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SM50 Repeat-Polypeptides Self-Assemble into Discrete Matrix Subunits and Promote Appositional Calcium Carbonate Crystal Growth during Sea Urchin Tooth Biomineralization

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Abstract

The two major proteins involved in vertebrate enamel formation and echinoderm sea urchin tooth biomineralization, amelogenin and SM50, are both characterized by elongated polyproline repeat domains in the center of the macromolecule. To determine the role of polyproline repeat polypeptides in basal deuterostome biomineralization, we have mapped the localization of SM50 as it relates to crystal growth, conducted self-assembly studies of SM50 repeat polypeptides, and examined their effect on calcium carbonate and apatite crystal growth. Electron micrographs of the growth zone of *Strongylocentrotus purpuratus* sea urchin teeth documented a series of successive events from intravesicular mineral nucleation to mineral deposition at the interface between tooth surface and odontoblast syncytium. Using immunohistochemistry, SM50 was detected within the cytoplasm of cells associated with the developing tooth mineral, at the mineral secreting front, and adjacent to initial mineral deposits, but not in muscles and ligaments. Polypeptides derived from the SM50 polyproline alternating hexa- and hepta-peptide repeat region (SM50P6P7) formed highly discrete, donut-shaped self-assembly patterns. In calcium carbonate crystal growth studies, SM50P6P7 repeat peptides triggered the growth of expansive networks of fused calcium carbonate crystals while in apatite growth studies, SM50P6P7 peptides facilitated the growth of needle-shaped and parallel arranged crystals resembling those found in developing vertebrate enamel. In comparison, SM50P6P7 surpassed the PXX24 polypeptide repeat region derived from the vertebrate enamel protein amelogenin in its ability to promote crystal nucleation and appositional crystal growth. Together, these studies establish the SM50P6P7 polyproline repeat region as a potent regulator in the protein-guided appositional crystal growth that occurs during continuous tooth mineralization and eruption. In addition, our studies highlight the role of species-specific

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polyproline repeat motifs in the formation of discrete self-assembled matrices and the resulting control of mineral growth.

Keywords

Sea urchin; tooth evolution; biomineralization; polyprolines; crystal growth; SM50; amelogenin

Introduction

Teeth are compact structures within the oral region that are equipped with sharp edges or abrasive surfaces to aid organisms with the partitioning of food. In some mammals, teeth also play a role in speech and esthetics. In most vertebrates, teeth are composite organs made out of hydroxyapatite and consisting of an ectomesenchymal portion (pulp and dentin) and an ectodermal portion (enamel epithelium and enamel). Animals in a few other vertebrate clades (e.g. juvenile salamanders, turtles, and birds) rely on horny crests or keratinous teeth of purely ectodermal-epithelial origin as food grasping organs. In contrast, many echinoderms (e.g. starfish, brittle stars, and sea urchins) feature teeth of mesodermal origin that are mostly composed of calcium carbonate or magnesium carbonate (Kniprath, 1974; Lawrence, 2013). While in vertebrates, cells secrete an extracellular matrix to shape the size and properties of the future tooth, mineral nucleation in echinoderms occurs within vacuoles inside of the odontoblastic syncytium, and mineral is deposited once syncytial vacuoles have come into contact with the growing tooth surface (Märkel, 1969; Kniprath, 1974, Märkel et al. 1977). A common element among vertebrate and invertebrate teeth is the use of unique biomineralization proteins that facilitate the assembly of biological mineral platelets into complex odontogenic minerals (Gopinathan et al. 2014). In the present study we have focused on the biomineralization process in echinoderm teeth and especially on role of polyproline repeat proteins such as SM50 in sea urchin tooth mineralization.

Echinoderms are marine organisms characterized by mineralized calcite skeletons and a five-fold radial symmetry. The phylum *Echinodermata* includes sea urchins, sea cucumbers, sea lilies, star fishes, brittle stars, and sea daisies. While many echinoderms rely on filter feeding mechanisms to access their food sources, sea urchins are grazers that use their penta-radial tooth organ to feed on kelp and other algae, barnacles, mussels, brittle stars, and other mineralized organisms inhabiting the sea floors and coral reefs (Larson et al., 1980; Wang et al., 1997; Pearse, 2006; Cook and Kelly, 2009; Killian et al., 2011; Lawrence, 2013). The ability of sea urchins to feed on shells and other mineralized tissues is a result of a sophisticated feeding apparatus and the hardness of their teeth (Killian et al., 2011).

