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Species-specific shells: Chitin synthases and cell mechanics in molluscs

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Abstract. The size, morphology and species-specific texture of mollusc shell biominerals is one of the unresolved questions in nature. In search of molecular control principles, chitin has been identified by Weiner and Traub (FEBS Lett. 1980, 111 : 311–316) as one of the organic compounds with a defined co-organization with mineral phases. Chitin fibers can be aligned with certain mineralogical axes of crystalline calcium carbonate in a species-specific manner. These original observations motivated the functional characterization of chitin forming enzymes in molluscs. The full-length cDNA cloning of mollusc chitin synthases identified unique myosin domains as part of the biological control system. The potential impact of molecular motors and other conserved domains of these complex transmembrane enzymes on the evolution of shell biomineralization is investigated and discussed in this article.

Chitin in organisms

Chitin in extracellular matrices of cells

In the end of the 19th century, there was a hot debate regarding the chemical nature of chitin. It lasted until acetylated glucosamine was found in alkaline melts of the carapace of diverse arthropods in contrast to tunicate cellulose [1], and „mycosin“ of fungi in contrast to „fungal cellulose“ (reviewed by [2]). Today, fungi are well accepted as one of the most prominent groups of eukaryotic organisms which contain chitin as part of their extracellular matrix [3]. Since chitin is usually associated with proteins, complex carbohydrates and sometimes mineral phases, it remains a challenge until today to characterize the diversity of cell walls and integuments in both, uni- and multicellular organisms [4–9]. One of the best examples for naturally pure chitin are cell wall appendices of diatoms [10, 11], and some enzymes involved in their formation have recently been identified [12, 13]. As originally determined by fiber diffraction studies [14–17], three major modifications of chitin are distinguished: α-, β-, and γ-chitin [18, 19], which differ from each other by the arrangement and molar fractions of differently oriented poly-*N*-acetylated linear $\beta(1\rightarrow4)$ glucosamine (*i.e.* chitobiose homopolymer, Fig. 1) backbones. They assemble into fiber crystals which are stabilized by H-bonds in either two or three dimensions [4, 20, 21]. Squids such as the European Squid *Loligo vulgaris* or the Humboldt squid *Dosidicus gigas*, which are members of the molluscan class Cephalopoda, perform the biosynthesis of different chitin polymorphs in a tissue-specific manner. The chitinous organs are associated with different sets of proteins [22]. The *N*-acetyl-glucosamine (GlcNAc) chains are assembled into fibrils and hierarchical structures in order to accomodate specific functions such as mechanical stiffness and strength [23, 24]. This is important for the functional design, *e.g.* of insect exoskeletons and additional functions such as structural colours and adhesive micro-pillar appendages [25, 26].

A major achievement is the abundant information regarding primary structures of enzymes involved in glycan metabolism, as revealed from cDNA library screenings and whole genome bioinformatic resources [27–30]. The most comprehensive information about all aspects of chitin synthesis and the biochemistry of chitin in different organisms can be found in two recently published articles [4, 31]. It is very likely that many aspects including the enzymatic mechanisms of the chitin biosynthetic pathway are similar in insects and molluscs, since the catalytic center of the glycosyltransferase domain is highly conserved [27, 32]. There-

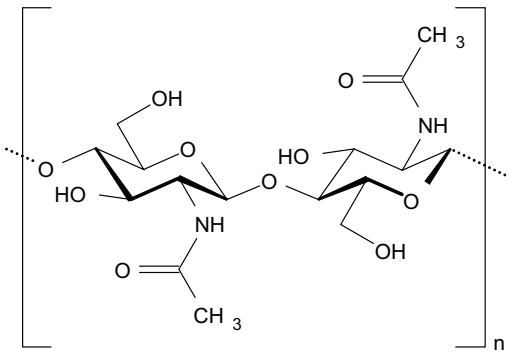

Fig. 1. Structure of the chitobiose molecule, two β-1,4 linked *N*-acetylglucosamine units that repeat to form long chains of chitin. Reproduced from http://commons.wikimedia.org/.

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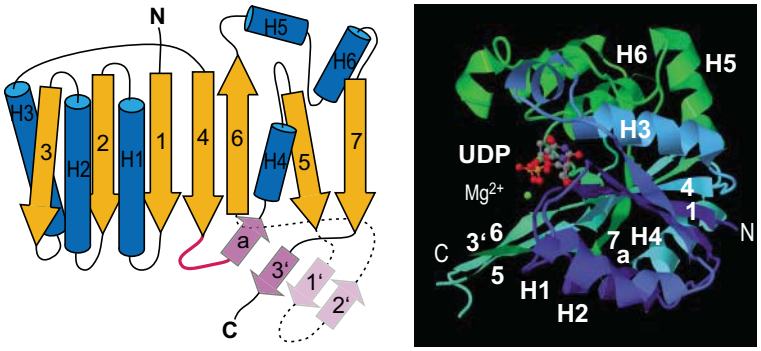

Fig. 2. The Rossmanoid fold [41] might have been present in the Last Common Universal Ancestor (LUCA) of all present day life forms [40, 44]. Redrawn, with permission, from [39]. Right, Jmol image of the nucleotide-diphospho-sugar binding site of SpsA (http://www.cazy.org/GT2_structure.html; PDB ID: 1QGS) [43].

fore, only some aspects of chitin metabolism with particular relevance for biomineralization will be covered here.

Enzymes, multicellularity and bio-mineralization

Polysaccharides with *N*-acetyl-glucosamine residues are part of the extracellular matrix of both, eukaryotic and prokaryotic organisms. This means that the ability to produce chitin-like polymers is one of the ancient capabilities of life on earth [33–35]. To date, we know more than 94 classified glycosyltransferase (GT) families (http://www.cazy.org/, Carbohydrate Active Enzymes database [28, 30]). These species-specific enzymes produce and degrade manifold simple and complex cell surface structures and are thus intimately linked to cell differentiation and developmental pathways in multicellular organisms [3, 36–38].

For example, the GT-2 family comprises 25,550 different enzymes contained in the CAZy database as of February 5, 2012. More than 22,200 are known from Bacteria, 1,249 from Archaea, and about 1,900 from Eukaryota. Only 256 of them were characterized so far, among them cellulose synthases (EC 2.4.1.12), chitin synthases (EC 2.4.1.16), and hyaluronan synthases (EC 2.4.1.212). For detailed information, the reader is referred to the CAZy database server (http://www.cazy.org/GT2_characterized.html), where all aspects of glycosyltransferases are covered and continuously updated [28, 30].

There is no natural classification that would suggest the evolutionary events leading to the emergence of the present-day glycosyltransferases [39]. Glycosyltransferase superfamilies differ especially with respect to the degree of conservation of a characteristic DXD (Asp-X-Asp) motif, which most probably mediates the binding of divalent cations such as Mg^{2+} or Mn^{2+} to the aspartic acid carboxylates, which then form complexes with the phosphate oxygens of UDP-activated sugar substrates [40]. Ubiquitous distribution of conserved 3-D motifs with 6 α-helices and several β-sheets, *e.g.* GT-A/Rossmann fold [41, 42] (Fig. 2) in all three domains of life [40, 43, 44] suggests the ancient origin of both superfamilies [39].

However, why should enzymatic considerations about polymerization of soluble sugar monomers into insoluble chitin across a biological membrane be so important for explaining the evolution of the so-called "controlled biomineralization" [45]? As a matter of fact, molluscs belong to one of the most ancient animal phyla which acquired the ability to not only perform, but actually make use of biomineralization. Only "controlled" biomineralization enabled them to form highly sophisticated, functional mineral composites such as statoliths, invertebrate teeth [46] and shells with outstanding mechanical and optical properties [47–49]. While calcium carbonate skeletons from corals are rather brittle, the calcareous shells produced by molluscs are remarkably tough (Fig. 3) [50].

The event of genetic encoding of calcareous skeletons occured more or less in the late Precambrian period at the Proterozoic/Phanerozoic Eon boundary about 542 Ma ago, shortly after the occurrence of multicellularity [47, 51–55].

