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The Dynamics of Secretion during Sea Urchin Embryonic Skeleton Formation

Fred H. Wilt^{1,3}, Christopher E. Killian¹, Patricia Hamilton^{1,2}, and Lindsay Croker¹

¹Department of Molecular and Cell Biology, University of California, Berkeley, 142 Life Sciences Addition, M/C #3200, Berkeley, California 94720-3200 U. S. A.

Abstract

Skeleton formation involves secretion of massive amounts of mineral precursor, usually a calcium salt, and matrix proteins, many of which are deposited on, or even occluded within, the mineral. The cell biological underpinnings of this secretion and subsequent assembly of the biomineralized skeletal element is not well understood. We ask here what is the relationship of the trafficking and secretion of the mineral and matrix within the primary mesenchyme cells of the sea urchin embryo, cells that deposit the endoskeletal spicule. Fluorescent labeling of intracellular calcium deposits show mineral precursors are present in granules visible by light microscopy, from whence they are deposited in the endoskeletal spicule, especially at its tip. In contrast, two different matrix proteins tagged with GFP are present in smaller post-Golgi vesicles only seen by electron microscopy, and the secreted protein are only incorporated into the spicule in the vicinity of the cell of origin. The matrix protein, SpSM30B, is post-translationally modified during secretion, and this processing continues after its incorporation into the spicule. Our findings also indicate that the mineral precursor and two well characterized matrix proteins are trafficked by different cellular routes.

INTRODUCTION

The formation of biomineralized hard parts by organisms presents many interesting problems for biologists. Formation of teeth, bones, shells, spicules and carapaces involves import and accumulation of large amounts of mineral precursors, which are then precipitated and/or crystallized with specific, secreted structural matrix proteins to form composites that possess remarkable forms and material properties. There is a tremendous variety in the composition and morphology of these structures [1]. The development of the skeletal spicule of the sea urchin embryo offers good opportunities to grapple with the cellular and molecular mechanisms that govern the formation of a calcified endoskeletal element. Indeed, the study of the sea urchin embryo skeleton figured prominently in the foundations of experimental embryology and cell biology [2,3]. We wished to characterize the relationship between the mineral precursor, calcium, and the matrix proteins during their synthesis, accumulation and transit through the cell to the skeleton.

The endoskeletal spicule of the larva is composed of more than 99% calcite, containing about 5% Mg and 95% calcium carbonate [4]. The matrix (< 1% of the mass) is primarily composed

³corresponding author, E-mail: wilt@socrates.berkeley.edu.

²present address: Genentech, Inc., 1 DNA Way, 12-332, South San Francisco, California 94080, U. S. A.

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of 40 or more water soluble proteins, many of which are acidic and glycosylated [5]. The proteins radically alter the material properties of the spicule, rendering it harder and more flexible than calcite [6]. The calcite originates from calcium of the sea water [7] and is accumulated and secreted by primary mesenchyme cells (PMCs) into the membrane limited space surrounded by the cytoplasmic filopodia of a syncytium of PMCs [8–11].

Several of the matrix protein genes have been cloned and the proteins characterized [11,12]. We will consider here the proteins SpSM50 and SpSM30B, two well characterized spicule matrix proteins. The SpSM50 protein is an integral matrix protein that has a basic pI and is not glycosylated. Its promoter has been characterized [13] and the protein has been shown to be present and occluded within the calcite as well as present on the surface of the mature spicule [5,14,15]. Deposition of SM50 is essential for spicule elongation [16]. SM30, an acidic glycoprotein, originally characterized by George et al.[17] and Killian and Wilt [5]. Recent studies have found that the originally isolated SM30 protein is a member of six closely related proteins designated SpSM30A-F [12]. This original SM30 protein is now designated SpSM30B in *S. purpuratus*.

There has been little direct examination of the dynamics of the several constituent cellular processes involved in endoskeleton formation. We have used vital labels to trace the constituents of the cell from the cell to the spicule. Trafficking of the calcium in PMCs was followed by use of the calcium fluorophor, calcein. GFP-tagged matrix proteins were used to follow deposition of protein in the spicule, and radioactive labeling of a matrix protein was used to follow the protein processing and secretion. We will show that the calcium and some matrix proteins are located in different vesicles. After secretion, calcium moves primarily to the elongating tip, while matrix proteins are restricted in their mobility in the filopodium. The prevalent spicule matrix protein, SpSM30B, is modified during its secretion, a process that continues even after it is incorporated into the spicule.

METHODS AND MATERIALS

Embryo Culture, Micromere Culture, and Microinjections

Strongylocentrotus purpuratus sea urchins were collected locally, and *Lytechinus pictus* were purchased from Marinus Scientific. Gametes were shed and embryo cultures were maintained at 15°C, at a concentration of 0.25 % (v/v) with stirring at 60 rpm.

