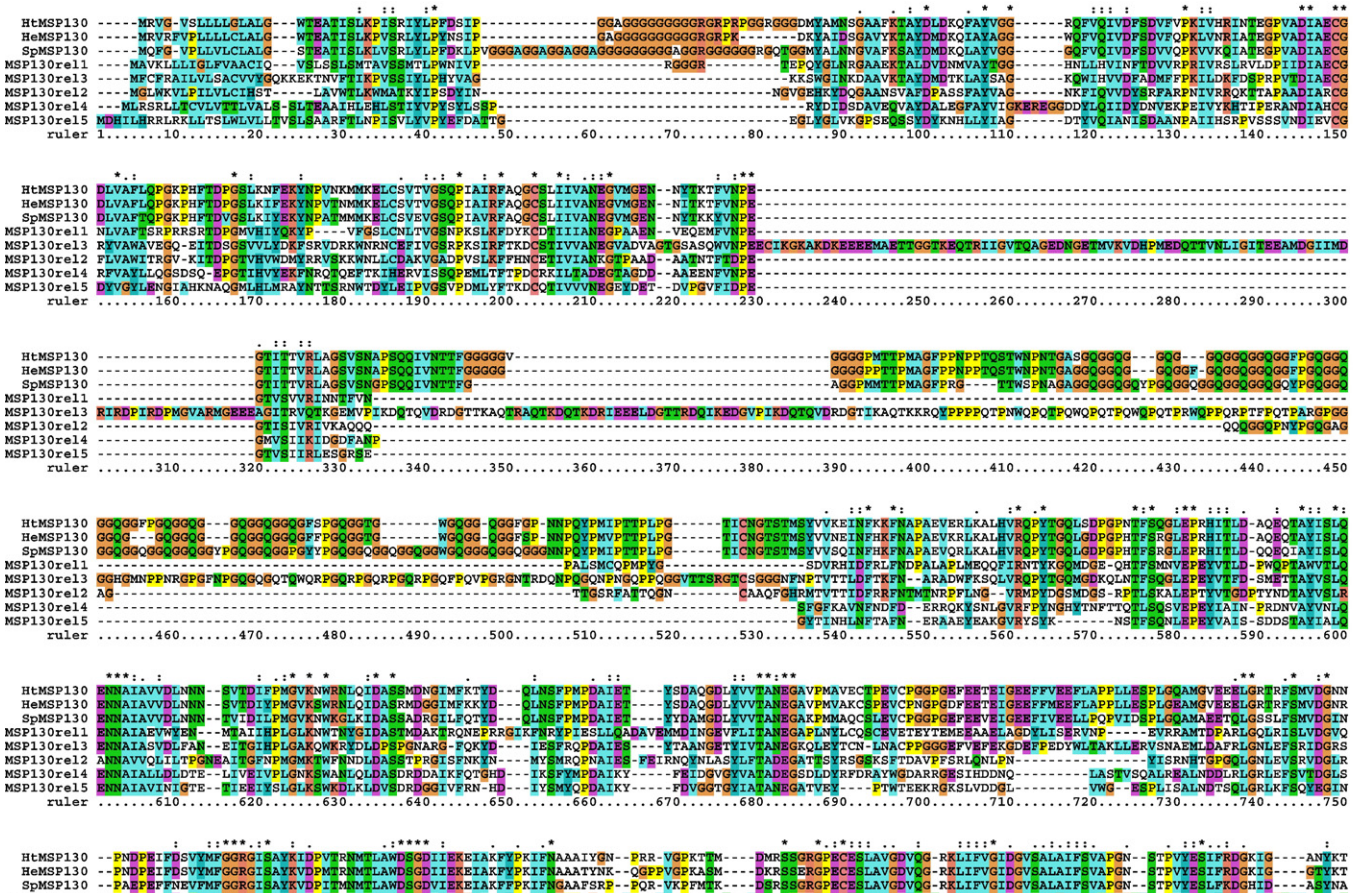

Fig. 4. ClustalX alignment of members of the MSP130 protein family.

represented haplotype variants or other assembly artifacts, they were not considered further. The current estimate of seven MSP130 family members should, however, be considered provisional and conservative.

Of the four new proteins, three (MSP130-related, 3, -4, and -5) contain a predicted signal sequence, a feature of all three known members of the family. Although the predicted protein sequence of MSP130-related-6 (*SPU_014492*) does not include a consensus signal peptide, there are very few cDNA sequences that align to this model and it is not possible to verify several features of the predicted protein sequence, including the N-terminus. A ClustalX alignment of the MSP130 family members (excluding MSP130-related-6) is shown in Fig. 4.

At least some of the MSP130 family genes are clustered in the genome. *SpMSP130*, *SpMSP130-related-1*, and *SpMSP-related-3* are arranged in tandem on Scaffold11 and *SpMSP130-related-4* and *SpMSP130-related-6* are adjacent on Scaffold1550. Scaffolds 11 and 1550 are both relatively large (624 and 231 kb, respectively), and each cluster of MSP130 family genes is flanked by at least one, apparently unrelated, gene on both sides. *SpMSP130-related-2* and *SpMSP130-related-5* are located on smaller scaffolds that also include small numbers of apparently unrelated genes. Future assemblies of the *S. purpuratus* genome may reveal higher order clustering of MSP130 family genes.