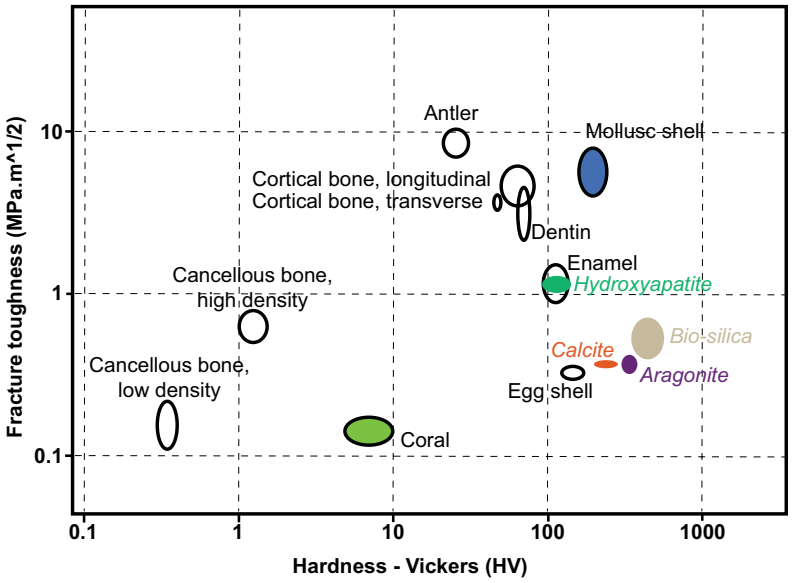

Fig. 3. Mechanical properties of calcium carbonate composite materials produced by corals and molluscs, in comparison to geological minerals and biominerals which evolved substantially later. Redrawn, with permission, from [50].

Sponges, probably the most ancient multicellular organisms [56, 57], also produce chitin and minerals [58, 59]. The shells of Brachiopods – regarded as "living fossils" – and gastropods contain chitin sheets interspaced by mineral phases [60, 61]. It is likely that the hard parts of many ancient members of the phyla Brachiopoda and Mollusca may have been intimately associated with chitin [46, 62, 63]. Also larval shells of contemporary bivalves contain a chitinous matrix and express chitin synthase in very early developmental stages [32, 64]. As outlined in the following section, an extremely species-specific level of chitin-based extracellular control for determining the directionality of aragonite crystal formation has been evidenced based on two breakthrough observations reported by Weiner and his colleagues and by Suzuki *et al.* [65–67]. For the moment, let's keep in mind that the Rossmanoid glycosyltransferase folds (GT-A) of the mollusc chitin synthase existed by far before the existence of controlled biomineralization.

Chitin and oriented mineral crystals

Polymer fibres are part of all organic matrix controlled biominerals. They form a "microenvironment" for crystallization and provide mechanical reinforcement. Fibers and minerals are arranged such that optimum strength and elasticity in certain directions are achieved [68, 69]. Biopolymers could also influence biomineralization in close neighbourhood to the secretory tissue, assuming that fibrous elements such as collagen or chitin bridge the extracellular space from the cellular epithelium towards the mineralizing front. In other words, cells do have the power to interfere mechanically with extracellular assembly. Waller realized the impact of such a concept as early as 1980, based on electronmicroscopic studies of shell forming tissue [70]. In mollusc shells, β-chitin is the most prominent biopolymer with a fibrous morphology [71–74]. The degree of alignment between mineral crystals and chitin fibers in mollusc shells is species-specific [65, 66, 75] (Fig. 4). A co-alignment with chitin was also observed for lamellar, plywood-like carbonate apatite in the barnacle *Ibla* (Cirripedia) [76]. The mechanical advantage of keeping hard, but brittle compounds below a critical size is obvious [77]. Such fundamental concepts of composite materials science are certainly more important on the length scales of metazoan organisms than on the micron scale of single cells. Therefore, evolutionary pressure to develop lamellar composites goes hand in hand with multicellular development. But how was a reasonably working recipe for shell formation genetically encoded and transferred from one generation to the next? One very successful strategy seems to involve the synthesis of an organic matrix which gains order and mineralizes with time [78–81].

In vitro experiments have shown that the formation of aragonite crystals can be controlled by means of a chitinous microenvironment [82–84]. Several studies have demonstrated that deacetylation, covalent cross-links, and chitin-binding proteins can alter the materials properties of chitin and chitosan gels [4, 85–89]. Such chemical and biochemical modifications of chitin are subject to enzymatic control [90, 91]. Many organisms have specific proteins with conserved peptide motifs for the recognition and interaction with chitin [92–96]. Some of them undergo conformational changes with Ca^{2+} and may also play a role in innate immunity [97, 98]. Chitin associated biomineralization proteins may interact with mineral phases on different levels of organization [99, 100]. In 2009, Nagasawa and his colleagues demonstrated in a landmark study that the chitin binding protein Pif97 associates with an aragonite inducing protein Pif80. Both proteins are expressed in equal amounts in vivo. In vitro, they serve as a three-dimensional nucleation site for oriented aragonite growth [67].

This fundamental observation from chitin-binding protein complexes would explain, to some extent, how a true 3D preferred orientation of crystals can be present in juvenile shells, while at a later stage of growth, for example in adult *Notosaria nigricans* (Brachiopoda), the texture looses most of its 3D ordering and becomes a 1D fibre texture [101–106]. In some shell regions, the texture becomes bi- or even multimodal as revealed by high-resolution electron backscatter diffraction (EBSD), also known as backscatter Kikuchi diffraction [107]. Obviously, species-specific and developmental-specific control mechanisms are active when different shell layers are formed [65, 108–117].

In the following, further essential requirements for producing functional chitin fibers, chitin layers and chitin bulk materials along with mineralized hard parts in a species-specific manner will be discussed.

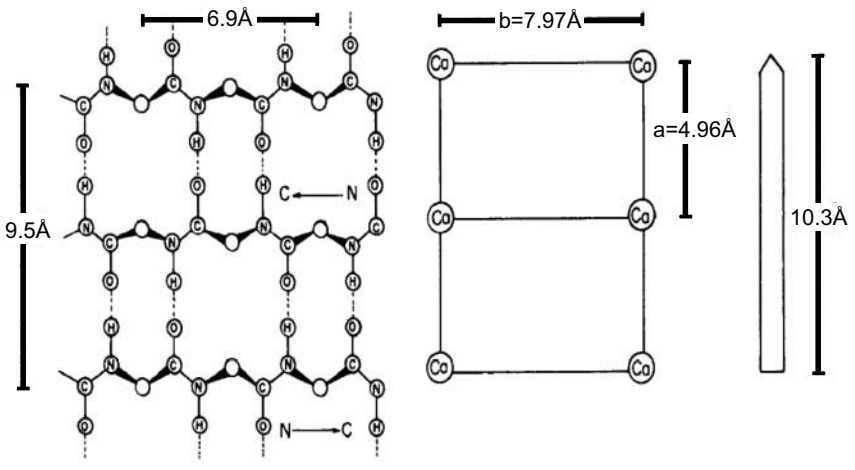

Fig. 4. Model for the alignment of crystals, protein (left) and β-chitin (right) in the nacreous shell of *Nautilus*. Reproduced, with permission, from Weiner & Traub [66].

Myosin chitin synthases and biomineralization

Chitin synthases and chitin self-assembly

Chitin synthases contain a minimum of three transmembrane helices [31, 118], which are located C-terminal with respect to the catalytic center, where *N*-acetyl-glucosamine monomers or GlcNAc dimers (chitobiose) are polymerized and translocated from the intracellular to the extracellular compartments. It is not exactly known so far to what extent additional transmembrane and membrane-associated proteins, or other factors, are involved in this process [119]. The publication of the first full-length invertebrate chitin synthase cDNA [27] shed some first light on molecular mechanisms of chitin formation in multicellular organisms. For example, invertebrate chitin synthases contain at least 15 transmembrane helices [31, 120] (Fig. 5). Both, extra- and intracellular protein domains are significantly different from the fungal enzymes. In fact, only the central glycosyltransferase domain (Rossmann fold) is highly conserved while *N*- and *C*-terminal domains are unique [120]. Interestingly, the chitin synthase domains of the respective molluscan enzymes are highly conserved over almost all the length (see supplementary file 1 in [121]).

It is inherently difficult to experimentally study the influence of cell cortices and native membranes on the transmembrane activities of these complex enzymes at sufficiently high resolution. For example, the directionality of chitin synthesis across the membrane was established using TEM techniques [122], with the respective experimental drawbacks of staining, ultra-high vacuum and the like. Many basic aspects regarding the biosynthesis of chitin were established in fungal model organisms [123–125], which are much easier to cultivate and cell walls can be purified and characterized in defined stages during the cell cycle. However, the organization of chitin and extracellular matrix proteins as a function of mineralization is physico-chemically a bit more complex [81, 126–129]. In the living animal, such non-linear processes should be regulated in some way. In the case of chitin fibril formation, distinct enzymatic activities such as chitinases and chitin deacetylases are eventually involved [130, 131]. The question arises whether the fine-tuning of mollusc shell structures requires monitoring of distinct materials properties by means of locally distributed sensors. If so, then what would such a sensor look like in its most basic form?