Micromeres were collected *en masse* on sucrose gradients and cultured at 15°C in bacteriological grade Petri plates following methods derived from those of Okazaki [9], as outlined by Wilt and Benson [18]. Microinjections were carried out by the methods established by McMahon et al. [19] and Arnone et al. [20].

Calcein Labeling

Isolated micromeres were cultured on coverslips and stimulated to form biomineralized spicules with 4% horse serum. Cultures were exposed to sea water containing 100µg/ml of calcein, then rinsed several times in plain sea water, fixed briefly with 5% formalin in sea water, and rinsed again. The coverslips were inverted on to a glass slide and kept in moist chambers. During labeling and processing of micromere cultures, the calcein labeled cells were kept in darkness at 4°C as much as possible. They were examined within 30 min by confocal microscopy.

⁴⁵Calcium Labeling

After 50–52 h of culture at 15° C, the sea water-horse serum medium was aspirated and artificial sea water containing 25 µCi/ml of ⁴⁵CaCl₂ (Amersham) was added. After 40 min of labeling

the label was removed and the culture plate gently rinsed with ice cold sea water. Non-radioactive sea water at 15° C was then added and the cells were harvested and processed for determination of protein and radioactivity in the whole cell and spicule by the method of Hwang and Lennarz [21] as modified by Ingersoll and Wilt [22].

GFP Constructs

The reporter constructs pGFP-SM50 promoter and pGFP-SM30 promoter were obtained from Koji Akasaka (University of Tokyo). Each contains the pGreenLantern-1 GFP reporter vector (Gibco-BRL) with the upstream controlling regions of either SpSM50 or SpSM30C subcloned just 5' to the region that encodes the GFP protein. pGFP-SM50 promoter was generated by inserting the 0.4 kb Pst1-Sal1 fragment of the SpSM50 upstream region into the Pst1 and Sal1 restriction sites of the pGreenLantern-1 multiple cloning site [13,20]. pGFP-SM30 promoter was generated by inserting the 1.6 kb Kpn1-Xba1 fragment of the *SpSM30C* upstream region [12,23] into the Kpn1 and Spe1 sites of pGreenLantern-1 vector.

Standard PCR and subcloning techniques were used to generate the eGFP-SM50 fusion and eGFP-SM30 fusion constructs. The upstream controlling regions described above were stitched together with the coding sequence of the *SpSM50* and *SpSM30B* spicule matrix protein genes and fused in frame to the amino end of the eGFP gene (pEGFP-N1 vector (Clontech)) with a linker sequence encoding eight alanine residues. *SpSM30B* and *C* genes encode amino acid sequences that are 99% identical and these forms are prevalent in the spicule [5,12]. Embryos were visualized using a Zeiss LSM 510 META/NLO Axioplan 2 confocal microscope. The slit width was adjusted the same for all samples, and allowed enough background for faint visualization of non-fluorescing cells and spicules.

³⁵S-Methionine Labeling and Analysis of PMC Cultures

After 48–60 h of culture at 15°C., the sea water-horse serum medium was aspirated and artificial sea water containing 25–40 µCi/ml of ³⁵S-methionine was added, and the culture was continued for various lengths of time. A chase was imposed by removal of radioactive medium, followed by a gentle rinse of the culture plate with sea water, followed by continued culture in sea water containing 1 mM non-radioactive methionine.

After pulse labeling or after the chase, the medium was removed from the plates and then rinsed with 10 ml of ice cold sea water. Plates, which still had adherent mesenchyme cells, were then exposed to plain sea water (mock extraction), or spicule extraction medium (CMFSW, 5 mM EDTA, 20 mM EGTA, 50 mM Pipes pH 6.0, protease inhibitors) for 1–2 h at 4° C. The protease inhibitors mixture contained: 5 mM benzamidine, 5 mM ε-amino-N-caproic acid, 5 mM N-ethylmaleimide, and 0.5 mM phenyl methyl sulfonyl fluoride. The demineralization of spicules in the PMCs was examined during this period by bright field and DIC microscopy to ensure demineralization was complete. The extraction medium was removed gently and designated as "spicule" extract. The plate was rinsed again with sea water, and the adherent cells were then loosened from the plate by scraping with a plastic policeman in homogenization medium (0.5 M NaCl, 50 mM Tris pH 8, protease inhibitors). The harvested cells were homogenized in a tight fitting stainless steel Dounce homogenizer and examined by phase contrast microscopy to ensure complete homogenization. The homogenate was centrifuged for 5 min at 1100 × g, and the supernatant removed and designated "cell". An aliquot of this fraction was used for protein determination. The pellet was resuspended in homogenization medium containing 0.5% (v/v) NP-40 detergent, suspended by vigorous pipetting with a 1.0 ml capacity "blue" tip, and centrifuged again at 12000 × g, for 10 min. There was no immunoprecipitable radioactivity in the pellet from this second round of centrifugation. This detergent soluble fraction is designated "deterg" in the text and figure 3. The fractionation thus results, when pH 6 and EGTA are used, in a "spicule" fraction containing soluble spicule matrix proteins, a low

speed supernatant of the homogenate designated "cell", and a detergent soluble fraction of the low speed pellet designated "deterg". No immunoprecipitable radioactivity was found in the sea water rinse of the plate after spicule extraction, or in the high speed pellet of the detergent solubilized material.