The first known description of the sea urchin feeding apparatus dates back to the great Greek philosopher and naturalist Aristotle, who in his book “*Historia Animalium*” compared the sea urchin jaw apparatus with a horn lantern, leading to the term “Aristotle’s lantern” (Brusca and Brusca, 2003; Killian et al., 2011). Aristotle likely saw similarities between the five elongated urchin teeth arranged in a radially symmetric fashion and the frames of horn lanterns, which were used in ancient times to protect candle lights in the evenings from the strong Mistral winds of the Mediterranean. In such a comparison, the horn covered spaces

between the lantern frames would be equivalent to the muscles, plates, and periodontal structures that anchor the five teeth within the echinoderm masticatory apparatus.

Echinoderms are deuterostomes, and their genome is surprisingly close to that of chordates in terms of complexity and because of the number of conserved genes between both phyla (approximately 70%, Sodergren et al., 2006). While many genes are conserved between vertebrates and echinoderms, there are substantial differences between vertebrate and echinoderm biomineralization proteins, and sea urchins do not have counterparts to secretory, calcium-binding phosphoproteins expressed by the SCPP gene cluster (Sodergren et al., 2006). Moreover, it has been demonstrated that the evolution of structural biomineralization proteins has occurred independently and multiple times among metazoans (Jackson et al., 2009). While biomineralization genes might have evolved independently to respond to different and rapidly changing dietary habits throughout the course of evolution, many deuterostome biomineralization proteins share polyproline tandem repeat elements as a common sequence motif (Gopinathan et al., 2014). These polyproline repeat elements commonly occur as polyproline type II helices and have been associated with their intrinsically disordered molecular structure and their suitability as biomineralization modulators (Delak et al. 2009). The most prominent sea urchin biomineralization protein, SM50, has a molecular weight of 50kDa (Sucov et al., 1987; Richardson et al., 1989; Killian and Wilt, 1996) and features a total of 15 PXX tripeptide repeats, 12 PXXXXXXQ-PXXXXXQ combined septa-hexa-peptide repeats, and 5 other polyproline tripeptide tandem repeats (Gopinathan et al. 2014). Previous studies using the vertebrate tooth enamel biomineralization protein amelogenin as a model have demonstrated that the length of these polyproline repeat motif stretches is a powerful regulator of crystal growth and habit through its effect on the compaction of matrix subunits (Jin et al. 2009). Furthermore, in support of our application of polypeptides as a biological agent suitable for mineralization studies, self-assembling peptides have been successfully employed to promote enamel remineralization and bone regeneration, (Kirkham et al.; 2007, Semino, 2008).

The purpose of the present study was to characterize key stages of intracellular tooth biomineralization in the Pacific sea urchin *Strongylocentrotus purpuratus* and to ask the question how the polyproline repeat protein SM50 contributes to this process. To address this question, the relationship between the odontoblast syncytium and the mineral phase was analyzed using electron microscopy. Presence and localization of key sea urchin biomineralization proteins SM30 and SM50 was verified using immunohistochemistry and Western blotting. We then compared the echinoderm SM50 repeat motif and the vertebrate enamel-related amelogenin repeat motif PXX24 in their ability to form matrix subunit compartments and to control calcium carbonate and hydroxyapatite crystal growth. Together, these data provide insights into the self-assembly dynamics of polyproline repeat element proteins and their effect on the biofabrication of crystalline calcium biominerals in deuterostomes.

Materials and Methods

Electron microscopy

For electron microscopy, Aristotle's lanterns of the Pacific sea urchin *Strongylocentrotus purpuratus* were prepared using surgical dissection tools. Individual teeth were further isolated, fixed in Karnovsky's fixative for two hours, and subsequently contrasted using 4% OsO₄. Following dehydration in a graded series of ethanol, samples were embedded in Epon 812 (EM Sciences, Hatfield, PA). Polymerized blocks were subjected to semithin sectioning for the selection of ideal tissue profiles, and ultrathin sections were cut using a diamond knife (Diatome, Hatfield, PA). Sections were then contrasted in uranyl acetate and lead citrate as previously described (Diekwisch et al., 1993; 1995). Sections were viewed using a JEM 1220 electron microscope.