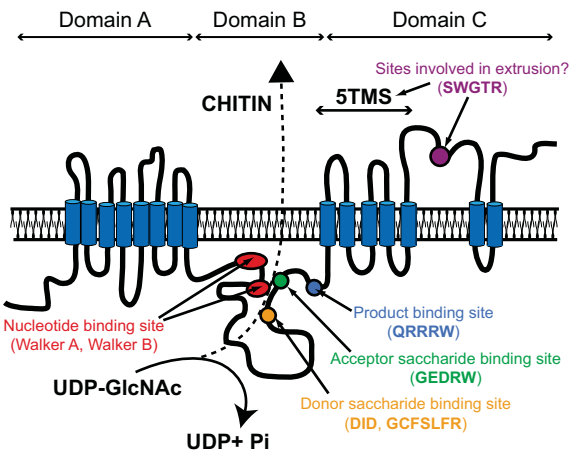

Fig. 5. Scheme of the transmembrane architecture of metazoan chitin synthases. Redrawn, with permission, from [120].

Biomechanics at the shell forming interface

Some biological aspects on chitin biosynthesis were studied in the yeast *S. cerevisiae* [132], the red flour beetle *Tribolium castaneum* [133], and the model nematode *C. elegans* [134]. In vivo experiments showed that chitin synthesis is closely linked to cell division, the development of tissues and organs, and the overall symmetry of the body plan. This explains to some extent, why in the particular case of mollusc larval shell development, the presence of tiny amounts of the chitin synthase inhibitor NikkomycinZ during growth prevented proper shell formation on different levels of hierarchy [135].

Signaling pathways related to the formation of extracellular matrices are complex. This certainly applies to mineralized coatings. As a matter of fact, the cytoskeleton has a strong direct influence on the microvilli architecture of the tissue interface where biomineralization takes place [70, 136] (Fig. 6). It has been reported that the activity of hyaluronan synthases, a GT-2 enzyme, induces the formation of microvillus-like cell surface protrusions [137].

The mechanical toolkit of cells based on cytoskeletal proteins such as actin filaments, actin-binding proteins, and motor proteins (*e.g.* myosin) is well understood [139–141]. X-ray scattering experiments revealed five different configurations of the myosin head geometry [142]. Each one of them is specifically related to the unbinding (ATP capture and hydrolysis) and binding to actin filaments (P_i and ADP release) in a sequential, cyclic manner. The step

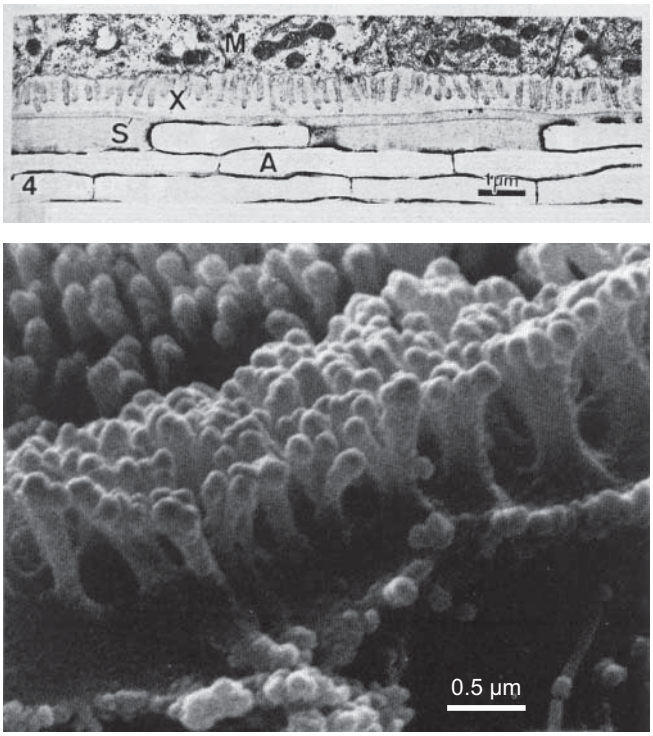

Fig. 6. Interface between a mollusc shell and shell forming tissue. Reproduced, with permission, from [70, 136, 138].

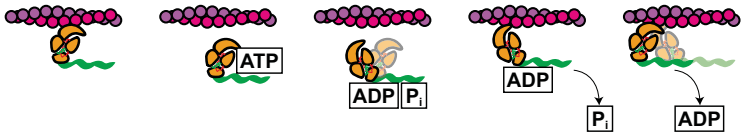

Fig. 7. Myosin power stroke. (**a**) Rigor position, (**b**) released by ATP uptake, (**c**) cocked by ATP cleavage, (**d**) weak binding by phosphate release, (**e**) power stroke by ADP releasse. Forces are in the pN range, distances in the range of few nm. For details, compare also [139, 142].

size of once cycle is in the range of few nanometers, forces generated by one myosin head per ATP cleavage are in the pN range (Fig. 7). The involvement of myosin in mechanical signal transduction receives increasing attention, since the life times of particular conformational sub-steps were discovered to be rate-dependent [143].

Conventional and "orphan" myosins

According to Foth and colleagues [144, 145], myosins are a complex superfamily. The species-specificity of glycosyltransferase superfamilies represents the diversity of oganisms, as discussed in the previous section. Likewise, more than ~24 major myosin classes are distinguished [145]. The evolutionary history of myosin paralogues has been reviewed by Richards & Cavalier-Smith [146], who established the divergence of prokaryotes, plants, amoebozoa and the fungi/metazoan lineages based on several highly conserved myosin domain motifs. Based on sequence homology searches, the myosins of choanoflagellates such as *Monosiga brevicollis* diverged clearly from fungal myosins, while sharing many conserved domains with metazoan myosins (Fig. 8) [147]. This means, that a huge variety of myosins must have existed at least 1,500 million years before present.

In summary, glycosyltransferases as well as myosins existed some 10^8 to 10^9 years before controlled biomineralization has been achieved and inherited by genomes of the metazoan lineage [54]. It also means that the pure presence of glycosyltransferases and the pure presence of myosins seemingly does not suffice to achieve controlled mineralization of extracellular chitin in multicellular organisms. Then, what were the limiting factors for the evolution of functional materials? The fact that the mollusc chitin synthase does contain a myosin domain in one and the same transmembrane glycosyltransferase molecule [32] motivated the myosin community to group this enzyme together with so-called "orphan" myosins (Fig. 9), each one of them classified separately from all other myosins known so far [147].

Chitin synthases with and without myosin motor domains

The insect chitin synthase [27] shares many features with the mollusc enzyme [32] in terms of basic enzymology. In contrast to fungal enzymes [148], the chitin synthases of invertebrates are all proteins with a complex transmembrane architecture [27, 120]. However, the mollusc chitin synthase is unique: it does contain a myosin domain, but it must not be classified as a class XVII myosin [146, 149, 150]. The entire chitin synthase molecule represents a motor protein which is able to transfer the chemical energy of ATP into directional motion or force by means of numerous transmembrane helices. To what extent the generation of mechanical forces by one or several proteins interferes with membrane traffic, chitin fibril assembly and mineralization still remains an open question.

The cloning of the complete mollusc chitin synthase sequence was based on a high quality cDNA library from adult mantle epithelium of the genus *Atrina* [151], kindly provided by Prof. Addadi & Prof. Weiner (Weizmann Insti-