The "spicule", "cell" and "deterg" fractions prepared from labeled PMCs were immunoprecipitated using an anti-SpSM30B antibody (polyclonal, made in rabbits) [5] and protein A-sepharose by the methods described by Harlow and Lane [24]. Labeled extracts were pre-adsorbed to remove adventitious binding by use of pre-immune serum and protein A-sepharose. Control experiments were performed using different antibody concentrations and successive reprecipitations to ensure that there was complete precipitation of the SpSM30B antigen under the conditions employed. Immunoprecipitates were dissolved in sample buffer and subjected to gel electrophoresis using the methodology of Laemmli [25]. Gels were impregnated with 1 M sodium salicylate, dried, and analyzed by autoradiography and/or phosphorimager.

Labeled PMCs extracted initially with plain sea water never showed any immunoprecipitable label in the extract, and this "mock" extraction control was not further considered. Gel analysis of various time points had lanes with many control samples, including the plain sea water extractions and reprecipitations of supernatants from the protein A sepharose. Figures 3B, C, and D were prepared from scans of these gels. Adobe Photoshop and Illustrator were used to crop irrelevant lanes from the raw image.

RESULTS

Ca⁺² is Precipitated in the PMC before Secretion into the Spicule

Calcein is a member of the fluorescein family that interacts with Ca⁺², and gives a strong fluorescent signal that is easily detected when the calcium bound to the fluorophor is precipitated or crystallized [26] in the presence of the fluorophor. It has been used to follow enamel deposition [27], the dynamics of sponge spicule formation [28] and the kinetics of elongation of the spicule in sea urchin embryos [29]. In these various experiments intense fluorescence is present only in the newly formed portion of the biomineralized tissue; tooth or spicule material formed before introduction of calcein is not labeled. We reasoned that calcein could be used to detect intracellular calcium precipitates or crystals prior to its deposition in the spicule, if such a process occurred.

In embryos the uptake of dye by surface cells is rapid, though the mechanism of transport of this anionic dye is not known. The penetration of the dye into the blastocoel is slow. The ability to wash out unprecipitated dye in order to reduce the background to reasonable levels severely limits kinetic studies. We therefore used cultures of mesenchyme cells developing on cover slips for these experiments. Calcein can be introduced easily into the culture medium, and is rapidly taken up by mesenchyme cells. Figure 1A shows a portion of a single spicule with some primary mesenchyme cells enrobing the already developed spicule. After 20 minutes of exposure to calcein the attached cells were gently washed, lightly fixed, and examined immediately by confocal microscopy. Distinct foci of fluorescence can be seen in PMCs attached to already formed spicules; the outline of the previously formed spicule is barely visible. Figure 1 B illustrates the situation after a longer exposure of 40 min to the dye. There is more fluorescence in some of the attached cells. The spicule is outlined by light labeling of cytoplasm of the syncytial cable, and the extending tip is now heavily labeled. The images are consistent with a primary precipitation event in the cell bodies followed by appearance in the filopodial cytoplasm, which in turn is followed by incorporation of the labeled calcium into the elongating and thickening spicule. After continuous exposure to calcein for 10 hours (figure 1C) the newly formed portion of the spicule is labeled, and there is very little fluorescence in

cell bodies. The sizes of punctate fluorescent foci in PMCs (Fig. 1A and 1B) are variable. These foci certainly exceed 200 nm in diameter since that is the approximate limit of resolution of the microscope used and the foci are seen clearly. Fifteen to twenty min pulses were the shortest exposure times we attempted. After such pulses, there was invariably labeling of cellular elements and little incorporation into the spicule itself. When calcein was washed out after a 20–30 min pulse, the punctate fluorescent foci in the PMC gradually dimmed taking a minimum of about 3 hours to disappear in all experiments (n= 12). Concomitant with waning of fluorescence in the cell body, fluorescence of the spicule, especially the tip [30], increased. Formation of intracellular fluorescent precipitates occurs prior to spicule deposition and wanes during a chase, a result consistent with the notion that the intracellular precipitates are precursors to the mineral of the spicule.

