ANNUAL PROGRESS REPORT TO THE OFFICE OF NAVAL RESEARCH

PROJECT TITLE:

MOLECULAR AND CELL BIOLOGICAL STUDIES ON BIOMINERALIZATION BY PRIMARY MESENCHYME CELLS OF THE SEA URCHIN

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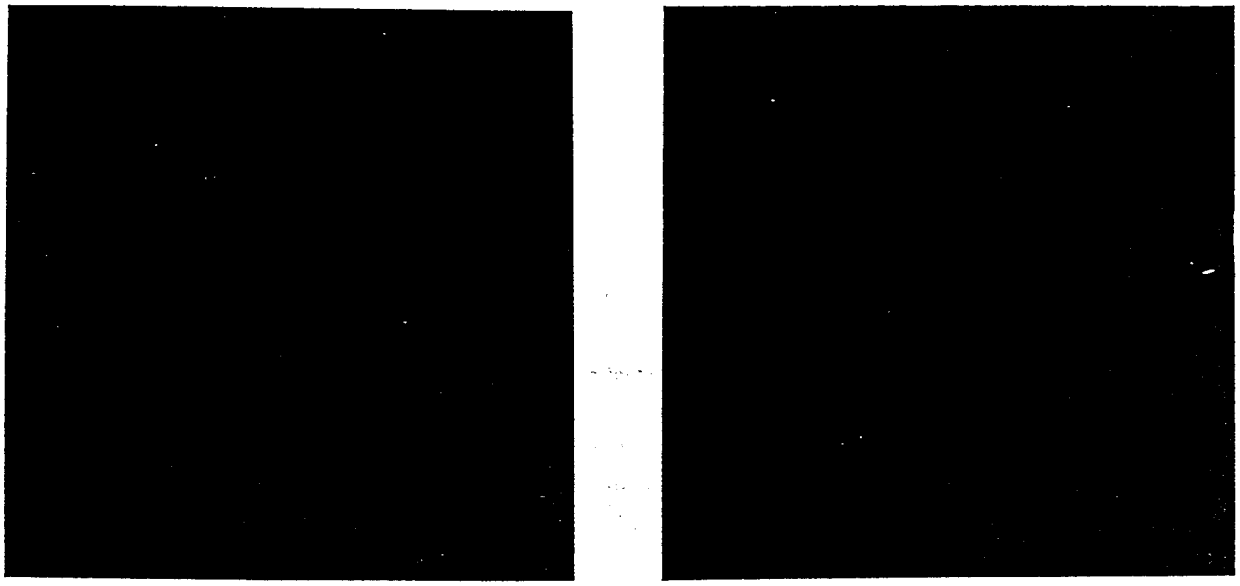
SUMMARY OF PROGRESS REPORT DURING THE PAST YEAR

Subproject I - Structure and Function of a Sea Urchin Homolog (suBMP) of Human Bone Morphogenetic Protein

Work on the function of sea urchin bone morphogenetic protein 1 (suBMP) in the sea urchin *S. purpuratus* has progressed in three areas. We are currently examining the developmental expression of the suBMP protein, its function in the developing sea urchin embryo, and its putative protease activity. Previous work had shown that suBMP protein first appears in the hatched blastula stage, rapidly accumulates to the mesenchyme blastula stage, then decays to a lower but persistent level throughout the rest of embryonic development. Using an affinity purified polyclonal antibody to suBMP, we have begun *in situ* analysis to localize its expression in the mesenchyme blastula immediately preceding gastrulation and prism stage at the onset of spiculogenesis. Preliminary results indicate that suBMP is found in all cells rather than being localized to one specific cell type (Figure 1).

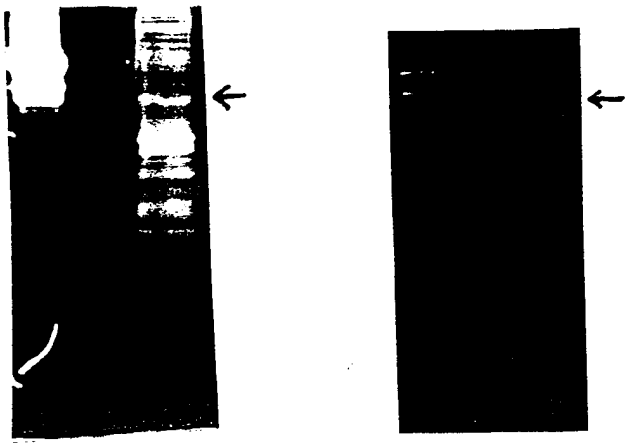


A B

Figure 1. Expression of suBMP protein in late gastrula stage embryo. A) Embryos stained with anti-suBMP antibody, B) Embryos stained with a negative control antibody, anti-GST.