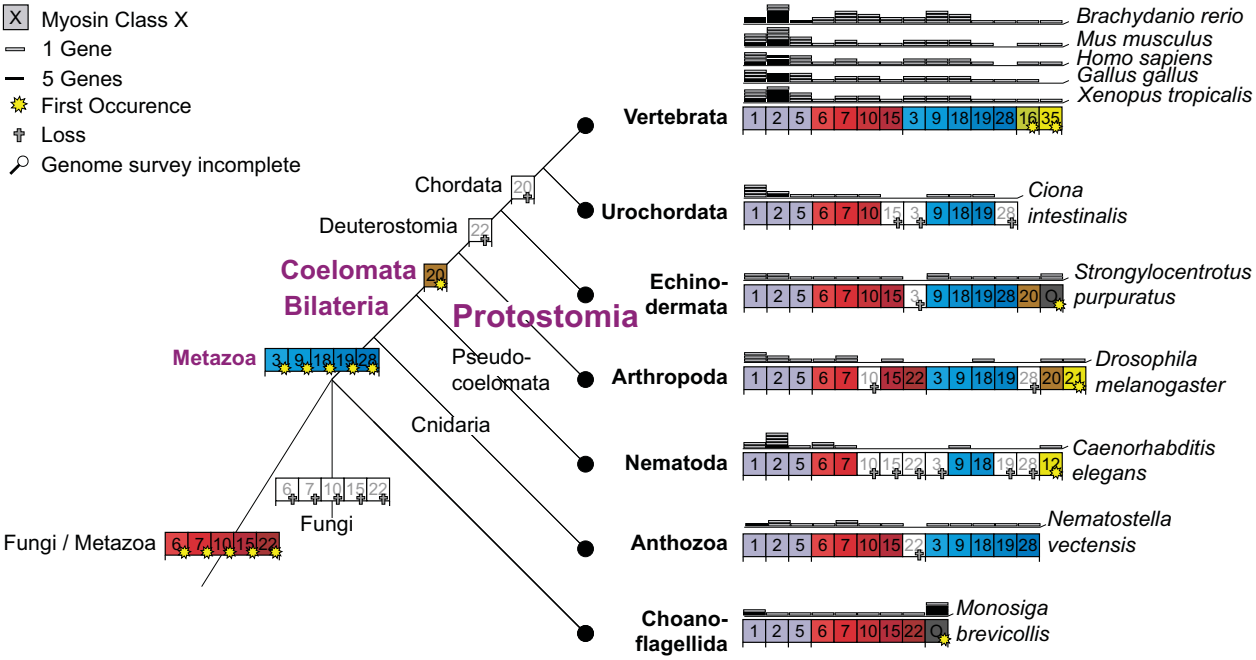

Fig. 8. Evolution of the myosin superfamily. The splitting of fungi and metazoan lineages are represented in the distribution pattern of specific myosin classes. Reproduced and adapted with permission from [147].

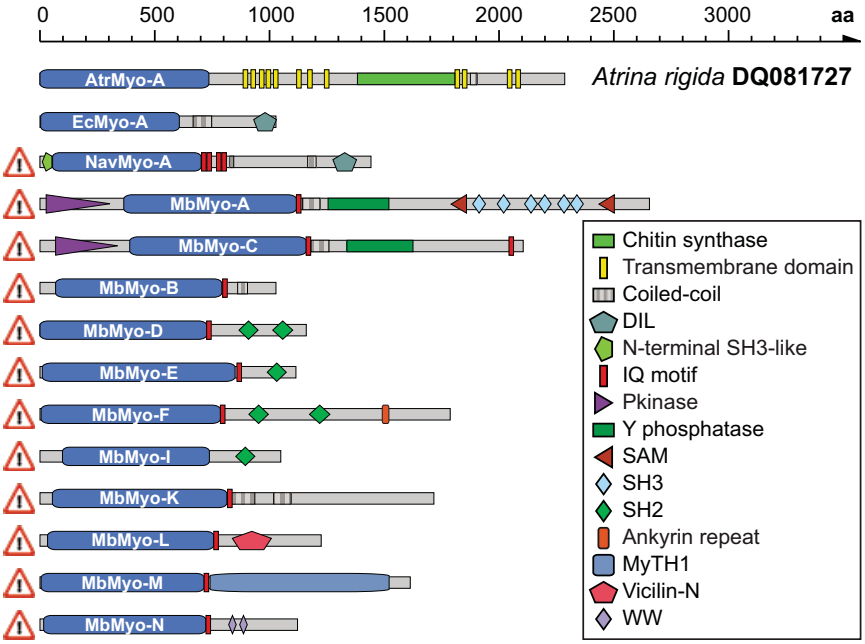

Fig. 9. Mollusc chitin synthase (*Atrina rigida* DQ081727) and other orphan myosins. The exclamation mark on the left side of some sequences signifies that the corresponding sequences (especially the tail regions) have not completely been validated because of missing comparative genome sequences. Reproduced and adapted with permission from [147].

tute of Science, Israel) and Prof. Tuross (Smithsonian Center for Materials Research and Education, Suitland, Maryland, U.S.A.). In summary, the first cDNA sequence of an invertebrate (*Atrina rigida*) myosin-chitin synthase *Ar*-CS1 [GenBank ID: DQ081727] has been determined and analyzed [32]. The molecular weight of this 2,286 amino acid protein is 264 kDa, including the 83 kDa *N*-terminal myosin domain (Fig. 10). Heterologous expression of this sequence yields the respective transmembrane protein which recovers chitin synthase activity [121, 152]. Sequence comparison with a second mollusc chitin synthase *Mg*-CS1 [GenBank ID: EF535882] showed that also the myosin domains of the two mollusc chitin synthases are homologous over large domains. This, together with the sequence information of pearl oyster *Pinctada fucata* myosin chitin synthase [100] and the finding of respective sequences in the gastropod *Lottia* (Prof. B. Degnan, University of Queensland, Australia, personal communication

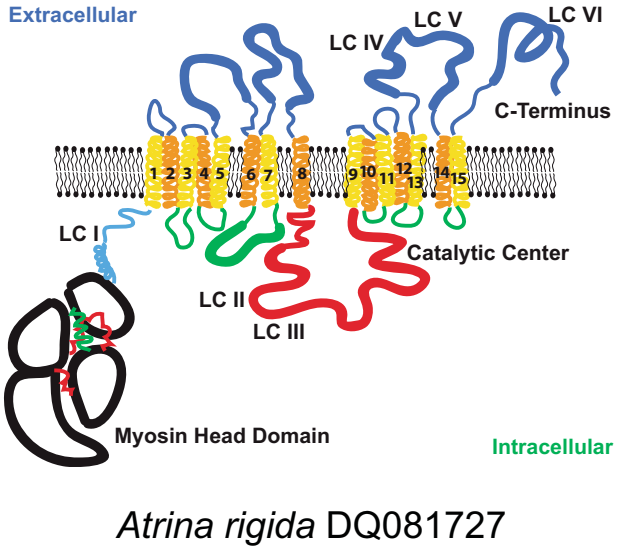

Fig. 10. Transmembrane architecture of the chitin synthase *Ar*-CS1 from *Atrina rigida* (GenBank ID: DQ081727). Reproduced and adapted with permission from [32].

2008) indicates that myosin motors as part of chitin synthases might be a core functionality in shell forming metazoan organisms.

In summary, the complex transmembrane architecture as well as the presence of the myosin domain suggest an important regulatory function of the cytoskeleton and membrane biophysics in the mechanisms of organic matrix assembly and shell biogenesis in molluscs.

Coordination with other shell constituents

There is clear evidence that a huge number of secreted gene products is involved in shell formation [130, 153–158]. This raises immediately serious questions: The molar ratios of all the different gene products need to be fine-tuned. The respective proteins may change their solubilities as a function of pH and ionic strength. Their interactions with solvents, organic interfaces, and minerals at the levels of solubilized ions, prenucleation clusters, amorphous phases, and macroscopic crystals may be extremely complex. The requirements in terms of spatially and timely responsive regulatory cascades, which accomodate an efficiently adaptive recipe for mineralizing complex extracellular matrices (ECM) under distinct cellular control, must be adequately covered. Glycosyltransferase activities connected to mechanical signaling via phosphotyrosine pathways offer the possibility to locally detect and, at the same time, influence the materials properties of the ECM. As outlined above, molluscs dispose of complex chitin synthases with myosin motor domains. This suggests that chitin serves not only as a mechanically favourable thin-layer material and shock-absorber in the final composite. To a certain extent, it may fulfil an additional role as a surface-active component while it is synthesized [159]. As a consequence, the materials properties could change as a function of density and alignment of chitin in the bulk. The local change in elasticity, in turn, could be immediately detected by the enzyme system which produces the chitin. A scenario, how such events can be translated into