Immunohistochemistry

For immunohistochemical localization, Aristotle's lanterns of the Pacific sea urchin *Strongylocentrotus purpuratus* were dissected using a surgical knife and fixed in 10% buffered formalin. Tissues were decalcified in disodium EDTA (Warshawsky and Moore, 1967) for six weeks, dehydrated, and embedded in paraffin as previously described (Diekwisch et al., 1997). Immunoreactions were performed following the instructions of the Histostain Plus IHC kit (Life Technologies, Grand Island, NY). All reactions were carried out in a humidified chamber at room temperature. Briefly, sections were treated against endogenous peroxidase using methanol and 3% hydrogen peroxide and then blocked using in 10% goat serum for 10 min. Sections were incubated with primary antibody for 2 hours. Primary antibodies were 1:100 diluted in phosphate buffered saline (PBS). As a methodological control, the primary antibody was replaced with normal serum. Sections were washed 3 times in PBS and subsequently incubated for 10 min with biotinylated anti-rabbit IgGs as secondary antibodies. After washing in PBS (3 times), sections were exposed to the streptavidin-peroxidase conjugate for 10 min and then washed again in PBS (3 times). Signals were detected using an AEC Substrate-Chromogen mixture of. Sections were counterstained using hematoxylin and mounted with GVA-mount.

Immunocytochemistry

Immunohistochemistry was carried out as described by Diekwisch (1998). Prior to immunoreactions, sections were blocked in a solution containing 1% Tween 20 and 1% Cold Water Fish Ganglion Gelatine (Sigma, St. Louis, MO) for 15 min. After washing in phosphate buffered saline (PBS), sections were incubated in 1:100 anti-SM50 or SM30 primary antibodies. Incubation in primary antibody was followed by three washes in PBS. Protein A gold particles (5nm diameter) served as secondary antibodies. Sections were incubated in a 1:100 dilution protein A gold solution for 30 min following three washes in distilled water. Sections were briefly counterstained using a 1% solution of uranyl acetate. For controls, primary antisera were replaced with 1:100 diluted pre-immune serum.

Western Blots

For Western blots, individual teeth were dissected and proteins were extracted using 10% acetic acid. Protein extracts were separated on SDS gels and transferred onto nylon membranes. Membranes were then incubated with primary SM50 or SM30 antibodies, washed 3 times in PBS, incubated with secondary anti-rabbit IgG, washed again, and signals were detected with NBT and BCIP as described (Diekwisch et al., 1993).

Antibodies

The antibodies used for immunohistochemistry, -cytochemistry, and Western blot were based on the primary polyclonal rabbit antisera against maltose-binding SM50 and SM30 fusion proteins (Killian and Wilt, 1996), which were generously provided by Dr. Wilt. SM50 has a deduced AA sequence with a molecular weight of 50kDa (Sucov et al., 1987; Richardson et al., 1989; Killian and Wilt, 1996). SM30 antisera detected 2 bands of 43 and 46kDa on Western blots of spine and 46 and 49kDa on Western blots of spicule matrix proteins (Killian and Wilt, 1996).

SM50 and PXX24 self-assembly studies

For the design of the SM50 repeat peptide, a 26 amino acid stretch between amino acids 227 and 252 containing four successive polyproline repeat motifs was selected (Gopinathan et al., 2014). SM50 227–252 was chosen as a representative polyproline type II repeat peptide because each of the four repeat motifs contained an N-terminal proline and a C-terminal glutamine, and repeat motif length alternated between six and seven amino acids. For comparison purposes, a PXX24 polypeptide containing eight successive PXX tripeptide repeats was derived from the mammalian amelogenin repeat region (Jin et al. 2009). This polypeptide contained eight complete repeats and was of equivalent length (24–26 amino acids). In comparison, the SM50 repeat peptide contained four alternating hexa- and hepta-peptides, while the amelogenin-based PXX24 contained eight successive tripeptide repeats. Peptides were synthesized at 98% purity using a commercial vendor (Elim Biopharm, Hayward, CA).