We used ^{45}Ca labeling to estimate a functional size of the calcium pool used for spicule formation. PMC cultures that were actively forming spicules were labeled for 40 min with $^{45}\text{CaCl}_2$, and then washed with sea water and maintained in culture in non-radioactive sea water. These experiments are an extension of those carried out by Ingersoll and Wilt [22]. They had previously shown active incorporation of ^{45}Ca from cellular pools into spicules during a one hour chase period. We repeated the experiments and monitored incorporation into spicules during a chase period of 5 hours. ^{45}Ca deposition into growing spicules continued with little diminution for more than 4 hours of chase period (data not shown), indicating a rather large functional pool of intracellular calcium for spicule formation. The result is concordant with the slow disappearance of calcein labeled material from the PMCs.

GFP Tagged Matrix Proteins are Incorporated into the Elongating Spicule

We wished to monitor the secretion of matrix proteins and compare their trafficking to the delivery of calcium. Plasmid DNA encoding spicule matrix protein-GFP fusion proteins driven by *SpSM30C* or *SpSM50* promoters was microinjected into zygotes of *S. purpuratus*, and expressed in PMCs of some embryos. We also found appropriate expression after injection of these same constructs into zygotes of *L. pictus*, indicating the *S. purpuratus* *SpSM50* and *SpSM30C* promoters are recognized by the transcription apparatus of *L. pictus*. This observation is consistent with the expression of these genes in interspecific hybrids [31].

The GFP fluorescence was observed by confocal microscopy, and is shown in Figure 2. The expression of spicule matrix protein-GFP fusion constructs was restricted to the PMCs. Figure 2A shows expression of the eGFP-SM50 fusion protein in a *S. purpuratus* embryo: There are three foci of high expression (arrows) in this particular embryo, and there are also a number of non-expressing PMCs. This pattern of expression is to be expected in a situation where expression is mosaic and descendants of only a few cells among the four to eight founder cells are competent to express the transgene at high levels. When GFP fluorescence in the expressing cells is viewed at lower intensities, peak fluorescence is often seen in a perinuclear pattern and located on the side next to the spicule itself. In contrast to calcein staining, GFP fluorescence is not speckled. Some staining of the spicule adjacent to labeled cells is evident, though it usually extends a limited distance (~10 μm) from the nearest labeled cell. Some fluorescence in filopodial cytoplasm is discernable (see figure 2A). *SpSM50* is known to surround the spicule, as well as embedded within it [15].

eGFP-SM30 fusion protein expression is also limited to PMCs and displays a mosaic pattern of expression within the PMCs. Foci of strong cellular expression are shown in figure 2C (arrows). Observations at lower intensity of fluorescence than figure 2 showed that the intracellular expression has primarily a perinuclear localization, consistent with its presence in the Golgi body. Fluorescence does not spread throughout the PMC syncytial array. Fluorescence of syncytial cytoplasm and spicules is faint, just as is the case when immunostaining procedures are used to localize endogenous *SpSM30B* protein [15]. However,

in a few instances eGFP-SM30 fluorescence could be seen in filopodial cytoplasm near expressing cells (figure 2E); this example (2E) is a spicule in a larva of *Lytechinus pictus*, indicating that the eGFP-SM30 fusion transgene is expressed with fidelity in another species, as was the eGFP-SM50 fusion transgene (data not shown). Most SpSM30B is found occluded within the mineral [15] and GFP fluorescence is probably less visible viewed, as it were, through a calcite shroud. We sometimes could see fluorescence of this occluded protein within the exposed mineral when a spicule was inadvertently broken (data not shown).

Expression of both fusion proteins was random with respect to the skeletal location, viz., there was no preference for the cells near the original ventrolateral site of initiation of biomineralization, nor preference for the elongating tip, though labeling of cells at the tip was seen occasionally. The SM50 and SM30-GFP fusion proteins are secreted and incorporated into the skeleton in the vicinity of the cell of origin.

For comparison, a construct of GFP alone (without the fusion protein) under the control of either *SpSM50* or *SpSM30C* promoter is shown in figure 2B (*SpSM50* promoter) and figure 2D (*SpSM30C* promoter). In these control experiments, GFP is present exclusively in PMCs, as would be expected for transgenes driven by these promoters. However, unlike the eGFP-SM50 and eGFP-SM30 constructs, all of the PMCs and spicules are labeled in these instances. This is similar to what has been described for SM50 promoter by Arnone et al. [20] and for SM30 promoter by Akasaka (personal communication). Since it is known that the inheritance of the transgene is mosaic, and only some PMCs are expressing the transgene, the presence of GFP throughout the skeleton indicates that the GFP is mobile within the syncytium, just as are lower molecular weight dyes [20].