The archenteron and the vegetal plate show a marginally higher level of expression. In tandem with these experiments, we are attempting to inhibit the function of suBMP by injecting the above mentioned antibody into the blastocoel of mesenchyme blastula stage embryos. Previous work has demonstrated that suBMP can be found in the blastocoel of the developing embryo (Hwang, et al., 1994).

Based on its homology to the crayfish metallo-protease astacin, it has been proposed that suBMP (and all of the BMP-1 homolog) have a proteolytic activity. Using zymography, we have been able to detect a 72 kDa EDTA-sensitive gelatinase in the embryos at prism stage (Figure 2). As shown in Figure 2, it has been previously reported that suBMP migrates as a 72 kDa band in embryo extracts (Hwang, et al., 1994).



5 mM EDTA	-	+
2 mM 1,10-Phenanthroline	-	+

Figure 2. Zymography of *S. purpuratus* embryo extracts. Tris buffered saline extracts of prism stage embryos were run on SDS-PAGE with 0.5% gelatin impregnated in the gel. Gelatinase activity was detected at pH 8.0 in the presence of 1 mM pefabloc, 50 μM E64, 10 μg/ml pepstatin with, or without 5 mM EDTA and 2 mM 1,10-phenanthroline. A) reference standard metalloproteinase, B) molecular weight markers, C) prism stage embryo extract. Arrow indicates the position of the 72 kDa EDTA/1,10-phenanthroline sensitive gelatinase activity.

In order to facilitate the study of suBMP, we have assembled a full length cDNA clone and we are using this clone to generate GST-suBMP fusion proteins. We are currently constructing a pro-suBMP (Figure 3C), and a factor Xa-activatible suBMP (Figure 3B). The boundary between the pro-region and the astacin-like protease domain was deduced based on the crystal structure of astacin and suBMP's homology to astacin at the amino acid level.