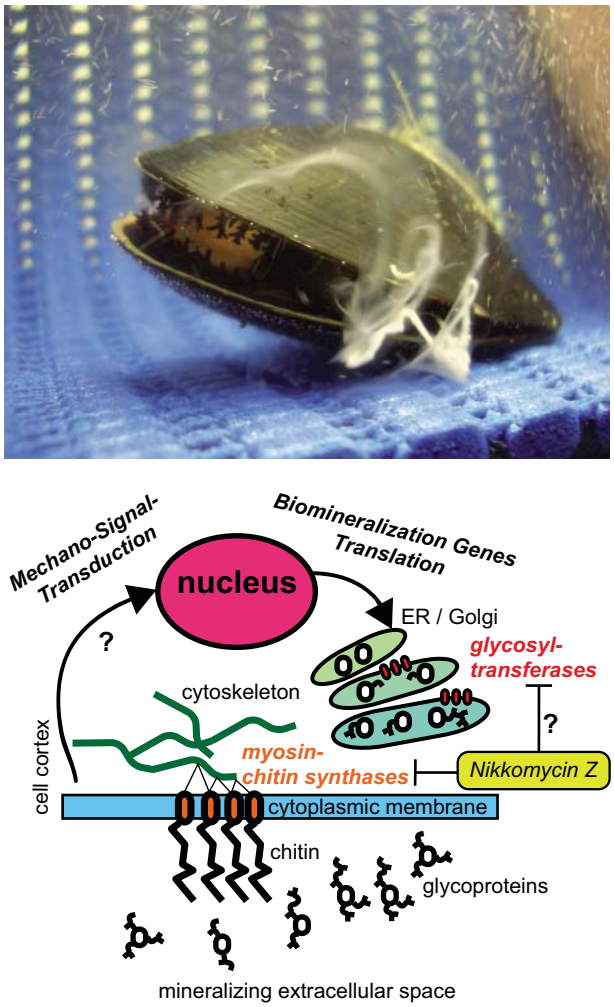

Fig. 11. Mantle shell-interface exposed by a spawning *Mytilus* (top). Bottom, Pathway model for coordinating the secretome of a biomineralizing cell in response to chitin self-assembly and mechanical signal transduction. Reproduced, with permission, from [135].

gene expression patterns for biomineralization proteins is schematically outlined in Fig. 11.

Mechanical forces and shell formation

A direct involvement of cytoskeletal forces in a sense as reviewed by Ingber and colleagues [160] in the regulation of mollusc shell chitin synthesis and mineralization is regarded to be likely. One century ago, Schmidt [161], pp. 194–203, already considered a possible overlap of "eigene Anziehungskräfte der Micelle" (colloidal forces) and "äußere Kräfte" (meaning: forces specifically applied by an organism), while referring to former studies on biological fibre formation from colloidal precursors performed by v. Ebner [162]. Their early concepts may find substantiation in the molecular architecture of the myosin chitin synthase.

The plant pathogenic fungus *Ustilago maydis* uses a myosin-chitin synthase in order to infect a plant. Myosin-chitin synthase (*Um*-Mcs1) deficient Δmcs1 mutants of *Ustilago* are unable to penetrate the host plant tissue. The hyphal shape of these mutants did, however, not change [163]. Obviously, a concerted interaction of forces, generated by the cytoskeleton while the fungal hyphae grow, is

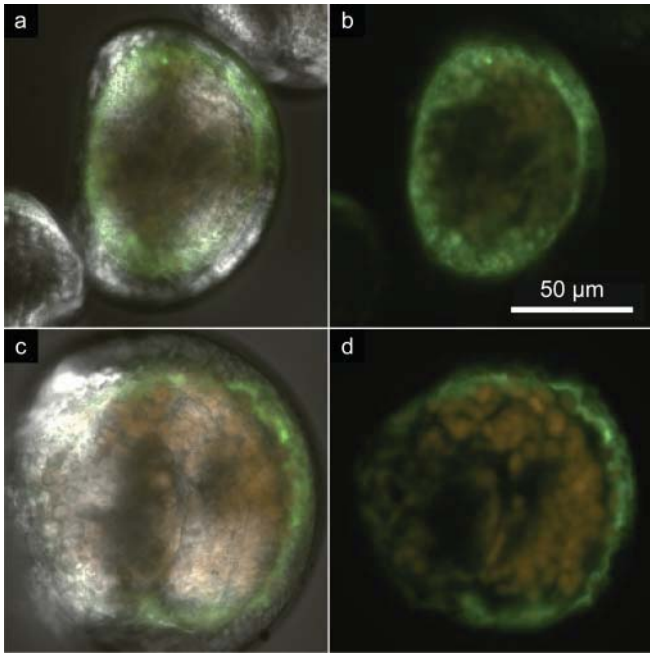

Fig. 12. Interface between *Mytilus* larval shells and shell forming tissue, demonstrating the expression of myosin chitin synthase *Mg*-CS1 (green signal). Red label, actin mRNA; (**a**, **b**) 6 days; (**c**, **d**) 15 days old larva. Reproduced, with permission, from [32].

required to penetrate the rigid plant cell walls. This means that this molecular motor has a direct regulatory function, in addition to intracellular transport and localization. Other *Ustilago* chitin synthases, which lack motor domains, are not relevant for this phenotype [163].

These observations altogether raise the central question, how closely chitin synthesis in molluscs is linked to shell biogenesis, while mediating peculiar mechanical interactions with the interior of secreting mantle cells and the cell cortex via cytoskeletal reorganization. This seems particularly important in early developmental stages of shell formation (Fig. 12). A direct coupling of chitin synthases with motor proteins does not appear to be a prerequisite as long as chitin deposition occurs in a more or less passive manner and the matrix and mineral components organize "themselves" by chemical self-aggregation. Only by exhibiting a motor protein domain, the mollusc chitin synthase gains dramatically in importance in terms of regulation. It offers new explanations for the precision of biomineralized structures in terms of cell adhesion and cytoskeleton mediated signal transduction pathways.

Structural diversity of mollusc shells

Signaling pathways and encoding capacities

Meanwhile, it is well accepted that mechanical signals play a major role in cell differentiation, development and many diseases [164–166]. Among the most important cellular switches in this context are Rho and Rac [167], a large family of GTPases which have profound effects on the actin cytoskeleton, and Arp2/3 complexes [141, 168]. These are involved in downstream signaling pathways originating from growth factor receptors including receptor

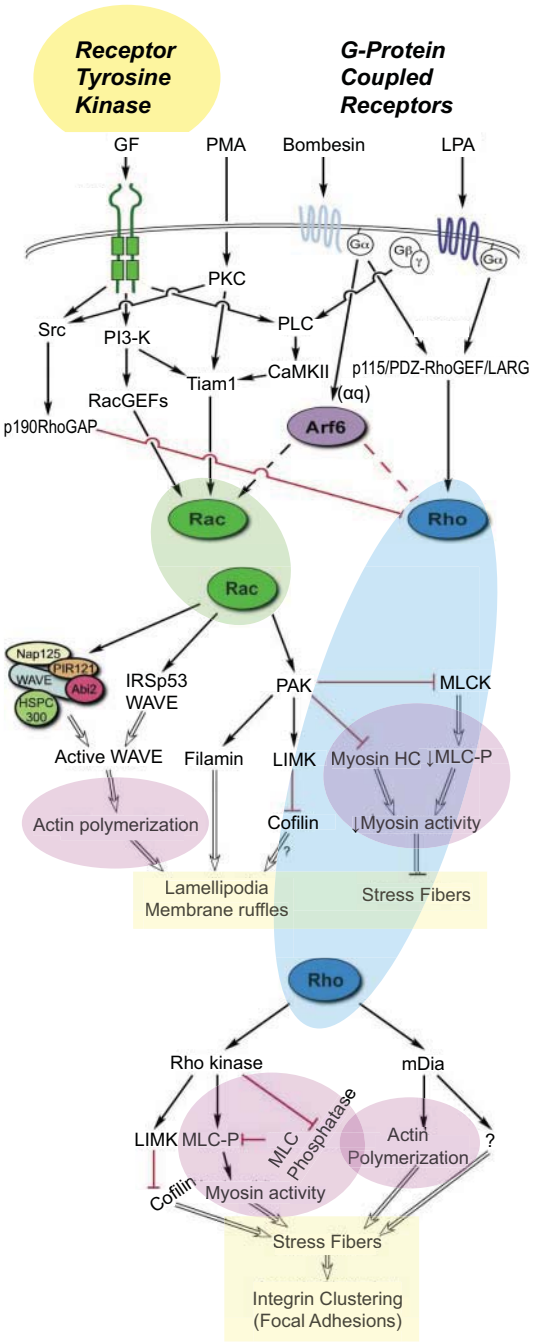

Fig. 13. Cellular signal transduction from extracellular soluble factors to intracellular cytoskeletal response across the cytoplasmic membrane. Rho and Rac take center stage. Reproduced and adapted, with permission, from [167].