Chip expression data and EST analysis show that *MSP130-related-3* and *MSP130-related-5* are expressed during embryogenesis. In situ hybridization studies confirm that both mRNAs are restricted to PMCs (Fig. 5D and data not shown). Thus, all three members of the small gene cluster containing *SpMSP130*, *SpMSP130-related-1*, and *SpMSP130-related-3* are expressed specifically in PMCs, although the corresponding mRNAs are present at different steady-state levels.

It seems unlikely that *SpMSP130-related-4* and *SpMSP130-related-6* are expressed during embryogenesis. Weak chip hybridization signal is associated with *SpMSP130-related-4*

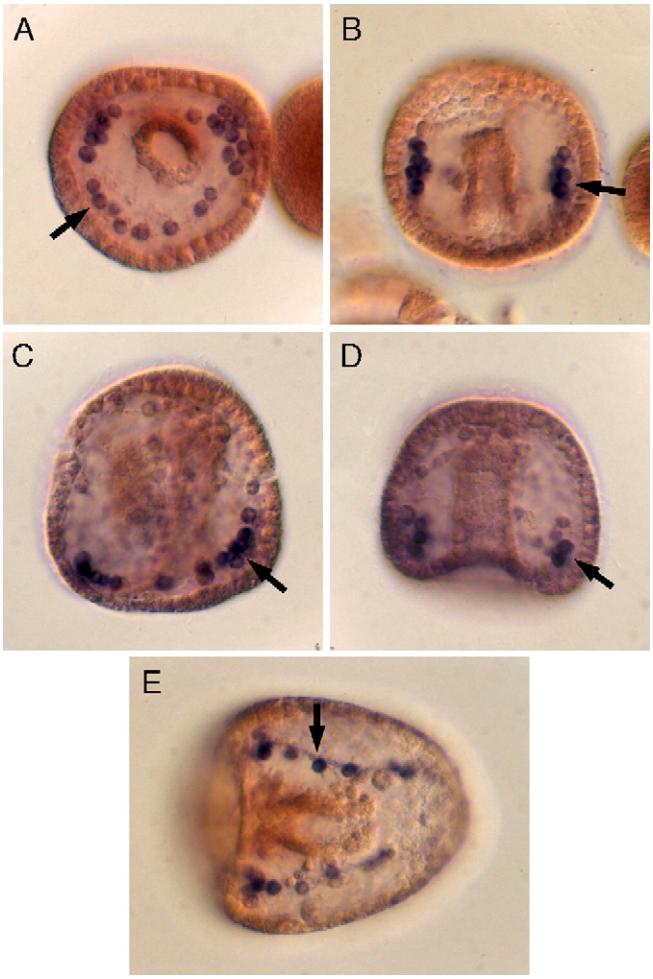

Fig. 5. In situ hybridization analysis of selected sea urchin biomineralization genes. (A) *SPU_018403*. This gene is closely linked to SpP16 and encodes a transmembrane protein with similar general features. (B) *SPU_018407*. This gene is also closely linked to SpP16 and encodes a glycine-rich transmembrane protein with proline-containing repeats. (C) *SPU_007944/025210*. This gene encodes a cyclophilin (peptidylprolyl *cis–trans* isomerase). (D) *SpMSP130-related-3*, a member of the MSP130 gene family. Panels A–D show embryos at the late gastrula stage; Panel E is a late prism stage embryo.

and there are no matching ESTs in the databases. *SpMSP130-related-6* shows substantial chip signal, but this signal does not align closely with the predicted exons of the gene and only a single matching EST (CD309446, derived from a larval cDNA library) was identified. *SpMSP130-related-4* and *SpMSP130-related-6* may be expressed in the adult or may represent pseudogenes.

Cyclophilins

Amore and Davidson (2006) recently identified *Sp-cyp1* (*Sp-cyclophilin1*), a gene encoding a member of the cyclophilin subfamily of peptidylprolyl *cis–trans* isomerases. The function of *Sp-cyp1* in biomineralization and other aspects of PMC morphogenesis is unknown, but the transcript is abundant and restricted to PMCs. The *Sp-cyp1* gene is positively regulated by ETS1 and DRI, two transcription factors of the PMC gene regulatory network. By searching the PMC EST database, we found that at least eight different cyclophilin genes are expressed by these cells. The previously identified *Sp-cyp1* (*SPU_007484*) is by far the most highly expressed based on the very large number of matching ESTs (124) in the PMC EST collection. Four other cyclophilin genes (*SPU_000637*, *SPU_015088*, *SPU_007944/025210*, and *SPU_013756/028874*) are expressed by PMCs at intermediate levels (10–30 matching PMC ESTs) and three (*SPU_008305*, *SPU_022479*, and *SPU_028896*) are expressed at low levels (1–4 matching ESTs). We examined the pattern of expression of *SPU_007944/025210* by in situ hybridization as this gene had the second largest number of matching PMC ESTs after *Sp-cyp1*. In situ hybridization shows that *SPU_007944/025210* mRNA is restricted to PMCs (Fig. 5C). Sp-CYP1 has a predicted signal sequence and is presumably secreted. Most of the newly identified cyclophilins expressed by PMCs, including SPU_007944/025210, do not appear to contain signal sequences, although in some cases it was not possible to verify predictions of the N-termini of the proteins from cDNA sequences.