Our observations that the GFP-SM50 and SM30 fusion transgene construct expression is not discrete but rather stains large whole portions of the PMCs in the light microscope is consistent with the observations of Ingersoll et al. [32]. They used immunoelectron microscopy to localize SpSM50 and SpSM30B to post Golgi vesicles that are less than 50 nm in size. These matrix-protein-containing vesicles appear somewhat smaller than the vesicles identified by calcein staining and electron microscopy [32] as likely containing the mineral precursors. The GFP-SM50 and SM30 fusion transgenes also labeled the spicule close to the PMCs were expressing the gene, while the calcein that was chased from the PMCs labeled the end of the growing spicule. These different patterns of expression further indicate that spicule matrix proteins SpSM50 and SpSM30B are trafficked in vesicles different from those that traffick the calcium carbonate mineral precursor.

SpSM30B Protein is Modified During Secretion

Since SpSM30B is a prevalent protein found occluded in spicules, we wished to follow its secretion by use of radioactive tracers; this is facilitated by the use of primary mesenchyme cell cultures. Immunoprecipitation using a polyclonal antibody against the SpSM30B protein was efficacious and backgrounds were very low. In a typical experiment, cultures actively forming spicules (~2.5 days in culture), were washed free of serum containing sea water, and then exposed to ³⁵S methionine in sea water for 15–30 minutes. Some of the culture plates were then quickly washed in sea water and culture continued in sea water containing non-radioactive methionine.

Radioactive proteins in the spicule were gently extracted using sea water adjusted to pH 6 and containing EGTA [33]; under these conditions the spicules could be seen to demineralize in 30–60 min at 4°C. A control was carried out in which labeled cells and spicules were exposed to plain sea water at pH 8. Under these conditions microscopic examination showed that the spicules were not demineralized; under such conditions any label present in the spicule would not be extracted and would therefore appear as intracellular. Both mock extracted (sea water)

and cells extracted at pH 6 + EGTA were scraped from the plates, homogenized, fractionated by centrifugation and treated with non-ionic detergent. A flow diagram of the fractionation is shown in figure 3A.

After 15 min of labeling there was no labeled SpSM30B in the spicule (data not shown). SpSM30B is exclusively present in cellular structures that sediment at relatively low speeds ($1100 \times g$), which are completely solubilized by treatment with detergent (Figure 3B, "Deterg"). The detergent solubilized material does not sediment at $100,000 \times g$ for 1 hour (data not shown). The intracellular SpSM30B solubilized by detergent migrates at ~ 45 kDa, if cells were not previously extracted at pH 6 and EGTA (Fig. 3B "Mock"). If, however, cells were exposed to the spicule extraction medium containing EGTA at pH 6, most of the immunoprecipitable SpSM30B in the detergent soluble fraction has been cleaved by proteolysis, forming specific bands at mobilities corresponding to 32, 30 and 25.5 kDa (Fig. 3B "Deterg"). This pattern of proteolytic degradation was highly reproducible, and it occurred only after the intact cells were labeled for very short times and were exposed to pH6 and EGTA. Nascent intracellular SpSM30B must be more susceptible to proteolytic degradation when cells are stressed by low pH and EGTA.

The situation seen after 15 minutes of labeling changes substantially after a ~ 3 hour chase in non-radioactive sea water containing 1 mM methionine (figure 3C). There is now substantial radioactive SpSM30B that has been secreted and is present in spicules, and it is present in two isoforms with mobilities corresponding to 45 and 42 kDa. These two bands are identical to the forms of SpSM30B extracted from highly purified pluteus larvae spicules. However, after this chase, the 42 kDa isoform is not present in detectable amounts in the intracellular pool of SpSM30B prior to its secretion; only the high molecular weight form is seen in cells after the chase (figure 3C "Deterg"). This intracellular SpSM30B is not sensitive to the proteolytic degradation (figure 3C "Deterg"), which was seen after the 15 min pulse (figure 3B). The results of the pulse-chase regimen indicate that SpSM30B is present in detergent sensitive structures, and is then delivered to the spicule. Beginning or concomitant with the delivery a process of slow conversion of a 45 kDa to a 42 kDa form occurs.

Extended periods of labeling show a secretion of SpSM30B into the developing spicule (figure 3D "Spicule"), and the 45 kDa and 42 kDa forms are both present, with the 42 kDa form predominating. The low speed supernatant (figure 3D, "Cell") shows low levels of both forms in this experiment, however, this was not so obvious in three other duplicate experiments. This may indicate the beginning of intracellular processing just prior to or concomitant with secretion. The intracellular form (figure 3D "Deterg") migrates at 45 kDa and is not susceptible to proteolytic degradation after prior extraction of spicule proteins.