For repeat polypeptide self-assembly studies, droplets containing 100µl of diluted (1mg/ml) pH7.5–8.0 SM50 or PXX24 peptide solution were placed on carbon coated copper TEM grids and incubated in a moisturized container at 37°C for 2 hours. Thereafter, grids were rinsed with double distilled water, stained with saturated uranyl acetate solution for 25 minutes, quickly rinsed 3 times with double distilled water, air dried, and analyzed using a Joel1220 TEM.

Apatite and Carbonate Crystal Growth

Crystal growth studies were performed as previously described (Beniash et al., 2005; Jin et al., 2009). In the present study, our SM50 and PXX24 repeat peptides (described above) were dissolved in double-distilled water at a concentration of 4 mg/ml and then adjusted to pH7.5–8.0 with 20mM NH₄OH at 4°C. Carbon coated copper TEM grids were immersed into the reaction mixture containing 1mg/ml SM50 or PXX24 peptide, 2.5mM CaCl₂ and 1.5mM (NH₄)₂HPO₄ for apatite and (NH₄)₂CO₃ for calcium carbonate, and incubated in a moisturized container at 37°C for 2 hours. Subsequently, grids were rinsed with double-

distilled water, blotted against filter paper, and air dried. Transmission electron microscopy was performed using a JEOL 1220 electron microscope.

Results

Aristotle's lantern provides the anchorage for five symmetrically arranged, self-sharpening, and continuously growing teeth

The Pacific sea urchins used for the present study featured a globular body encapsulated by a mineralized urchin endoskeleton, which in turn was surrounded by sharp spines arranged in a radial and perpendicular orientation toward the test (Fig. 1A). Surgical removal of test and spicules exposed the jaw apparatus (Aristotle's lantern) containing five continuously growing teeth arranged in a radially symmetric fashion (Figs. 1B,C). The oral aspect of the urchin featured the chewing tip of the five teeth surrounded by a concentric ring of podia, organelles that aid in locomotion and feeding (Fig. 1D–F). The soft tips of the five plumulae of the ever-growing sea urchin teeth extended beyond the anal boundary of Aristotle's lantern (Figs. 1B,C). The occlusal aspect of the sea urchin mouth revealed the unusual five-fold symmetry of the protruding teeth (Figs. 1D,E), which were in continuous motion in living sea urchins (Fig. 1F). After further dissection of the Aristotle's lantern, an individual sea urchin tooth was isolated, revealing three key elements, chewing tip, shaft, and plumula (Fig. 1G). Semithin sections through the shaft illustrate the sea urchin odontoblast syncytium adjacent to mineralized primary plates (Figs. 1H,I).

Continuous mineralization of continuously growing sea urchin teeth occurs through the deposition of mineral platelets from secretory vesicles onto the tooth surface

Electron micrographs documented a series of spatially adjacent events indicative of intracellular mineralization, including (i) mineral accumulation in vesicles within syncytial odontoblasts (Figs. 2A,D), (ii) vesicle deposition at the tooth mineralization front (Figs. 2B,E), and (iii) fusion of mineral platelets generated within the vesicular lumen with the sea urchin tooth primary plates (Figs. 2C,F). Spherical mineral/protein assemblies in immediate proximity to the mineralization front were no longer surrounded by a vesicular membrane (Figs. 2C,F).

Immunolocalization of SM50 and SM30 mineralization proteins in the plumula and cytochemical detection of SM50 at initial mineralization sites in sea urchin teeth

Immunolocalization of the SM30 glycoprotein and the polyproline-rich embryonic spicule protein SM50 was performed in the growth zone of continuously erupting sea urchin teeth and confirmed by Western blotting (Fig. 3). Antibodies against SM30 and SM50 proteins specifically reacted with the cytoplasm of cells associated with the developing tooth mineral as well as remaining mineral crystallites, but not with muscles and ligaments (Fig. 3). SM50 was widely distributed throughout the odontoblast syncytium and especially in secretory vesicles (Fig. 3A,B) as well as in the immediate proximity of initial mineralization sites (Fig. 3C). SM30 was detected at the tip of the odontoblast syncytium (Figs. 3D,E). Immunoreactions were confirmed using Western blots, revealing 53kDa, 48kDa, and 35kDa bands for SM50, and 46kDa and 35kDa bands for SM30 (Fig. 3F).