Collagens

Biochemical studies indicate that PMCs secrete collagen and that this ECM molecule functions as an essential substrate for spiculogenesis (Blankenship and Benson, 1984; Benson et al., 1990; Wessel et al., 1991; reviewed by Ettensohn et al., 1997). Several collagens have been cloned from sea urchins and shown to be expressed by PMCs and secondary mesenchyme cells. These include COLP1alpha, COLP2alpha, COLP3alpha, and COLP4alpha (Exposito et al., 1994; Suzuki et al., 1997) and Spcoll (Angerer et al., 1988, Wessel et al., 1991).

Analysis of the PMC EST collection reveals sequences corresponding to eight different collagen genes. We find that the previously described Spcoll and COLP3alpha are products of a single gene (*SPU_003768/005187*). *Spcoll/COLP3alpha* mRNA is the most abundant collagen transcript in PMCs at the gastrula stage and one of the most abundant mRNAs overall in these cells (161 matching ESTs). The next most abundant collagen mRNA is encoded by *SPU_015708/027250* (83 ESTs) and corresponds to the previously identified *COLP4alpha* gene (Exposito et al., 1994). *Spcoll/COLP3alpha* and *COLP4alpha* both encode non-fibrillar collagens, and our EST analysis therefore suggests that non-fibrillar collagens are the most abundant class of collagen protein produced by PMCs during gastrulation. Six other collagen genes, mostly encoding fibrillar collagens, are represented at lower abundance in the PMC EST database: *SPU_006067/022116* (8 ESTs), *SPU_013557* (2 ESTs), *SPU_026008* (corresponding to the *COLP1alpha* gene) (2 ESTs), *SPU_009076* (1 EST), *SPU_000142* (1 EST), and *SPU_021235/017184* (1 EST). The PMC EST database does not contain ESTs that corresponded to *COLP2alpha*, probably because this gene and *COLP1alpha* are expressed at high levels only at later developmental stages (Suzuki et al., 1997).

Carbonic anhydrases

Carbonic anhydrases, zinc-containing enzymes that are found ubiquitously in nature, catalyze the reversible hydration

of carbon dioxide ($CO^{2+}\ H_2O \leftrightarrow HCO_3^- + H^+$) (Lindskog, 1997). Biochemical studies have demonstrated an increase in carbonic anhydrase activity during sea urchin embryogenesis beginning at about the mesenchyme blastula stage, and inhibitors of carbonic anhydrase block spicule formation in vivo (Chow and Benson, 1979; Mitsunga et al., 1986).

We identified 19 genes in the *S. purpuratus* genome that encode carbonic anhydrases. Of these genes, only three (*SPU_012518*, *SPU_008658*, and *SPU_007745*) match sequences in the PMC EST database. *SPU_012518* is expressed at the highest level in PMCs based on the number of matching PMC ESTs (20 ESTs). The amino acid sequence of SPU_012518 includes a predicted N-terminal signal sequence and a C-terminal GPI anchor, indicating that this protein is an extracellular carbonic anhydrase (Sly and Hu, 1995). RT-PCR studies show that *SPU_012518* mRNA is present at very low levels in the fertilized egg but is expressed at much higher levels by the prism stage, when overt skeletogenesis is taking place (Fig. 3). In situ hybridization reveals that *SPU_012518* mRNA is restricted to PMCs during embryogenesis (Fig. 5E). In the adult, *SPU_012518* is expressed at much higher levels in spines than in coelomocytes, a non-mineralized tissue (Fig. 3).

Novel proteins

SpP16 (*SPU_018408*) is a novel, small (172 aa) transmembrane protein (Illies et al., 2002). Morpholino knockdown studies show that SpP16 plays an essential role in spicule growth and acts at a late step in this process; i.e., downstream of PMC specification, migration, and fusion (Cheers and Ettensohn, 2005). *SpP16* mRNA is very abundant in PMCs based on the number of matching ESTs (137) in the PMC EST collection and the intensity of in situ hybridization signal (Illies et al., 2002). In the present study, we found no evidence by BLAST analysis of proteins highly similar to SpP16 in the *S. purpuratus* genome or evidence of homologues of P16 in hemichordates, cephalochordates, or vertebrates. When we examined the scaffold containing the *SpP16* gene (Scaffold236), however, we found two closely linked genes with relatively large numbers of matching PMC ESTs (15–30 ESTs). One gene, *SPU_018403*, encodes a protein with general features similar to those of P16. SPU_018403 is a small (276 aa), novel protein with a predicted signal sequence, a possible transmembrane domain located near the C-terminus, and an intervening region rich in glycine, serine, and acidic residues. In situ hybridization analysis confirms that *SPU_018403* mRNA is restricted to PMCs (Fig. 5A). A second gene, *SPU_018407*, immediately adjacent to *SpP16*, encodes a somewhat larger protein (369 aa), again with the shared features of a predicted, N-terminal signal sequence and a transmembrane domain near the C-terminus. In this case, however, the intervening region is basic rather than acidic. SPU_018407 is highly glycine-rich and contains proline- and glycine-rich repeat elements that bear some resemblance to those found in spicule matrix proteins. In situ hybridization analysis reveals that *SPU_018407* mRNA is also restricted to PMCs (Fig. 5B). A third gene, *SPU018405*, located in the same cluster is a predicted to encode a small, highly acidic, secreted protein and is a candidate for further analysis, although fewer PMC ESTs (2) matched this gene.