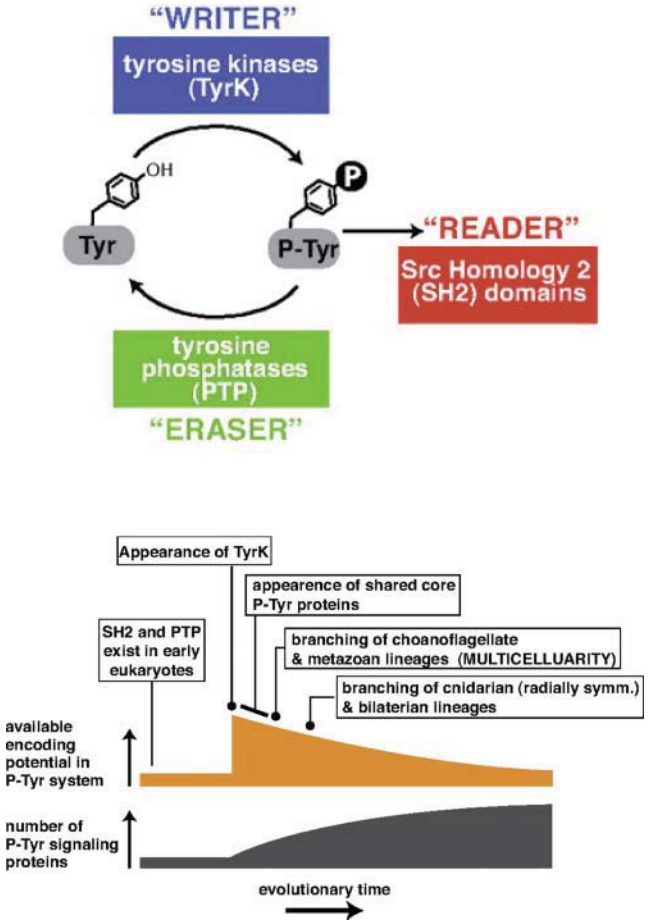

Fig. 14. Evolution of the RTK pathway. Reproduced and adapted, with permission, from [169].

tyrosin kinases (RTK pathways) and cell adhesion molecules (CAM) as outlined schematically in Fig. 13.

The RTK pathways are particularly interesting in terms of the capacity of signaling systems and, hence, the evolution of multicellularity. The genetic model organism for studying the basic toolkit of early eukaryotic organisms on their way to multicellularity (metazoan lineages) is the choanoflagellate *Monosiga brevicollis*. It turned out that Src Homology 2 domains (SH2 domains) and phosphotyrosin rich protein domains (PRP) were present in early eukaryotes [169, 170]. All of a sudden, the number of shared core P-Tyr proteins increased with the appearance of tyrosine kinase (TyrK) (Fig. 14). This clearly indicates the evolutionary advantage of developing the RTK pathway, in addition to common GPCRs (G-protein coupled receptors), which usually provide a fast response to external stimuli. The efficient enzymatic on/off switching of phosphorylation states and coupling to conserved read-out systems such as SH2 domains caused a tremendous increase in encoding potential.

The RTK pathways are also coupled to Rho and Rac, which both interfere with actin polymerization, myosin activities and, subsequently, with stress fibers, integrin clustering into focal adhesions as well as lamellipodia formation and membrane ruffels [167]. By means of cytoskeletal rearrangements, the architecture of shell forming tissues is thus under direct developmental control of the whole organism. Receptor tyrosine kinases may well be locally activated by biomineralization proteins such as IGFBP-like perlustrin, which has been isolated natively from the nacreous part of *Haliotis* shells [171].

Encoding mollusc shell structure

In molluscs, the step-wise mineralization process must integrate into cell cycles of the growing tissue, leading – in the case of mollusc larvae – to a radial arrangement of aragonite crystals [161, 172, 173]. In the adult shell, there are several prominent and some less common shell ultrastructures [174]. All of them are produced in a species-

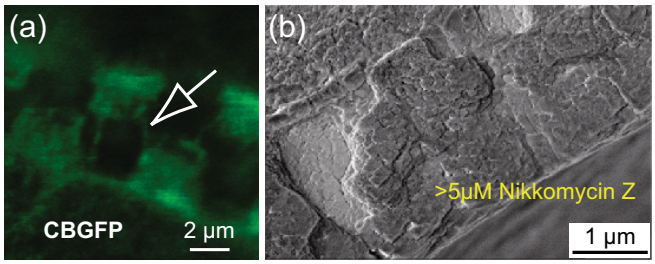

Fig. 15. Distribution of chitin (left) in the larval shell hinge of *Mytilus* and detrimental effect of chitin synthase inhibitor on hinge mineralization (right). See Refs. [64] (left) and [135] (right) for details.

specific manner: The nacre, the prismatic, and the crossed-lamellar structure [175–178]. While the crossed-lamellar structure appeared comparably late in the evolutionary history [179], nacre seemed to be present in the earliest molluscs [47]. Chitin is apparently associated with different minerals in different metazoan organisms such as brachiopods, molluscs and barnacles [46, 60, 61, 76, 100, 128]. Here, only one example is briefly mentioned because it serves as a model system for the transformation of amorphous calcium carbonate into crystalline aragonite: The larval mollusc shell [64, 172].

A remarkably homogeneous larval shell is formed, irrespective of dynamic cell movements and reorganization of the developing tissues. Within few hours or days, the shell covers the whole organism and exhibits a high performance in terms of accuracy of fit at the hinge and shell edges. These shell regions also revealed some characteristic chitin structures. At the shell edge, chitin fibres were oriented as well in parallel as perpendicular (radial) toward the lateral growth front. At the hinge region, the functional impact of chitin becomes obvious. In the early stages of shell formation, chitin forms a linear connection between the two shell valves. Chitin nodes are formed at points, in which the two valves are beginning to interlock by the hinge teeth. These nodes finally pervade the growing hinge teeth completely. Especially the hinge requires a restrictive control of mineralization in order to guarantee its functionality. Some possible scenarios how chitin could be functionally involved in the coordination of cells and tissues are discussed in [64, 135, 180].

The chitin synthase inhibitor NikkomycinZ interferes with larval shell formation in vivo [135]. This "small-molecule" drug, a nucleosid peptide with structural similarity to UDP-GlcNAc, is transported into the cells via cellular peptide transport systems. The inhibition of chitin synthesis effects larval shell formation at various hierarchical levels (Fig. 15). Due to the natural rate of malformation, sometimes similar effects were observed in individuals grown in the absence of NikkomycinZ. In the presence of NikkomycinZ, however, all the organisms showed one or several characteristic shell abnormalities. The grade of harmfulness or toxicity was found to depend mainly on the developmental age of the organisms, the duration of treatment, and the concentration of chitin synthase inhibitor. There was only a narrow range of inhibitor concentration (~5–10 µM) that allowed observing significant effects on larval shell formation, while keeping a considerable amount of larvae alive for the duration of the experiments. This evidences a central link between chitin synthesis and shell development. For example, shells were formed asymmetrically and much slower than the organism grew. As a consequence, there were partly "naked" larvae. Surprisingly, they were still alive and active. The shell remnants were irregular, sensitive to etching by water and mechanically instable [64, 135].

Physiological control of shell structure

Interfacial cellular control, cytoplasmic membranes and microvilli

The cell membrane is a central checkpoint in the formation of extracellular matrix and, thus, also at the interface between an organism and the biomineral it forms [70, 136] (see also Fig. 6). The membrane consists of ion channels, receptors, and regulatory elements [141, 181]. It is the location of secretion of structural proteins and polysaccharides. There are protein anchors connected with cytoskeletal fibres that are involved in the mechanics and shapes of animal cells and tissues [182, 183].

Spot and belt desmosomes together with the cytoskeleton mediate mechanical interactions between cells in tissues. On the single cellular-level, multiple focal adhesion complexes are often connected to an extracellular substrate via integrins [165, 183] and thus offer the possibility to transmit mechanical forces into the extracellular space. In this way, cells could also interfere mechanically with biomineral formation. The versatile function of myosin chitin synthases in biomineralization may depend on controlled lateral organization, from where tunable chitin networks with pre-defined properties emerge.

It becomes obvious that chitin synthesis can not be seen as an isolated enzymatic process. Moreover, it is extremely important how the chitin synthases are organized within intracellular chitosomal vesicles, how fusion with the cytoplasmic membrane is achieved, and whether and where they eventually find their final destinations on cell surfaces. This may vary from species to species.

Formation of chitin-mineral composite materials in vivo

Let's assume that mantle epithelial cells are able to recognize the fine structure of the shell: How would this help if shell formation was temporarily interrupted? Once shell formation is continued, any sudden change in terms of structure and stability of the overall composite should be avoided. Otherwise, the formation of cracks would be favoured, and subsequently the survival of the individual animal would be less likely.