We followed the immunoprecipitable radioactivity in SpSM30B over an extended period of time (figure 4). A 45 min pulse of exposure to ^{35}S methionine was followed by washing and subsequent culture in sea water with non-radioactive methionine. Samples were processed in this example at 2, 4, 6, and 24 hours after cessation of the pulse. By two hours, a decrease of radioactive, intracellular SpSM30B has occurred together with an increase in radioactivity in the spicule. Thereafter, the radioactivity in the cell continues to slowly decrease, while radioactivity in the spicule remains approximately level. We interpret this to mean that turnover of SpSM30B in the mature spicule is low, but that the SpSM30B in the cell that is not secreted is subject to intracellular turnover. Whether the intracellular SpSM30B is only subject to turnover under these culture conditions, or whether it also occurs in intact embryos, is not known. Also shown in figure 4 is the ratio of 45 to 42 kDa isoform of SpSM30B present in the matrix of the secreted spicule. There is a gradual lessening of the amount of the 45 kDa isoform and a concomitant increase in the lower 42 kDa form. The exact ratio of 45 kDa to 42 kDa forms varied in different experiments, but always showed a decrease in the 45 kDa form and

an increase in the 42 kDa form as chase time progressed. We believe it is likely that the 45 kDa form is slowly processed to form the 42 kDa form, concomitant with and also subsequent to its actual secretion into the forming spicule.

DISCUSSION

Our experiments reveal some novel features of secretion of a calcareous endoskeleton. The processing and delivery of calcium and two different matrix proteins are: 1) Localized in different organelles, 2) Secreted with different kinetics, and 3) Are shuttled to distinct terminal locations. Furthermore, a prevalent spicule matrix protein, SpSM30B, is slowly modified at the time of delivery to the spicule and this modification continues after incorporation of protein into the mineralized spicule. We should note that the discovery of distinct pathways for calcium versus SpSM30B and SpSM50 does not exclude the possibility that some other matrix protein(s) are associated with precipitated calcium, and one candidate would be the matrix protein(s) shown by Raz et al. [34] to stabilize amorphous calcium carbonate.

The Mineral

Previous work [7] showed that calcium in sea water is the ultimate source of calcium for spicule construction. Inhibitors of calcium (and other ions) transport interfere with spicule formation (reviewed in [10]). Hwang and Lennarz [21], using 30 minute labeling periods, showed directly that ^{45}Ca was imported into PMCs, *in vitro*, and then rapidly secreted into forming spicules. The present results of the ^{45}Ca chase experiments show that radioactive spicule deposition continues many hours after withdrawal of the label from the medium. These findings along with the findings of Ingersoll and Wilt [22] indicate that the intracellular pool of calcium that is the proximate source for spicule formation must be rather large, when compared to the rate of withdrawal for spicule secretion.

Previous attempts to visualize the intracellular sources of calcium for spicule formation, notably staining experiments of Decker et al. [33] and Beniash et al. [35], were able to identify calcium deposits in fixed, processed material; Beniash et al. [35] showed the deposits were probably a non-crystalline metastable intracellular precipitate. We have shown here, using a vital fluorescent tag specific for precipitated calcium, that calcium is present in rather large intracellular precipitates. The mechanisms by which the anionic dye is taken up by cells engaged in mineralization is not known, even though it has been observed in many instances [28,29]. Cellular labeling was seen without fixation, but the background was too great to characterize it; very brief fixation with formalin lowered background sufficiently to reveal the speckled appearance of the PMCs. One cannot dismiss the possibility of fixation artifacts, but the conclusion that the cells are labeled before spicules is clear.

The fluorescent precipitates in lightly fixed cells are very labile, often disappearing in 30–40 minutes during examination on the confocal microscope, as would be expected for amorphous calcium carbonate. The intracellular fluorescent label slowly disappears during a chase and labeled calcium precipitates subsequently appear in and on the developing spicule. In our judgment, this constitutes strong support for the idea that the source of calcium for the spicule is exocytosis of amorphous calcium carbonate. We should note that the data do not exclude dissolution of the intracellular amorphous calcium carbonate followed by transport of the calcium ion to the hydrophobic space in which the spicule is formed.

Matrix Proteins

Our picture of matrix protein secretion for spicules, and indeed, generally for skeletal elements of invertebrates, is based on static images using immunocytochemistry and other cyto- and histochemical techniques. The introduction of GFP tags and microinjection of sea urchin