SpP19 (SPU_004136) encodes a small, PMC-specific protein of unknown function (Illies et al., 2002). The *SpP19* mRNA is one of the most abundant transcripts in PMCs based on a qualitative impression of whole mount in situ hybridization signal and on the large number of matching ESTs (573) in the PMC EST collection. Protein and nucleotide BLAST searches reveal no related proteins in the *S. purpuratus* genome and no evidence of homologous genes in other organisms. *SpP19* is located on a small scaffold (Scaffold1517) that contains only two other genes, a carbonic anhydrase (*SPU_004135*) and a epoxide hydrolase (*SPU_004137*), complementary to 0 and 2 PMC ESTs, respectively.

A search for counterparts of vertebrate biomineralization proteins in S. purpuratus

As an additional strategy to identify new biomineralization-related genes in sea urchins, we searched the *S. purpuratus* genome for counterparts of genes that have been implicated in biomineralization in vertebrates, the only other deuterostome clade that exhibits extensive biomineralization. These genes encoded extracellular matrix (ECM) proteins, ECM-modifying enzymes, transcription factors, and other proteins.

Secreted calcium-binding phosphoproteins

Vertebrates form three types of mineralized tissues: bone, dentin, and enamel. In all three cases, mesenchymal cells secrete extracellular matrix proteins that regulate mineral deposition. It has recently been proposed that many of these extracellular proteins constitute a family of secreted calcium-binding phosphoproteins (SCPPs) that arose through gene duplication (Fisher et al., 2001; Qin et al., 2004; Kawasaki et al., 2004, 2005; Kawasaki and Weiss, 2006). We searched the set of predicted *S. purpuratus* proteins but were unable to find homologues of vertebrate SCPPs (Table 1). Using BLAST searches, we identified 22 proteins in *S. purpuratus* that initially appeared to be related to mammalian dentin sialophosphoprotein (DSPP). Further examination of these proteins, however, indicated that the BLAST similarity was the result of an alignment of a highly repetitive region of DSPP that consists almost entirely of serine and aspartic acid residues with serine- and aspartic acid-rich regions of several sea urchin proteins. There was no significant alignment outside of the repetitive region of DSPP with any of the sea urchin proteins that appeared similar to DSPP based on BLAST analysis. None of the putative sea urchin DSPP-like proteins aligned closely with each other. These sea urchin proteins were therefore designated Sp-serine-rich proteins. Transcripts corresponding to four of the proteins, encoded by *SPU_007081*, *SPU_016032*, *SPU_018432*, and *SPU_008499*, were detected in PMCs. They were present at relatively low levels based on the numbers of matching PMC ESTs (3–6 ESTs/gene). Another gene, *SPU_015338*, was expressed in other embryonic tissues based on EST and chip data.

Table 1
Vertebrate mineralization genes not detected in the sea urchin genome

Tissue distribution	SCPP proteins	Other proteins
Dentin and bone	Dentin sialophosphoprotein	Osteonectin
	Dentin matrix acidic phosphoprotein	Osteoadherin
	Bone sialoprotein	Osteocalcin
	Matrix extracellular phosphoglycoprotein	Osteoprotegrin
	Osteopontin	Osteopontin
		Osterix
Enamel	Amelogenin	Enamelysin
	Enamelin	
	Ameloblastin	

The vertebrate *SCPP* genes are thought to have arisen from duplications of the *SPARCL1* (secreted protein, acidic, cysteine-rich-like 1) gene. In mammals, the *SCPP* genes, including *SPARCL1*, are found in two clusters on chromosome 4. *SPARCL1* is thought to have arisen via a duplication of *SPARC* (*osteonectin*), which is located on a different chromosome (Kawasaki et al., 2004, 2005). We searched the sea urchin genome for SPARC-related genes and found two, *Sp-osteonectin* (*SPU_028275*) and *Sp-SPARC-like* (*SPU_012548*). Phylogenetic trees generated using SPARC-related genes from sea urchin, mammals, fish, nematode, *Drosophila*, and Artemia are shown in Fig. 6. The results confirm that *osteonectin* (represented in sea urchins by *Sp-osteonectin*) was basal to both the chordate and protostome clades. The sea urchin gene *Sp-SPARC-like* is not closely related to vertebrate *SPARCL1* or to other *SPARC* genes in protostomes or deuterostomes. This suggests that the sea urchin *SPARC-like* is a sea urchin-specific gene that is likely due to an independent duplication of the *SPARC* gene. Chip hybridization data confirm that *Sp-osteonectin* is expressed during embryogenesis and in situ hybridization analysis shows that *Sp-osteonectin* mRNA is expressed widely in the embryo (data not shown). *Sp-SPARC-like* is expressed at larval stages based on EST analysis but sequences corresponding to this gene are not represented in the PMC EST collection.