Biomineralizing cells seem to have a narrow time-window for being conditioned to form the shell. We know from "wound-healing" studies that a secondarily formed biomineral is structurally different from the original one [184]. Furthermore, cell–cell communication is required in order to guarantee the continuity of the overall skeletal structure (Fig. 16). One prominent example is the radial orientation of aragonite crystals in larval mollusc shells

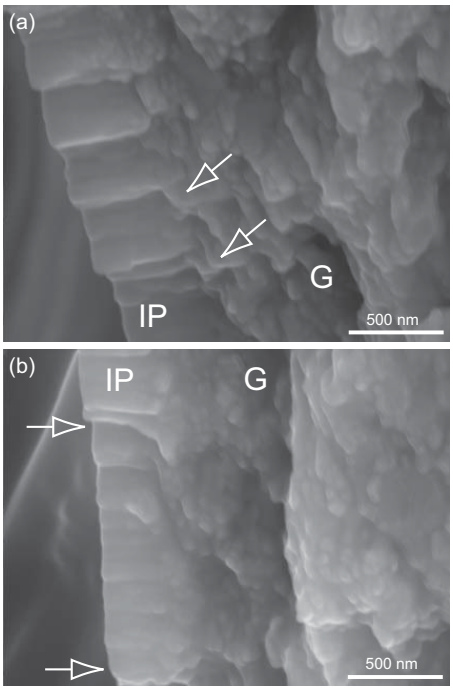

Fig. 16. Larval *Mercenaria* shell exposing the developing mantle-shell interface. IP, inner prismatic shell layer; G, granular shell layer. (**a**), The matured prismatic structure develops continuously at the granular interface (arrows). (**b**), The forming shell edge shows the gradual structuring of the prisms (upper arrow). The interface of the larval shell, which is exposed to the larval mantle tissue close to the newly deposited mineral at the edge, is significantly less structured (lower arrow). Reproduced, with permission, from [172].

[161]. The fact that the aragonite needles emerge from an amorphous precursor phase [172] raises the question, whether and how the mineral phase is molded prior to crystallization. One can think of both, co-organization with organic matrix components and, active participation of cells and tissues.

Experimental tools for studying mollusc chitin synthases

Many current approaches to understand mollusc shell formation target the "material", meaning the final shell, once biogenesis has been completed. From a biochemical point of view, several key processes happen during the time course of shell formation. Larval and juvenile molluscs are promising model organisms for in vivo studies. Their size is microscopic. Their shell is semi-transparent and bears a signature of biogenesis in terms of fine structure and mineralogy [172, 185]. Genetic tools can be applied [32].

Chitin originating from native chitosomal membranes can be quantified on solid supports [186]. Expression systems for genetically tailored mollusc myosin chitin synthases are now available [121, 152]. Cell mechanics may depend on glycan polymer length [187, 188]. Chitin synthesis, in turn, may depend on membrane curvatures. A newly forming native shell interface is certainly difficult to mimic. Young's moduli, molar ratio of shell constituents, viscosity, osmolarity, crystalinity, topography and other read-outs are frequently changing on different length scales. Computational methods are currently developed for a number of biomineralization proteins and their interactions with mineral phases [189]. As outlined in Fig. 17 with respect to mollusc chitin synthases, biophysical cell cortex models, and suitable living systems such as the larval shell should help to identify additional key principles of controlled biomineralization [159, 186, 190, 191].

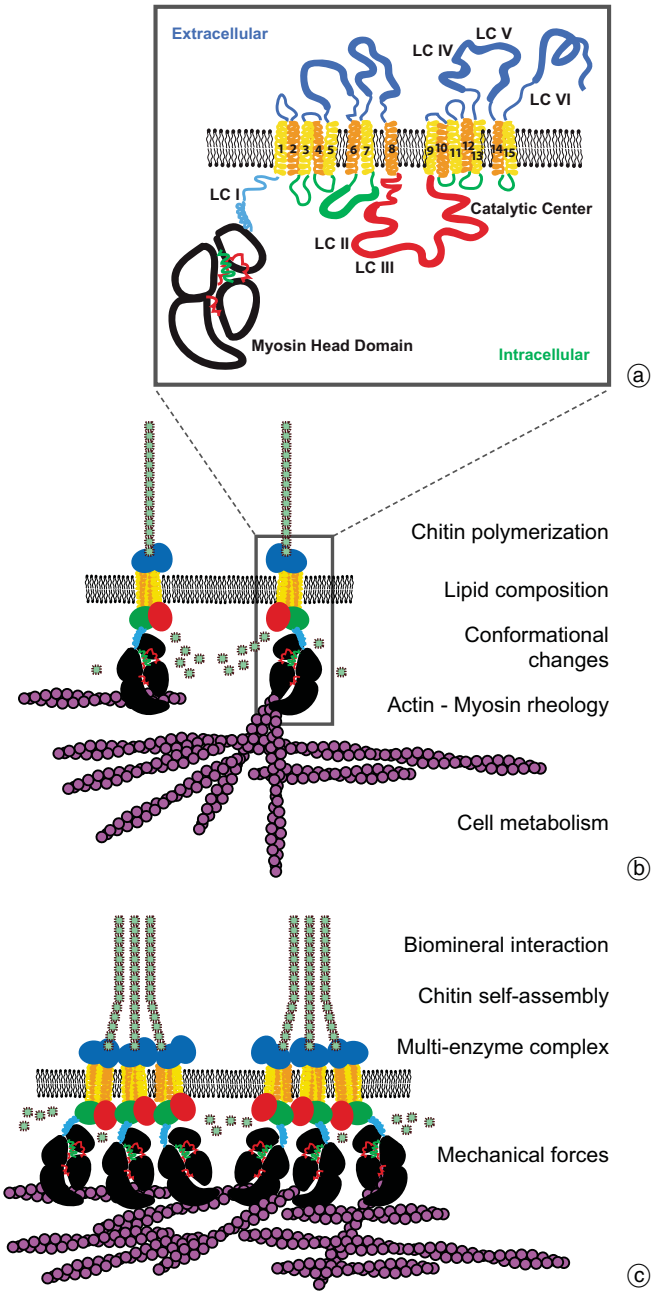

Fig. 17. Many parameters determine shell formation at the tissue interface. Understanding the structure and function of the 264 kDa chitin synthase *Ar*-CS1 (**a**) may help to answer open questions. Chitin polymers can grow at different speed (**b**), they can be further modified by subsequent enzymatic modification, and glued together by chitin binding proteins. Mechanical signals from the extracellularly forming shell could be transmitted to the underlying cell cortex close to the site of enzymatic chitin production and molding (**c**). Interferences between polymer synthesis, self-assembly and mineralization provide a tremendous number of hypothetical plug-ins for cellular, enzymatic and genetic control at various levels of hierarchy – a prerequisite for the evolution of species-specific shell ultrastructures.

Concluding remarks

Biology meets materials science – about 540 Mio years ago

It took Nature relatively long time for the evolution of complex biominerals [47, 192]. This happened when multicellular organisms evolved. It lead in relatively short periods of time to highly organized, stiff and tough extracellular matrices [51]. Along with the supramolecular arrangements of shell constituents, the principles of evolution got hands on materials properties.

Now, what are the consequences of the previous considerations? How would cellular mechanical forces contribute to mollusc shell biogenesis micro- and macroscopically? How can molecular forces be translated into the fine structure of functional materials? Can the overall process be constantly fine-tuned on the genetic level by employing modular elements of mechanical signal transduction as well as signals related to structure?

"It is the special properties of polymeric materials in amorphous phases that render them uniquely suited to many of the functions they perform both in biological systems and in technological applications. These properties are intimately related to the nature of the spatial configurations of the constituent molecules." (Paul J. Flory: Spatial Configuration of Macromolecular Chains. Nobel Lecture, 1974)

As Flory pointed out in a general context, the spatial arrangement of the chitin relative to the other shell constituents is indeed important. The direct cytoskeletal coupling of the mollusc chitin synthase located at the interface to the shell forming extracellular compartment – including intracellular vesicles – provides new conceptual perspectives for understanding the formation of composite biomaterials. The chitin biopolymer may act in a janus-faced manner: Due to its stiffness (see Flory 1953 [1931]) it may transduce forces from the cell cortex into the extracellular space. On the other hand, chitin represents a surface active molecule with its specific interfacial chemistry and functionality for biomineralization. Dynamic interaction between polymer synthesis, polymer self-assembly, and subsequent mineralization requires a versatile feedback control element from the site of mineralization upstream to the levels of the cell cortex, intracellular signal transduction and gene transcription. From a biological viewpoint, a general balancing of forces between intracellular and extracellular physical contraints could have been a major breakthrough during the evolution of the mollusc shell as a complex mineralizing extracellular matrix. This points towards one of the possible explanations for the cambrian diversification in the evolution of "biologically" controlled biomineralization [45, 55], once several genetic breakthroughs were manifested: Not only the creation of self-assembling structures one beneath the other, but keeping track of the molecular forces during the process of self-assembly. Not more than 600 Mio years ago, biomineralization processes [51, 53, 55], as well as fungal and animal chitin synthases [3, 118, 120] diverged. At this point, a versatile genetic toolkit may have allowed the evolution of mineral phase associated proteins, and framework polymer synthases [28]. Cell mechanics and signal response to extracellular matrix properties were genetically encoded by a motor protein directly coupled to extracellular matrix synthases [32]. Then, basic principles of materials science [77] may have paved the way for the evolution of complex mineralized composites such as nacre and crossed-lamellar shell types [174–176, 178, 179].