Highly defined ring-shaped SM50 repeat peptide self-assembly patterns

Electron micrographs indicated that SM50 sea urchin repeat proteins self-assembled into donut-shaped circular subunits with an outer diameter of approximately 50 nm and a central hole measuring 20 nm in diameter (Fig. 4A). In contrast, vertebrate amelogenin-derived PXX24 self-assembly subunits were fairly diffuse and contained spherical or circular subunits with a distance of approximately 25 nm in between individual subunits (Fig. 4B).

SM50 repeat peptide-mediated calcium carbonate crystal growth resulted in appositional growth patterns and fusion into expansive crystal networks

In electron micrographs, our SM50 repeat peptide mediated calcium carbonate crystal growth studies resulted in the formation of expansive calcium carbonate crystal webs (Fig. 4C). These lacey crystal webs consisted of a network of 100 nm thick rods of fused calcium carbonate crystals which were interspersed by circular spaces devoid of any crystals (Fig. 4C). In some areas, individual crystal platelets were distinguished, while other areas were too electron dense to identify individual crystallites (Fig. 4C). In contrast, calcium carbonate crystal growth conditions subjected to PXX24 peptide solution resulted in the formation of fairly thin (approximately 20nm) crystal alignments in which individual crystallites remained distinguishable (Fig. 4D).

PXX24-mediated apatite crystal growth featured elongated thick crystals while SM50-repeat peptide-mediated apatite crystal growth yielded parallel needle shaped crystals

In conjunction with our hydroxyapatite crystal growth solution, the SM50 sea urchin repeat peptide caused the formation of needle-shaped and parallel oriented crystals resembling those found in biological apatites of initial enamel (E). The lacey overall arrangement of apatite needle bundles was similar to those found in our SM50/carbonate growth studies (C). In contrast, the combination of the amelogenin-derived PXX24 tripeptide repeat peptide and apatite growth conditions resulted in the formation of 100–200nm long and 20–50nm thick composite crystals in which individual precursor crystallites remained visible (F).

Discussion

In the present study we have used the sea urchin tooth organ as a model system to determine parallels and differences between echinoderm and vertebrate tooth development as it relates to their protein-guided biomineralization strategy. Common elements included (i) the formation of mineral precursors within secretory vesicles, (ii) the gradual deposition of mineral precursors onto the developing tooth surface, and (iii) the use of polyproline-repeat element containing biomineralization proteins to control mineral growth and deposition. Differences between vertebrate and echinoderm tooth mineralization included (i) the types of mineral employed (calcium carbonate in sea urchins versus hydroxyapatite in vertebrates), (ii) the developmental origin of the odontogenic tissues (mesoderm in sea urchins versus ectomesenchyme (dentin/cementum) and ectoderm (enamel) in vertebrates), and (iii) the mode of mineral deposition in relationship to the secretory cells (intracellular in sea urchins versus extracellular in vertebrates), and (iv) the types of genes involved in biomineralization (SM50 and others in sea urchin versus amelogenin, DMP1, and others in

vertebrates). Together, this analysis provides unique insights into the fundamental mechanisms involved in the design and biological manufacture of mineralized teeth.

Macroscopic observation identified five symmetrically arranged, self-sharpening and continuously growing teeth within Aristotle's lantern of the sea urchin. Individual sea urchin teeth grow throughout the life of an animal, and mineralization occurs progressively, starting from the soft portion of the tooth, the plumula, to the plates and needle-shaped prisms of the tooth shaft, and further progressing toward the highly mineralized tip at the incisal edge of the tooth (Stock, 2014). With a Vickers microhardness of 200–360 kg/mm⁻², the tips of sea urchin teeth are as hard as or harder than human tooth enamel (Wang et al., 1997; Gutierrez-Salazar and Reyes-Gasga, 2003). Sea urchin tooth nanohardness data between 3.5GPa and >8 GPa even exceeds the 3.5GPa reported for human tooth enamel (Roy and Basu, 2008; Goetz et al., 2014). The gradual continuum between the soft plumula and the highly mineralized incisal tip resembles the gradual progression in enamel mineralization along the incisal face of the rodent incisor (Nanci et al., 1989). However, while the continuous differentiation of enamel secreting cells from the stem cell niche to the secretory and transitional ameloblast is well established, it is not known whether the continuous growth of the sea urchin tooth is paralleled by a similar differentiation of tooth-lining cells within the odontoblast syncytium. Our study revealed a gradual progression in differentiation from the syncytial periphery to the secretory front of the odontoblast continuum.