Other proteins

We also searched the sea urchin genome for genes that have been shown to play a role in the early specification, differentiation, or morphogenesis of the mesenchymal cells that in vertebrates give rise to mineralized tissues. Many of the molecules that function in these processes in vertebrates are members of larger gene families that have diverse functions in a wide range of tissue types. In many cases, gene duplications have generated paralogous genes that have diverged in function and expression. These include genes encoding transcription factors, ECM molecules, and proteins involved in cell–ECM interactions, such as secreted proteases. The sea urchin genome has representatives of all these gene families and some are expressed by PMCs. Relationships between the vertebrate and sea urchin genes are often not simple to establish, however, due to independent duplications of these genes in echinoderms or vertebrates (or both) following their evolutionary divergence.

In vertebrates, runt box transcription factors trigger development of mineralized tissues (Runx2), hematopoiesis (Runx1), and growth of the gut (Runx3). Sea urchins have two runt genes but only one, *SpRunt-1* (*SPU_006917*), is expressed during embryonic development (Robertson et al., 2002). *SpRunt-1* is expressed in several tissues and is required throughout the embryo for cell differentiation (Coffman et al., 2004). The vertebrate *osterix* gene, which is regulated by *Runx2*, is not present in the sea urchin genome. Two other vertebrate genes encoding transcription factors that play a role in bone development, *Ets1* (Raouf and Seth, 2000) and *Alx4* (Beverdam et al., 2001, Mavrogiannis et al., 2001) have counterparts in the sea urchin genome that play a role in spicule formation. Sea urchin *Ets1* (Kurokawa et al., 1999) and *Alx1* (Ettensohn et al., 2003) are expressed selectively in PMCs and are required for skeleton formation.

Vertebrates have a large number of genes encoding secreted proteases that modify extracellular matrix molecules. Some of these proteases are involved in the formation of mineralized tissues, including matrix metalloproteinases (MMPs) (reviewed by Ortega et al., 2003), ADAMTS (reviewed by Porter et al., 2005), and kallikrein serine proteases (reviewed by Clements et al., 2004). The sea urchin genome contains members of all of these gene families, although relationships between the echinoderm and vertebrate genes are often unclear (Angerer et al., this issue). Eight different MMP genes are expressed by PMCs, encoding both soluble and membrane-type MMPs. Three ADAM genes and one ADAMTS gene are also expressed by PMCs, as are two kallikrein protease genes.

The sea urchin genome contains a gene, *SPU_017585*, that encodes a calcium receptor similar to one that has been

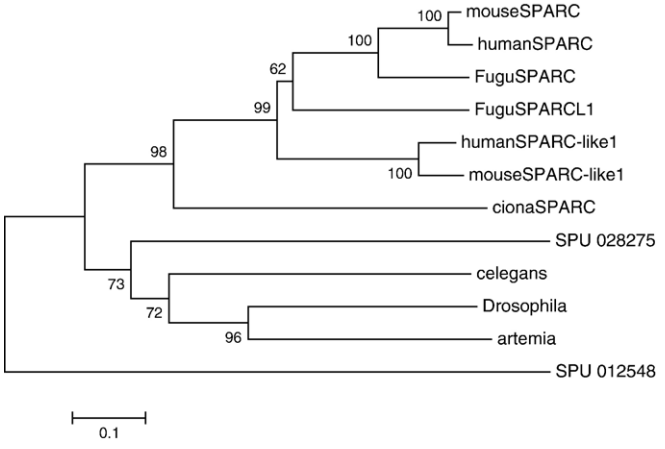

Fig. 6. Neighbor-joining tree comparing sea urchin SPARC-related genes (*Sp-osteonectin* and *Sp-SPARC-like*) to SPARC genes identified in other organisms. Sequences were aligned using ClustalW, and tree construction was carried out with MEGA. NCBI Locus numbers: Human osteonectin (SPARC)=AAA60993, Human SPARC-like=AAH33721, Mouse SPARC=CAJ18514, Mouse SPARC-like=NP_034227, Fugu SPARC=AAT01214, Fugu SPARC-like=AAT01216, *Ciona* SPARC=AAT01212, *C. elegans* SPARC=AAB88325.1, *Drosophila melanogaster* SPARC=CAB39319, Artemia SPARC=BAB20042.1.

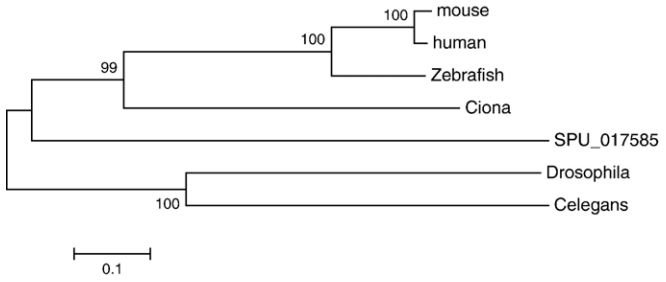

Fig. 7. Neighbor-joining tree comparing sea urchin Casr (SPU_017585) to existing Casr genes. Sequences were aligned using ClustalW, and tree construction was carried out with MEGA. NCBI Locus numbers: Human=NP_000379, Mouse=AAD40638, Zebrafish=XP689097, *Drosophila melanogaster*=NP_610386.1 (best BLAST hit), *C. elegans*=NP_509674.1 (best BLAST hit). *Ciona intestinalis* Genome Project locus=Ci0100130340.