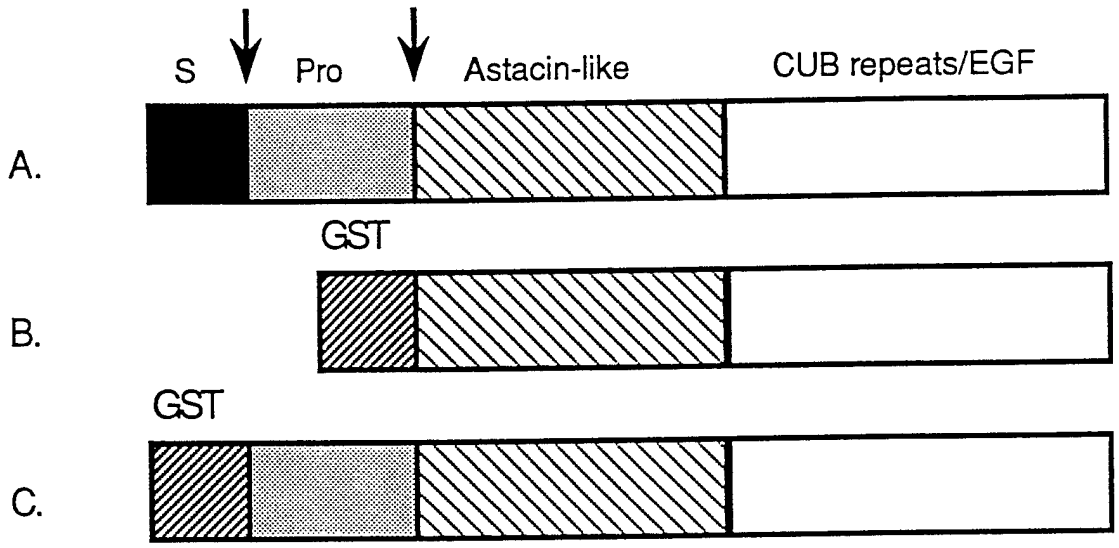


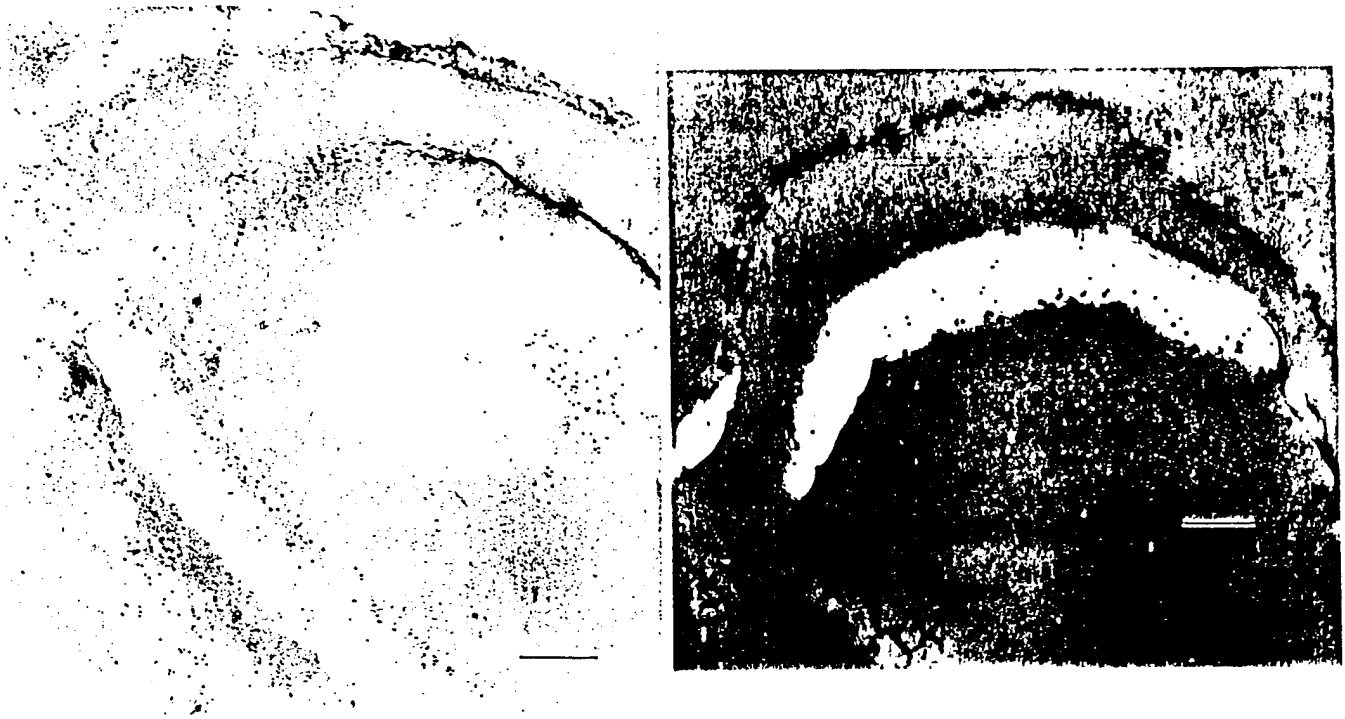
Figure 3. Recombinant suBMP Constructs. A) Schematic of the full length suBMP protein. Arrows designate the predicted cleavage site between the signal peptide and the pro-domain, and the cleavage site between the pro-domain and the astacin-like protease domain. B) Factor Xa-activatible GST-suBMP fusion protein structure. Factor Xa will be used to remove the GST tag and concomitantly activate the protease domain of suBMP. C) GST-Pro-suBMP construct. S=signal sequence, Pro=pro-domain of putative protease domain, Astacin-like=putative protease domain, CUB repeats/EGF=putative C-terminal protein-protein interaction domains.

Subproject II - Distribution of the Unique 1223 Epitope in a Spicule Matrix Protein

When proteins isolated from spicules of *S. purpuratus* embryos were examined by Western blot analysis, a major protein of approximately 43 kDa was observed to react with the monoclonal antibody, mAb 1223. Previous studies have established that this antibody recognizes an asparagine-linked, anionic carbohydrate epitope on the cell surface glycoprotein, msp130. This protein has been shown to be specifically associated with the primary mesenchyme cells involved in assembly of the spicule. Moreover, several lines of evidence have implicated the carbohydrate epitope in Ca^{2+} deposition into the growing spicule. The 43 kDa, spicule matrix protein detected with mAb 1223 also reacted with a polyclonal antibody to a known spicule matrix protein, SM30. Further characterization experiments, including deglycosylation using PNGaseF, two-dimensional electrophoresis, and immunoprecipitation, verified that the 43 kDa spicule matrix protein had a pI of approximately 4.0, contained the carbohydrate epitope recognized by monoclonal antibody mAb 1223 and reacted with anti-SM30. Electron microscopy confirmed the presence of proteins within the demineralized spicule that reacted with mAb 1223 and anti-SM30. We conclude that the spicule matrix protein, SM30, is a glycoprotein containing carbohydrate chains similar or identical to those on the primary mesenchyme cell membrane glycoprotein, msp 130.