Our electron micrographs documented that the mineralization of continuously growing sea urchin teeth occurred through the deposition of mineral platelets from secretory vesicles onto the tooth surface. Our series of electron micrographs suggests that initial sea urchin mineral crystallites are first nucleated within odontoblast vesicles, which are then transported toward the surface of the ever-growing tooth. In proximity to the tooth surface, vesicles then lose their membranes, and platelet-shaped mineral deposits are fused with the sea urchin tooth primary plates through appositional crystal growth mechanisms. Intracellular mechanisms of sea urchin tooth mineralization have been well established (Märkel, 1969; Kniprath, 1974; Märkel et al. 1977) and are distinguished from the extracellular secretory mechanisms that are employed by vertebrates. Moreover, sea urchin and other echinoderm teeth are composed of mineralized calcium carbonate (calcite), and contain up to 43.5mol% magnesium (Wang et al., 1997). These magnesium-enriched calcium carbonates of corals, sea urchins, and other marine invertebrates are referred to as protodolomite (Schroeder et al., 1969; Veis et al., 2011), while vertebrate teeth are distinguished through the use of hydroxyapatite (dahlite) as the primary biomineral (Diekwisch et al., 1995). The mechanical data mentioned above indicate that sea urchin and vertebrate teeth achieve similar levels of hardness independent from the mode of mineralization and the type of biomineral employed.

The sea urchin biomineralization proteins, SM30 and SM50, were localized in the plumula of the sea urchin tooth, suggesting their involvement in sea urchin odontogenesis. Both are known to contain similar motifs, including a C-type lectin domain and an intrinsically disordered repeat domain (Livingston et al., 2006; Hussain and Livingston, 2009). Earlier studies have suggested that the SM50 polyproline repeat fragment plays a role during early mineralization and formation of calcium carbonate particles with distinct morphologies (Rao

et al., 2013). Both proteins, SM30 and SM50, appear to increase the flexural strength and fracture resistance of the mineral (Xu and Evans, 1999; Seto et al., 2002; Wustman et al., 2002; Wilt et al., 2013). In the present study, SM30 was mostly detected at the tip of the plumula, while SM50 staining intensity was substantially higher, and the protein was specifically localized in the mineral secreting vesicles and in proximity of initial mineralization sites, further supporting the concept of a significant involvement of SM50 in sea urchin tooth biomineralization. In previous studies we have pointed to the similarities between the SM50 polypyrrol repeat protein and other polypyrrol repeat proteins that are expressed during vertebrate enamel and bone formation, such as amelogenin and collagen. Our studies suggested that such intrinsically disordered proteins with long repeat stretches aided in the elongation of individual apatite crystals and facilitated enamel prism organization (Jin et al., 2009; Gopinathan et al., 2014), while they resulted in a compaction of calcium carbonate crystals (Gopinathan et al., 2014). The strong presence of SM50 in secretory vesicles close to the mineral apposition site observed in the present study suggests that long polypyrrol repeat stretches may play a role in the deposition and fusion of nucleated carbonate crystals onto the continuously growing sea urchin tooth surface as supported by our transmission electron microscopic data.

Our polypeptide self-assembly studies revealed that our SM50P6P7 polypeptide assembled into discrete donut-shaped patterns. The overall structure of the resulting matrix caused by the regular arrangement of neighboring donut-like subunits somewhat resembled the organizational structure of the early enamel matrix (Diekwisch et al., 1993). Moreover, subunits were delineated by sharp boundaries and were substantially more discrete than those observed in our amelogenin repeat polypeptide studies (Jin et al., 2009), in the native enamel matrix, or in the PXX24 subunit shown in the present study. Moreover, subunits were twice the size of those found in developing enamel (Diekwisch et al., 1995). The reasons for this highly discrete sea urchin SM50 repeat polypeptide self-assembly pattern are not entirely clear but may be related to the unique physical properties of the alternating hexa- and hepta-polypyrrol repeats of the SM50 protein. Together, these data demonstrate that polypeptides from the repeat region of biomineralization proteins self-assemble into matrices of unique structural organization that are characteristic of the individual protein and do not require the N- or C-terminus to be present. This ability of the repeat region to function independently from N- or C-terminal domains is likely of biological significance because of the well-established N- or C-terminal cleavage mechanisms that facilitate the release of a biologically active biomineralization protein at an early stage of crystal formation (Nagano et al., 2009; Sun et al., 2010).