implicated in osteoblast differentiation in mammals (Chang et al., 1999; Yamauchi et al., 2005). Matching ESTs were found in the PMC EST database, albeit at low frequency (2 ESTs). An analysis of the phylogenetic relationships of this gene to homologues in both deuterostomes and protostomes is shown in Fig. 7. We also identified a sea urchin homologue (encoded by *SPU_028181*) of the vertebrate protein CEP-68 (chondrocyte expressed protein-68), a protein implicated in cartilage differentiation (Steck et al., 2001). Matching ESTs were detected in mesenchyme blastula, PMC, and larval EST sequence databases. The vertebrate and sea urchin proteins contain an ASPIC domain (Fig. 8). Proteins with this domain could not be identified in the *Ciona* and amphioxus genomes or EST databases, or in existing protostome genome sequence databases, but were found in two species of cyanobacteria. The *S. purpuratus* genome also has a gene encoding a fibulin-like protein, *SPU_026629*. Fibulins interact in the ECM with C-type lectins and have been shown to play a role in formation of vertebrate bone (Aspberg et al., 1999). Matching ESTs were found in the whole mesenchyme blastula collection but not in the PMC EST database.

Discussion

Biomineralization genes in sea urchins

Biomineral formation is regulated by secreted proteins that are closely associated with the inorganic component of the material. The proteins occluded within the sea urchin endoskeleton make only a minor contribution to its overall mass, but they probably play an important role in regulating the growth and physical properties of the biomineral. Our findings show that the genes encoding these occluded proteins, the spicule matrix proteins, constitute a small family of sixteen echinoderm-specific genes. It seems likely that our computational analysis has identified all or almost all of the sea urchin proteins that have the canonical structure of a signal sequence, a single CTL domain, and (in most cases) proline-rich repeats. Previous two-dimensional gel electrophoresis studies, however, indicated that as many as 40–50 different proteins are present in the spicule matrix (Killian and Wilt, 1996). This apparent complexity may reflect post-translational modifications of the known spicule matrix proteins and alternative splicing of some of the transcripts (i.e., *SpSM50*). It is also possible, however, that proteins with other structures are occluded within the spicule. The canonical spicule matrix protein structure used in our computational searches and in previous analyses (Illies et al., 2002) was originally defined based on the sequences of SpSM30-B and SpSM50. Both proteins were first identified by screening a cDNA expression library using a polyclonal antibody raised against total spicule matrix proteins (Sucov et al., 1987; George et al., 1991). Other proteins occluded in the spicule might not have been detected by these methods.

At present, only three of the likely spicule matrix proteins, PM27, SM50, and SM30-B, have been localized to the spicule matrix by immunological methods (Harkey et al., 1995; Killian and Wilt, 1996; Kitajima and Urakami, 2000; Urry et al., 2000; Seto et al., 2004). It will be important to analyze the distributions of the proteins encoded by the other members of the spicule

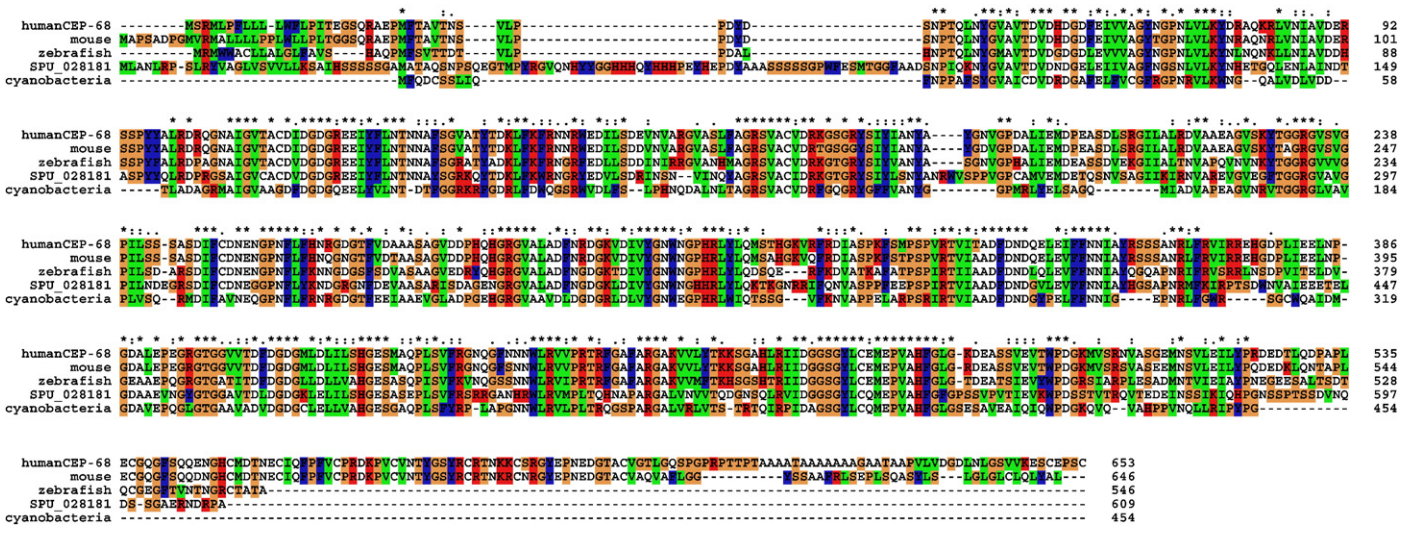

Fig. 8. BLAST alignment of SPU_028181 (ASPIC-containing protein) with vertebrate and cyanobacterial genes. NCBI locus numbers: Human=CAC0845, Mouse=NP_660105, Zebrafish=XP_686023, cyanobacteria (*Synechococcus*)=YP_474389.

matrix gene family to confirm that they are occluded within the spicule and to provide a clearer picture of their possible functions in biomineral deposition.