Subproject III - Ultrastructural Localization of Spicule Matrix Protein

We are attempting to localize spicule matrix proteins within developing spicules in the sea urchin, and to determine whether there is a discernable pattern to this localization. As was found by previous investigators, isolated spicules fracture in a manner that exposes a concentric ring pattern on the broken surface. We hypothesize that these concentric rings reflect the organization of microcrystals of calcium carbonate with fibrillar bands of matrix proteins interspersed. When isolated spicules are demineralized and the remaining material embedded in resin and thin sectioned, concentric rings of fibrillar protein are seen and these fibrillar rings can be gold labeled using antibodies to spicule matrix proteins such as SM30 and Mab 1223 (Figures 4A, 4B).



A B

Figure 4. When isolated sea urchin spicules are demineralized and the residual matrix is embedded in resin, thin sectioned, and reacted with anti-spicule matrix antibodies, concentric rings of fibrillar material label. A) Mab 1223 labels two concentric rings of this cross-sectioned demineralized spicule "ghost". B) Antibody to SM30, a spicule matrix protein, labels the inner concentric ring greater than the outer ring. Magnification bar =200 nm.

In a recent effort, we are using a Scanning Transmission X-ray Microscope, Beamline X1A at Brookhaven National Laboratories, in collaboration with members of the Physics Department, to further study the physical characteristics of the sea urchin spicule. By examination of semi-thick sections (500 nm) of resin embedded spicules at two different wavelengths, we observe a pattern of concentric bands of decreasing calcium density, which may correspond to the concentric fibrillar bands seen by standard transmission electron microscopy of demineralized spicules.

PLANS FOR THE COMING YEAR

Subproject I - Bone Morphogenetic Protein

Future work will continue in each of the areas discussed above. We will expand the *in situ* analysis of suBMP protein to all stages of embryonic development through the pluteus stage. If available, we will also examine newly metamorphosed juveniles. Experiments to knock out the function of suBMP will continue using both anti-suBMP and antisense oligonucleotides microinjected into the blastocoel. Recombinant suBMP will be used to examine its proteolytic activity in zymogels, in combination with immuno-precipitated suBMP. We will also be examining the substrate specificity of suBMP using synthetic peptides substrates. Co-immunoprecipitation and crosslinking experiments will be used to find proteins interacting with suBMP, with special attention paid to members of the TGFβ family.

Subprojects II and III - Structure Localization of Spicule Matrix Proteins

Over the next year, we will carry out gold labeling of broken spicules with antibodies to the SM30 spicule matrix protein and to Mab 1223 at the level of the scanning electron microscope. Preliminary studies show that the broken cross-sectional surfaces of the spicule can be labeled with Mab 1223. A very important issue will be to determine if there is some regular order to the distribution of these matrix proteins within the spicule. In addition, we will continue to characterize matrix macromolecules, in an attempt to determine whether one component of the matrix is a banded form of collagen.

PUBLICATIONS

1. Hwang, Sheng-Ping L., Partin, Jacqueline S. and Lennarz, William J., Characterization of a Homolog of Human Bone Morphogenetic Protein 1 in the Embryo of the Sea Urchin, *S. purpuratus*. Development 120, 559-568 (1994).

2. Lennarz, William J., Fertilization in Sea Urchins: How many Different Molecules are Involved in Gamete Interaction and Fusion. Zygote 2, 1-4 (1994).

3. Brown, Martin F., Partin, Jacqueline S. and Lennarz, William J., Spiculogenesis in the Sea Urchin Embryo: Studies on SM30 Spicule Matrix Protein. Development Growth and Differentiation, In press (1995).

Zygote 2 (February), pp 1–4. Printed in Great Britain

Commentary

Fertilisation in sea urchins: how many different molecules are involved in gamete interaction and fusion?

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Summary

It has been established that fertilisation in the sea urchin involves binding of acrosome-reacted sperm to an egg cell surface receptor. The structure and function of this receptor, as well as the possible involvement of other cell surface molecules in the binding, fusion and activation events, is discussed.