zygotes by the Davidson lab [20] provides another set of tools. We followed two well characterized spicule matrix proteins, SpSM30B and SpSM50. These proteins are processed in the Golgi apparatus, trafficked in small vesicles, and secreted into the space enclosing the forming spicule [32]. Immunocytochemical staining of SpSM30B and SpSM50 in PMC bodies is diffuse and is not punctate, consistent with their localization in numerous small vesicles. Only very small amounts of SpSM30B, a prominent spicule matrix protein, are located on the surface of the spicule, and static images of immunostaining usually only show protein in the primary mesenchyme cell bodies [15]. The GFP tagged SpSM30B shows a similar behavior, appearing mainly in cell bodies, and in favorable instances showing a clear perinuclear intracellular location on the side of the nucleus facing the spicule, consistent with a predestined vectorial secretion. SpSM50, on the other hand, is found predominantly on the surface of the spicule (though some is occluded), and the GFP tagged SpSM50 is present in the filopodia, as well as cell bodies. Neither tagged protein behaves as does GFP alone. Arnone et al. [20] showed that GFP, even though it is synthesized in only a few PMCs and is not secreted, diffuses throughout the entire syncytial chain of the primary mesenchyme cells. Both SpSM50 and SpSM30B fusion proteins are restricted to a portion of the syncytial filopodial chain that is close to the labeled PMCs expressing the transgene, never further than 5–10 μm from the cell of origin. Hence, cells along the spicule are contributing matrix protein to increases in girth, while cells near the tip contribute to increases in length. SpSM30B is only expressed at low levels in PMCs of the ventral transverse rod, while SpSM50 is expressed in all PMCs. We observed this same restriction in eGFP-SM30 fusion expression, never observing it in cells associated with the ventral transverse rod. The GFP tagged matrix proteins will probably be adequate surrogates for further detailed study of the mechanisms of vectorial secretion in these cells.

SpSM30B Processing

^{35}S -methionine labeling of newly synthesized SM30 in PMCs, *in vitro*, allowed us to directly follow its secretion. Pulse-chase experiments revealed that it takes at least 30 min to detect new protein in the spicule, and secretion of labeled protein ceases within 30–45 min after cessation of a pulse label. ^{45}Ca begins to label the spicule in 10–15 min [21] and continues to be deposited into the spicule for many hours after removal of the isotope (figure 4).

The spicule proteins were extracted from living PMC syncytia by the use of pH 6 and chelation of calcium. This is consistent with the idea [33] that the spicule is located in an extracellular compartment, albeit one that is almost completely enrobed in filopodial cytoplasm. This does not rule out the likelihood that the lower pH and some EGTA are admitted into the cell, and in fact, the limited and specific proteolysis of intracellular, pulse-labeled SpSM30B only occurs after extraction of spicules. The insult to cells of pH 6 and EGTA is not fatal, however, because after such extraction for 15 minutes the PMC cultures can be rinsed and cultured in sea water and continue to initiate new spicules.

Two features of SpSM30B secretion are worth noting. First, the size of the SpSM30B in the spicule, as measured by mobility on SDS gels, shows two forms, a feature noted from the beginning of the study of this protein [5,17,36]. The intracellular form of SpSM30B in PMCs is unimodal (45 kDa), and the form that migrates more rapidly on gels (42 kDa) is only seen in spicule extracts. The proportion of the 42 kDa form increases during a chase period, and this lower molecular weight form is very low or absent in the PMC cytoplasm. This behavior is consistent with a precursor-product relationship, the 45 kDa form giving rise to the 42 kDa form. Prior studies failed to distinguish between the several ways that two isoforms could arise. In spicules of plutei of *S. purpuratus*, this putative conversion is incomplete, some 45 kDa form being present in mature larval spicules. Kitajima et al. [36] reported that spicules from PMC cultures derived from *H. pulcherrimus* also display two bands of SM30, but matrix

protein from the mature spicule of the larvae of *H. pulcherrimus* has only the lower molecular weight form. We would interpret this as indicating a complete conversion of higher to lower molecular weight form of SM30 in that species. The difference between the two forms is not due to differences in extent of glycosylation [5]. Nor is it likely to be due to translation of different SM30 family members [12] because the principal SM30 genes being transcribed and translated at this time (*SpSM30B* and *SpSM30C*) have almost identical amino acid sequences and lengths.

The second feature of *SpSM30B* is that it is subject to turnover as well as secretion. During the chase period, deposition of *SpSM30B* to the growing spicule ceases, but turnover of *SpSM30B* in the PMC proper continues. In the whole embryo the transcription of *SpSM30B* waxes and wanes along the length of the spicule [29], but *SpSM30B* protein in the cell body, as revealed by immunostaining, remains fairly constant. It is possible that turnover of the *SpSM30B* in the PMC is a consequence of culture conditions, although the measurements were made during a time when spicule elongation was robust and continuing, so that we provisionally favor hypothesis that turnover is occurring.

Overall Conclusions

Some new and interesting features of the cell biological underpinnings of endoskeletal formation are apparent. As usual, they all lead to further questions. The calcium and at least two integral matrix proteins are processed by the cell in distinct pathways. Are some other matrix proteins found associated with the amorphous calcium carbonate in the cell, perhaps stabilizing it? Are there amino acid sequence tags for different matrix proteins that ensure vectorial secretion? Collagen, for example, is also secreted by PMCs, but is not found in or around the spicule [37]. *SpSM30B* and *SpSM50* have different distributions in the spicule, *SpSM50* prominent near the surface, *SpSM30B* primarily occluded within the spicule [15]. Are there signatures for that? Are there signature sequences that prevent matrix proteins from diffusing throughout the matrix? The GFP-matrix protein tag should be a useful tool to approach some of these questions. Another important question raised by the present studies is what is the importance of post-secretory processing of the *SpSM30B* matrix protein? Can *SpSM30B* be engineered so that it cannot be processed, and what is the consequence?