We have documented a developmental shift in the expression pattern of the SM30 subfamily of genes during larval development and adult tissue formation. *SpSM30-D* and *-F* show little or no expression in the embryonic endoskeleton but are the most abundant SM30-related mRNAs in adult spine. Whether this change in SM30 expression has functional consequences is presently unknown. In general, cellular and molecular features of skeletogenesis in the adult appear similar to those observed in the embryo (Heatfield and Travis, 1975; Markel et al., 1986; Emlet, 1985; Drager et al., 1989; Richardson et al., 1989; George et al., 1991; Harkey et al., 1995; Killian and Wilt, 1996). With more specific molecular probes, however, other examples may be uncovered of differences in the embryonic and adult programs.

The MSP130 proteins constitute another small family of echinoderm-specific proteins implicated in biomineralization. We have shown that this family consists of at least six members. Transcripts encoding at least four of these proteins (MSP130, MSP130-related-1, -2, and -3) are selectively expressed in PMCs, although at different levels. The original member of the family, MSP130, is glycosylated and a complex, N-linked oligosaccharide chain on MSP130, binds divalent cations, including Ca^{2+}, with relatively high affinity (Farach-Carson et al., 1989). A monoclonal antibody (1223) that recognizes this oligosaccharide chain blocks uptake of radiolabeled calcium and spicule formation by cultured micromeres (Carson et al., 1985). Based on these findings, it was proposed that MSP130 sequesters Ca^{2+} ions from the blastocoel fluid and facilitates their uptake into PMCs. It was found subsequently, however, that other PMC-specific proteins, including SM30, share the carbohydrate moiety recognized by the 1223 antibody (Kabakoff et al., 1992; Brown et al., 1995). Although the biochemical functions of MSP130 and other members of the family are not well understood, the expression of these protein at high levels specifically by PMCs and in calcified tissues of the adult, as well as their calcium-binding properties, points to a role in biomineralization.

Our work reveals that many of the proteins implicated in biomineralization in sea urchins are members of small families of coordinately expressed genes clustered in the genome. There are several known clusters of spicule matrix genes. The *SpSM30* gene cluster contains all six known members of this subfamily, most (perhaps all) of which are expressed selectively by biomineralizing cells in the embryo or adult. *SpSM50* (an alternatively spliced gene that also encodes the *SpM32* transcript) and *SpSM37* are clustered and expressed only by PMCs. *SpSM29* is flanked by several putative spicule matrix genes, although we have not yet confirmed that the flanking genes are expressed specifically by biomineralizing cells. *SpMSP130* family genes are clustered as well; for example, *SpMSP130*, *SpMSP130-related-1*, and *SpMSP130-related-3* are arranged in tandem and each is expressed selectively by PMCs. *SpP16* is clustered with at least two genes, *SPU_018403* and *SPU_018407*, which are also expressed specifically by PMCs. Further studies may reveal an even greater extent of gene clustering than is evident from the current WGS assembly. The clustering of coordinately expressed genes suggests that there may be shared *cis*-regulatory elements that control the transcription of multiple genes in the clusters. In some cases, such as the switch from embryonic to adult forms of SM30, separate, gene-specific temporal regulatory elements must be present.

The evolution of biomineralization in deuterostomes

Biomineralization is observed in most metazoan phyla. The fossil record of biomineralization in metazoans extends back at least to the Neoproterozoic, when tubular fossils with calcareous skeletons appear, just prior to the Cambrian explosion (Bengtson, 1994). Thus, there is ancient and far-flung evidence of biomineralization within multicellular animals. What remains unclear is the extent to which this reflects the independent invention of biomineralization in various taxa or, instead, is evidence of ancient, ancestral programs of biomineralization. Even if biomineralized structures *per se* appeared independently in different lineages, components of a common set of genetic and developmental mechanisms (i.e., a biomineralization "toolkit") may have been repeatedly employed.

Within the present-day deuterostomes, biomineralization is common in echinoderms and vertebrates but rare in other groups. All adult echinoderms, and the embryos and larvae of many species, have a calcite-based endoskeleton (a skeleton encased in an epithelial integument, in contrast to an exoskeleton). Vertebrates, with the exception of present-day jawless vertebrates (hagfishes and lampreys), have an extensive, mostly apatite-based endoskeleton. Examples of biomineralization in other deuterostomes are few. Some tunicates secrete small, biomineralized spicules in the body wall or gut that can be calcium phosphate or calcium carbonate (Lambert et al., 1990). Cephalochordates and hemichordates, groups that are represented by very few living species, do not possess biomineralized skeletons, although both have cartilage-like elements that have been poorly characterized biochemically.