In comparison to the tripeptide repeat protein PXX24 based on the vertebrate enamel protein amelogenin, the sea urchin SM50 derived hexa-/hepta-repeat peptide turned out to be a much more effective mediator of crystal growth. While PXX24 only resulted in sparse precipitates of calcium carbonate crystals on the grid surface as well as thick and undefined apatite crystal patterns, SM50P6P7 interaction with the mineral lead to the formation of expansive carbonate or apatite crystal conglomerates. Specifically, our electron micrographs document that SM50P6P7 induced the nucleation of carbonate crystals and caused adjacent crystals to fuse in an appositional manner as evidenced by the many calcium carbonate crystal arranged next to each other in our SM50/calcium carbonate crystal growth

experiment. Biophysical properties of the SM50 repeat region related to crystal nucleation, appositional growth, and adjacent crystal fusion would also explain the assembly of multiple calcium and magnesium carbonate crystals at the secretory aspect of the odontoblast syncytium and their deposition onto the continuously erupting sea urchin tooth surface.

Most remarkable was the ability of the invertebrate sea urchin repeat domain peptide SM50P6P7 to form needle-shaped and parallel arranged apatite crystals resembling those of developing vertebrate enamel (Diekwisch et al., 1995), albeit in a lacey overall arrangement. Based on our previous study revealing a direct link between peptide assembly and crystal growth (Jin et al., 2009) we propose that the formation of defined apatite crystals in our study is the direct result of the discrete SM50P6P7 polypeptide matrix structure. In support of this concept, the amelogenin-derived PXX24, which featured a much more diffuse matrix arrangement, lacked the capability of generating expansive carbonate precipitates or needle-shaped apatite crystals, even though PXX24 was derived from the vertebrate protein most associated with enamel formation. From an evolutionary perspective, the high effectiveness of SM50P6P7 repeat peptides in their ability to trigger calcium carbonate crystal assemblies and even to promote apatite parallel needle-shaped crystal growth might be explained by a putative evolutionary advantage of a highly effective protein machinery to facilitate the growth of sea urchin teeth capable of withstanding highest levels of abrasion and mechanical loads. As mentioned in the introduction, sea urchins have fairly evolved genomes and invertebrates are capable of generating species-specific biomineralization mechanisms, suggesting that throughout the course of deuterostomian evolution, the biomineralization machinery of individual clades may have rapidly evolved with unique sets of genes to respond to changing requirements for nutrition or protection. While evolving independently in vastly different organisms and in different mineralized tissues, many of these mineralization genes express intrinsically disordered proteins, a direct result of the polyproline type II helices within their protein structure (Kalmar et al., 2012). This utilization of polyproline repeats across deuterostomian mineralization genes is just one more example of the heterochromia of common sequence motifs in bones, enamel, dentin, and other mineralized tissues (Slavkin and Diekwisch, 1997; Diekwisch et al., 2002; Jin et al., 2009). The comparably lesser effectiveness of PXX24 as a crystal growth mediator when compared to SM50 may be simply an indication of the substantial complexity of vertebrate enamel development; including a significantly longer repeat region with 10–20 polyproline tripeptide repeats and elaborate posttranslational modifications (Gopinathan et al., 2004). Alternatively, with their hexa-/hepta- polyproline tandem repeat motif controlling tooth mineralization, sea urchins might have developed an exceptionally capable sequence motif to respond to the evolutionary pressure of their around-the-clock dependency on a functional tooth apparatus.