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Figure 1. Calcein labeling of PMCs

[A] A 30 min pulse of calcein was administered to the PMCs in culture at 65 h post-fertilization. One end of a spicule is shown, demonstrating considerable labeling of PMCs attached to the elongating spicule, and barely discernable deposition along the spicule surface. 600 X. [B] Another culture was labeled in a 40 min pulse at about 78 h post-fertilization. PMC labeling is evident and the elongating tip of the spicule is intensely labeled. 400X. C. A culture was labeled continuously from 62 to 72 h of development. The unlabeled central portion of the spicule had been deposited prior to the addition of calcein. 300X.

Figure 2. The expression of GFP-spicule matrix expression constructs in pluteus larva stage sea urchin embryos

[A–E] are projections of through-focus optical sections using fluorescent illumination. [A] eGFP-SM50 fusion construct expression in a *S. purpuratus* pluteus embryo. Arrows indicate foci of expression. [B] GFP-SM50 promoter construct expression in *S. purpuratus* pluteus embryo. [C] eGFP-SM30 fusion construct expression in *S. purpuratus* pluteus embryo. Arrows indicate foci of expression. [D] GFP-SM30 promoter construct expression in *S. purpuratus* pluteus embryo. [E] eGFP-SM30 fusion construct expression in *L. pictus* pluteus embryo.

[A]

[B] 15 min

[C] 3h chase

[D] 17h

Figure 3.

[A] A flow diagram of the processing of labeled PMCs. Cells are treated to extract extracellular matrix proteins, then homogenized and centrifuged at low speeds. The sediment of this low speed centrifugation is treated with non-ionic detergent and centrifuged at higher speeds. Most of the intracellular SpSM30 is present in this supernatant resulting from detergent extraction. [B, C, and D] Secretion of radiolabeled SpSM30B. A robust PMC culture (54 hr post-fertilization) was labeled with 40 $\mu\text{Ci/ml}$ of ^{35}S -methionine for 15 min. Some of the plates were then harvested [B], while others were chased for an additional 2 hr 45 min after the pulse [C]. Other plates from the same culture were labeled overnight (17 hr, [D]) with 25 $\mu\text{Ci/ml}$. All lanes (except "mock") show labeled proteins from culture plates extracted at pH 6 and chelators in order to demineralize the spicules and release the soluble proteins from the spicules. The lane labeled "Mock" [B] was prepared from a culture plate extracted with sea water only, which fails to extract any matrix protein from the spicules, thereby leaving all radiolabeled protein in the PMC. No radioactive protein was present in spicule extracts after a 15 min pulse of label (lane not shown). Notice that extraction of the spicule with pH 6 and chelators renders the intracellular SpSM30B susceptible to degradation ("Deterg" [B]), but if only sea water is used to extract the spicule, the intracellular labeled SpSM30 is not degraded. [C] A 15 min pulse followed by a chase (3h) shows the appearance of both the 42 and 45 kDa forms of SpSM30B (3h, "Spicule" [C]). The intracellular form of SpSM30B is 45 kDa (3 h, "Deterg" [C]). [D] After a much longer labeling period of 17 hr the SpSM30B of the spicule

is present as two bands. The intracellular SpSM30B precursor resides in an NP40 sensitive structure as a 45 kDa band. (17 h, "Deterg" [D]); the white line traversing this labeled band is due to a crack in the dried gel and does not indicate a doublet. Low levels of both 45 and 42 kDa forms can be seen in the low speed supernatant ("Cell", [D]), indicating the beginning of conversion may be occurring in a larger form prior to secretion.

Figure 4. Time course of ^{35}S -methionine incorporation into SpSM30B

A robust culture of PMCs was labeled for 45 min with ^{35}S -methionine, and then portions of the culture were chased with cold methionine for an additional 2, 4, 6, or 24 hours. The immunoprecipitated proteins were resolved on acrylamide gels similar to those shown in figure 3, and the radioactivity in SM30 bands quantitated by use of a phosphorimager. Phosphorimager units/ μg of total cell protein is plotted (left ordinate) for the SpSM30B extracted by low pH (spicule) and the residual SpSM30B within the PMC (cell). The ratio of 45 kDa SM 30 to the 42 kDa SM30 (spicule) shows (right ordinate) the slow conversion of the 45 to the 42 kDa form. Note the change in the time (abscissa) between 6h and 24h of "chase".