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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

Yelin Mao[a], Paul G. Satchell[b], Xianghong Luan[a,c], Thomas G.H. Diekwisch[c,d,*,1]

[a] UIC College of Dentistry, Department of Orthodontics, USA
[b] Baylor College of Dentistry, Department of Endodontics, USA
[c] UIC College of Dentistry, Department of Oral Biology, USA
[d] Baylor College of Dentistry, Department of Periodontics, USA

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

1. 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

* Corresponding author at: Texas A&M University, Baylor College of Dentistry, 3302 Gaston Avenue 75206 Dallas, TX, USA. Tel.: +1 214 828 8459.
E-mail addresses: diekwisch@bcd.tamhsc.edu, tomdkw@uic.edu (T.G.H. Diekwisch).
[1] Address: UIC College of Dentistry, 801 South Paulina, Chicago, IL, USA.

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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 the role of the polyproline repeat proteins such as SM50 in sea urchin tooth mineralization.

Echinoderms are marine organisms characterized by mineralized calcite skeletons and a fivefold 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., 2007). 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., 2007). Moreover, it has been demonstrated that the evolution of structural biomineralization proteins has occurred independently and multiple times among metazoans (Jackson et al., 2010). 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 50 kDa (Sucov et al., 1987; Richardson et al., 1989; Killian and Wilt, 1996) and features a total of 15 PXX tripeptide repeats, 12 PXXXXXQ-PXXXXQ 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. The 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.

2. Materials and methods

2.1. Electron microscopy

For electron microscopy, Aristotle's lanterns of the Pacific sea urchin *S. purpuratus* were prepared using surgical dissection tools. Individual teeth were further isolated, fixed in Karnovsky's fixative for 2 h, and subsequently contrasted using 4% OsO_4. 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.

2.2. Immunohistochemistry

For immunohistochemical localization, Aristotle's lanterns of the Pacific sea urchin *S. purpuratus* were dissected using a surgical knife and fixed in 10% buffered formalin. Tissues were decalcified in disodium EDTA (Warshawsky and Moore, 1967) for 6 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 h. 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 three times in PBS and subsequently incubated for 10 min with biotinylated anti-rabbit IgGs as secondary antibodies. After washing in PBS (three times), sections were exposed to the streptavidin-peroxidase conjugate for 10 min and then washed again in PBS (three times). Signals were detected using an AEC Substrate-Chromogen mixture of sections were counterstained using hematoxylin and mounted with GVA-mount.

2.3. Immunocytochemistry

Immunohistochemistry was carried out as described by Diekwisch (1998). Prior to immunoreactions, sections were blocked

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