A reconstruction of the evolution of biomineralization within the deuterostomes is challenging for several reasons. First, although molecular phylogenies clearly place the vertebrates, urochordates, cephalochordates, hemichordates, and echinoderms within the deuterostomes (Peterson and Eernisse, 2001; Hanalych, 2004), debate continues concerning phylogenetic relationships among some of the deuterostome taxa (Delsuc et al., 2006). Clarification of these relationships will be important in reconstructing the evolution of developmental processes, including skeletogenesis, within the group. A second challenge is that the fossil record of the most ancient, ancestral deuterostomes is sparse and subject to diverse interpretations (Jefferies, 1986; Gee, 1996; Holland and Chen, 2001; Dominguez et al., 2002; Shu et al., 2003, 2004; Smith, 2004). The scarcity and problematical nature of the fossil record of stem group deuterostomes do not presently allow, for example, a clear answer to the question of whether

these animals had biomineralized structures. One highly controversial view, the "calcichordate hypothesis", is that the ancestral deuterostome had an extensive, calcitic endoskeleton much like that of a modern echinoderm (Jefferies, 1986; Gee, 1996). According to this hypothesis, the skeleton was subsequently lost in most deuterostome lineages (including that which gave rise to vertebrates) but was retained in echinoderms. It is also possible that a cartilaginous extracellular matrix was present in the ancestral deuterostome, and this matrix later became mineralized in both echinoderms and vertebrates.

Donoghue and Sansom (2002) have proposed that the earliest vertebrates lacked biomineralized tissue and that the primitive vertebrate skeleton consisted of a non-collagenous, unmineralized, cartilaginous endoskeleton associated with the pharynx. Calcification of this primitive, oral skeleton is thought to have occurred first in the conodonts, a group of ancient, jawless vertebrates. These fossil animals possessed a complex array of biomineralized dental elements but lacked a dermal skeleton. Calcification of the oral skeleton was followed by the appearance of mineralized dermal armor and finally by the axial and appendicular skeleton. If this scenario is accurate, then a mineralized skeleton arose independently in the vertebrate lineage and may have been based on a more ancient, cartilaginous endoskeleton, probably derived from mesoderm.

The proteins that mediate late events in biomineralization in vertebrates and sea urchins are very different, at least with respect to their primary structures. There are no apparent homologues of sea urchin spicule matrix proteins, MSP130 proteins, or P16 in vertebrates. Conversely, recent studies suggest that many of the *SCPP* genes involved in vertebrate tissue mineralization appeared after the echinoderm–chordate split via duplication of an ancestral *SPARC-like 1* gene (Kawasaki et al., 2004, 2005, Fisher et al., 2001). *S. purpuratus* lacks a homologue of vertebrate *SPARC-like 1*, although an independent duplication of *SPARC* apparently occurred in the echinoderm lineage. The primary sequences of the SCPP proteins have diverged considerably, and it has been proposed that this is made possible by relatively loose constraints imposed on proteins that support crystal growth (Kawasaki et al., 2005). The structures of bone sialoprotein and osteopontin are highly flexible and disordered, lending support for this view (Fisher et al., 2001). These same relaxed constraints may have allowed echinoderms to evolve a different set of proteins that carry out similar biochemical functions. Support for this hypothesis will require a better understanding of the structures and biochemical properties of both the vertebrate and echinoderm proteins. We cannot completely rule out the possibility that one or more of the serine-rich proteins in *S. purpuratus* that show limited similarity to dentin play a role in mineralization. Veis et al. (2002) showed that proteins in sea urchin lantern react with antibodies against dentin matrix proteins, suggesting that their may be some structural similarities to DSPP in proteins found in echinoderm mineralized tissue.

Although the primary structures of proteins that mediate biomineral formation are very different in vertebrates and echinoderms, some of the cellular and molecular processes involved in earlier steps of skeletogenesis appear similar. In both groups of organisms, mesenchymal cells deposit a calcium-based biomineral within an extracellular space. Vertebrate bone and dentin are collagen-based biominerals, and in sea urchins, secretion of collagen by PMCs plays an important role in spicule formation (Wessel et al., 1991). PMCs, like the mesenchymal cells that produce mineralized tissue in vertebrates, secrete proteases that can modify collagen and other ECM components, as well as glycoproteins that associate with collagens and carbohydrates in the ECM and can bind calcium. Lastly, there are similarities in certain of the transcription factors (ETS and ALX family members) that control the differentiation of skeleton-forming cells in both taxa. These various observations suggest that the common ancestor of echinoderms and vertebrates may have had mesenchymal cells that secreted and processed collagen to form a type of structural support. These cells may have secreted proteins, like SPARC, that could interact with collagen and bind calcium with low affinity. Following the divergence of echinoderms and vertebrates, each may have independently evolved distinct proteins with similar biochemical properties that facilitated mineral crystallization. Subsequent genome and gene duplications in the vertebrate lineage may have supported the diversification of cell types (osteoblasts, odontoblasts, chondrocytes, and osteoclasts) associated with vertebrate biomineralized tissues. To develop a clearer picture of the evolution of biomineralization in the deuterostomes, it will be important to analyze biomineralization-related genes in other invertebrate members of this clade, including representatives of other classes of echinoderms.

Acknowledgments

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