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Appendix A. List of Abbreviations

5TMS	5-**T**rans**m**embrane **S**pans (highly conserved region in chitin synthases)
Å	Ångstrom (10^{-10} m)
A	**A**ragonite shell layer
aa	**A**mino **a**cid
Abi2	**Ab**elson **i**nteractor 2, contains SH3-domains and proline-rich motifs, forms a multiprotein complex consisting of WAVE, PIR121/Sra-1, Nap1, Abi-2 and HSPC300 mediates responsiveness of WAVE to upstream regulators such as Rac
Active WAVE	Member of the WASP family of scaffolding proteins (WASP, N-WASP and WAVE / **W**iskott-**A**ltein (WASP)-family **ver**prolin homologous protein), relays signals from Rho-family GTPases to the actin remodelling machinery, interacts with actin-

	binding proteins and the Arp2/3 complex. WAVE also recruits cAMP-dependent protein kinase and tyrosine kinase in response to Rac activation, and interacts with profilin and IRSp53
ADP	**A**denosine-**d**i-**p**hosphate
Arf6	**A**DP-**r**ibosylation **f**actor **6**, localized to the plasma membrane and belongs to the ADP ribosylation factor family of GTP-binding proteins and the RAS superfamily. Involved in vesicular trafficking of biological membranes, transmembrane protein localization and endocytosis, and activates phospholipase D.
Arp2/3	**A**ctin-**R**elated **P**roteins Arp**2** and Arp**3** are part of the Arp2/3 complex with seven-subunits. They closely resemble the structure of monomeric actin and serve as nucleation sites for new actin filaments at a distinctive 70 degree angle from the existing actin filament.
ATP	**A**denosine-**t**ri-**p**hosphate
AtrMyo-A	***At**rina **r**igida* (bivalve mollusc) **Myo**sin-A (A: *Variant designation*)
C	**C**-terminus of a protein
CaMKII	**Ca**$^{2+}$/cal**m**odulin-dependent protein **k**inase **II**. Serine/threonine-specific protein kinases, regulated by the Ca^{2+}/calmodulin complex and essential for Ca++ homeostasis. CaMKII are multifunctional CaM kinases in contrast to MLCK.
CBGFP	**C**hitin-**b**inding **GFP**
EcMyo-A	***E**ncephalitozoon **c**uniculi* (microsporidia) **Myo**sin-A (A: *Variant designation*)
ER	**E**ndoplasmic **r**eticulum
G	**G**ranular shell layer
GF	**G**rowth **f**actor such as PDGF, EGF, or insulin
Gα, Gβ, Gγ	heterotrimeric **G** proteins, made up of alpha (α), beta (β) and gamma (γ) subunits
H1	**H**elix, alpha-helix (protein secondary structure)
HSPC300	**H**ematopoietic **S**tem **P**rogenitor **C**ells **300** protein, forms a multiprotein complex consisting of WAVE, PIR121/Sra-1, Nap1, Abi-2 and HSPC300 mediates responsiveness of WAVE to upstream regulators such as Rac
IP	**I**nner **p**rismatic shell layer
IRSp53	**I**nsulin **R**eceptor tyrosine kinase **S**ubstrate **p53** links Rac and WAVE and has been implicated in lamellipodia protrusion and essential in the regulation of membrane ruffling
LARG	**L**eukaemia-**a**ssociated **R**ho**G**EF
LC	**L**ow **c**omplexity domain (LC I to LC VI, six different LC domains)
LIMK	Protein **k**inase consisting of N-terminal **LIM** domains with highly conserved cysteine-rich structures containing 2 zinc fingers. LIM stands for **L**in-11, **I**sl-1 and **M**ec-3.
LPA	**L**yso**p**hosphatidic **a**cid
M	**M**antle epithelium
MbMyo-X	***M**onosiga **b**revicollis* (choanoflagellate) **Myo**sin-X *(X: Variant designation)*
mDia	**m**ammalian **Dia**phanous-related formin, ubiquitously expressed superfamily with conserved polyproline rich domains
Mg^{2+}	**Mag**nesium ion
MLC	**M**yosin **L**ight-**C**hain, regulatory domain of myosin II
MLCK	**M**yosin **L**ight **C**hain **K**inase, a calcium/calmodulin-dependent serine/threonine kinase that phosphorylates the regulatory light chain of myosin II. These are specialized CaM kinases in contrast to CaMKII.
MLC-P	**P**hosphorylated **M**yosin **L**ight **C**hain, regulatory domain of myosin
Myo	Official abbreviation for **Myo**sin according to www.cymobase.org
Myosin HC	Myosin **H**eavy **C**hain
N	**N**-terminus of a protein
Nap125	**Nap125** and PIR121 are both direct Rac targets, which form a multiprotein complex consisting of WAVE, PIR121/Sra-1, Nap1, Abi-2 and HSPC300 mediates responsiveness of WAVE to upstream regulators such as Rac
NavMyo-A	***Na**sonia **v**itripennis* (wasp) **Myo**sin-A (A: *Variant designation*)
p115RhoGEF	**G**uanine nucleotide **E**xchange **F**actor for **Rho**, p115RhoGEF has N-terminal similarity to regulators of G protein signaling (RGS) proteins and activates the GTPases $G\alpha_{12}$ and $G\alpha_{13}$
p190RhoGAP	**p190**, a **Rho** family **G**TPase-**a**ctivating **p**rotein
PAK	**P**21 protein (Cdc42/Rac)-**A**ctivated **K**inase, a family of serine/threonine kinases, link RhoGTPases to cytoskeleton reorganization and nuclear signaling
PDZ-RhoGEF	**P**ost synaptic density protein, **D**iscs large protein, **Z**onula occludens
P_i	**I**norganic **p**hosphate
PI3-K	**P**hosphatidyl**i**nositol **3-k**inase
PIR121	Nap125 and **PIR121** are both direct Rac targets, which form a multiprotein complex consisting of WAVE, PIR121/Sra-1, Nap1, Abi-2 and HSPC300 mediates responsiveness of WAVE to upstream regulators such as Rac
PKC	**P**rotein **k**inase **C**
PLC	**P**hospho**l**ipase **C**, it hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP2) to diacyl glycerol (DAG) and inositol triphosphate (IP3)
PMA	**P**horbol-12-**m**yristate-13-**a**cetate
PTP	**P**hospho-**T**yrosine **P**hosphatases
P-Tyr	**P**hospho-**Tyr**osine
Rac	subgroup of the Ras superfamily of GTP hydrolases (→ **Ra**s-related **C**3 botulinum toxin substrate)
RacGEFs	**Rac** **G**uanine nucleotide **E**xchange **F**actors
Ras	small GTPase, prototypical member of Ras superfamily (→ **Ra**t **s**arcoma)
Rho	subgroup of the Ras superfamily of GTP hydrolases (→ **R**as **ho**molog gene family, small GTPase proteins)
RTK	**R**eceptor **t**yrosine **k**inase
S	**S**heet (organic)
SH2	**S**rc **H**omology **2** domains
Src	Proto-oncogene tyrosine-protein kinase (short for "**Sarc**oma")
SWGTR	peptide motif, Ser-Trp-Gly-Thr-Arg
Tiam1	**T**-cell lymphoma **i**nvasion **a**nd **m**etastasis-inducing protein **1**, with Ras-binding RBD domain. Human Tiam1 modulates the activity of Rho GTP-binding proteins and connects extracellular signals to cytoskeletal activities. TIAM1 activates Rac1, CDC42, and to a lesser extent RhoA
Tyr	**Tyr**osine
TyrK	**Tyr**osine **K**inases
UDP	**U**ridine-**dip**hosphate
UDP-GlcNAc	**U**ridine-**dip**hosphate-**N**-**Ac**etyl-D-**Glu**cosamine
WAVE	**W**iskott-**A**ldrich syndrome protein (WASP)-family **ver**prolin homologous protein
X	**E**xtrapallial space
αq	G**αq**, heterotrimeric G protein subunit that activates phospholipase C (PLC).