We conclude that sea urchin tooth mineralization is an intracellular process mediated by organic matrices within the odontoblast syncytium. Our data suggest that the embryonic spicule proteins SM30 and SM50 are involved in adult sea urchin tooth mineralization and that the highly abundant polyproline repeat protein SM50 is present in the odontoblast syncytium, at the site of crystal nucleation, and at the site of crystal deposition onto the continuously eruption sea urchin tooth. Polypeptides derived from the SM50 polyproline hexa-/hepta-peptide repeat region formed highly discrete, donut-shaped self-assembly

patterns ideally suited as crystal growth templates. In our studies, these SM50P6P7 peptides emerged as powerful mediators of calcium carbonate and apatite crystal growth. Their ability to promote carbonate crystal growth and fusion of adjacent crystals into a widespread network across a surface likely provides the molecular basis for the intracellular deposition of carbonate crystals onto the continuously mineralizing sea urchin tooth surface.

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Figure 1. Anatomy of the Pacific sea urchin *Strongylocentrotus purpuratus*

(A) Macrograph of an individual measuring 2 inches in diameter. The position of the mouth opening is marked by an arrow. (B and C) are preparations illustrating the structure of Aristotle's lantern with (B) illustrating the five teeth pointing upward and (C) revealing the position of the five plumulae of individual teeth at the anal aspect of Aristotle's lantern. The chewing tip (C) is surrounded by a concentric ring of podia (B). (D–F) are three different micrographs illustrating the incisal tips of the five individual teeth. (D) is a light micrograph of a fixed specimen, (E) is a scanning electron micrograph of a fixed specimen, and (F) is a

macrophotograph of the occlusal tip of a living organism. Note the sharpened incisal tips as a result of constant occlusal abrasion between adjacent teeth. The circular ring of bulbous protrusions represents the podia, small organelles involved in locomotion, adhesion, and feeding. (G) Macrograph of a *Strongylocentrotus* tooth, measuring 3cm in length. The position of plumula, shaft, and incisal tip of the tooth are indicated. (H,I) Thin sections of *Strongylocentrotus* illustrating the position of primary plates in relationship to the odontoblast syncytium.

Figure 2. Ultrastructure of sea urchin tooth mineralization

This figure contains two sets of electron micrographs (A-C, D-F) illustrating key stages of sea urchin intracellular tooth mineralization. (A,D) are low magnification electron micrographs of a secretory syncytial odontoblast, (B,E) illustrate the accumulation of mineral-filled secretory vesicles at the tooth mineralization front, and (C,F) illustrate the deposition of mineral platelets (min) from secretory vesicles (ves) and protein/mineral assemblies (abl) directly onto primary plates (pp) residing on the continuously growing sea urchin tooth surface. Note the lack of a membranous boundary surrounding the secretory vesicles in immediate proximity to the tooth surface (C,F).

Figure 3. SM30 and SM50 Biomaterialization protein localization in sea urchin teeth
 (A,B) Immunolocalization of the SM50 protein in the plumula syncytium of continuously growing sea urchin teeth. Note the pronounced reaction in the secretory vesicles (ves) at the lateral aspects of the plumula positioned adjacent to the mineral layer (not visible due to decalcification). (C) Immunohistochemical localization of SM50 adjacent to initial mineral deposits (arrow), (D,E,) SM30 protein localization in the syncytium (E is a higher magnification of figure D), (F) Western blots illustrating SM50 and SM30 protein recognition in sea urchin tooth extracts.

Figure 4. Sea urchin SM50 and amelogenin PXX24 self-assembly patterns and effects on carbonate versus apatite crystal growth

(A,B) illustrate differences in self-assembly patterns and subunit dimensions between SM50 and PXX24. The diameter **A** of a representative subunit (arrow) is highlighted as the distance between two parallel bars. (C,D) Representative electron micrographs of calcium carbonate crystal growth structures after 2 hour growth in SM50 (C) and PXX24 (D) protein solutions on holy carbon grids. (E–G) Representative electron micrographs of calcium hydroxyapatite crystal growth structures after 2 hour growth in SM50 (E) and PXX24 (F)

protein solutions on holy carbon grids. The combination of SM50 protein and hydroxyapatite growth solution yielded approximately equal numbers of the fused platelet (E) and the parallel needle (F) crystal habits. All electron micrographs were taken at the same magnification (bar = 100nm, F).