Keywords: Bindin, Egg surface receptor, Fertilisation – sea urchin, Sperm–egg binding

Introduction

Although in nature there are wide variations in the number and complexity of biological events that culminate in formation of a zygote, one process common to fertilisation in most if not all animals is the binding of sperm to the surface of the egg. The purpose of this perspective is to summarise very concisely the current status of understanding of this binding process in one organism, the sea urchin. In fact the review will be even more narrowly restricted, because for the most part it is limited to studies in only one species of sea urchin, *S. purpuratus*. For a detailed review the reader is directed to Foltz & Lennarz (1993). In *S. purpuratus* it is now clear that subsequent to the acrosome reaction in sperm a ligand–receptor pair involving the sperm protein, bindin, as the ligand (see reviews by Trimmer & Vacquier, 1986; Minor *et al.*, 1989) and an egg cell surface glycoprotein (Foltz & Lennarz, 1990, 1992; Foltz *et al.*, 1993; Ohlendieck *et al.*, 1993) as the receptor plays a key role in gamete binding. However, a number of important questions remain to be resolved: Are these two molecules the only cell surface components involved in gamete binding? Is this ligand–receptor interaction directly coupled to the subsequent events, namely fusion of a region of the plasma membranes of the two gametes, induction of a Ca^{2+} wave in the egg and metabolic activation of the fertilised egg? And are related pairs of proteins involved in fertilisation in higher organisms?

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Bindin: the receptor ligand

It has been clear from the very early work using gametes, as well as *in vitro* studies with isolated jelly coat and sperm (see review cited above), that bindin becomes exposed at the tip of the sperm as a result of an exocytotic event involving jelly coat as the secretogogue. This event apparently involved degradation (dehiscence) of a portion of the plasma membrane and the acrosomal membrane at the tip of the sperm, and subsequent fusion of the remaining membranes. As a consequence of this exocytotic event bindin inside the sperm acrosome becomes exposed at the tip of the sperm. Why, unlike a typical secretory event, bindin remains associated with the new cell surface membrane at the tip of the sperm is unknown, but Glabe and co-workers have shown that isolated bindin interacts with phospholipids in non-bilayer form, and have speculated that this may be the mechanism of retention at the plasma membrane (Kennedy *et al.*, 1989).

A number of lines of evidence either directly or indirectly implicate bindin as the protein on the sperm that mediates binding to the egg receptor. First, use of antibody to bindin revealed that immunoreactivity is observed in the region where the sperm contacts the egg (Moy & Vacquier, 1979). Second, isolated bindin, which exists as aggregates, promotes egg agglutination (Glabe & Vacquier, 1977; Glabe & Lennarz, 1979). This agglutination exhibits species preferentially, although the level of specificity is less than that of binding or fertilisation (see below). Finally, bindin particles bind the isolated sperm receptor (see below) in a species-specific fashion (Foltz *et al.*, 1993). On the basis of these three observations there is little doubt about the key role that bindin plays in the gamete binding process.

The bindin receptor

The bindin receptor was identified as a result of a long-term effort that started with unsuccessful attempts to isolate a pure cell surface molecule. When this approach failed to yield a pure protein the strategy was modified; instead fragments of the extracellular domain of the putative receptor were generated and attempts were made to purify one or more of them. Following promising initial experiments with trypsin to generate fragments of the receptor (Ruiz-Bravo & Lennarz, 1987*a*,*b*), lysoendoprotease C was used to produce a 70 kDa fragment of the receptor that inhibited fertilisation. Subsequently this fragment was purified to homogeneity and used to prepare an antibody that also inhibited fertilisation. Use of the antibody in expression screening of an immature ovary–oocyte library yielded clones that led to deduction of the amino acid sequence of the receptor (Foltz *et al.*, 1993). The deduced sequence indicated a 131 kDa protein with one well-defined transmembrane domain, a long extracellular domain with multiple potential *O*- and *N*-linked glycosylation sites, a cysteine-rich domain near the N-terminus and a short C-terminal intracellular tail. A model of the receptor is shown in Fig. 1. An interesting feature of the deduced sequence is the presence of a large segment of the extracellular domain that exhibits moderate homology with the hsp 70 family of proteins. The functional significance of this homology remains to be established.

Several lines of evidence confirmed that the sequence obtained by cloning is actually the receptor. First, amino acid sequencing of peptides generated from the extracellular domain revealed that they were identical to portions of the deduced amino acid sequence of the putative receptor. Second, when a construct encoding for the extracellular domain of the receptor was expressed in *E. coli*, the resulting purified protein was found to: (1) inhibit fertilisation in a binding assay, (2) bind to bindin particles and (3) cause adhesion of acrosome-reacted sperm to beads coated with it.

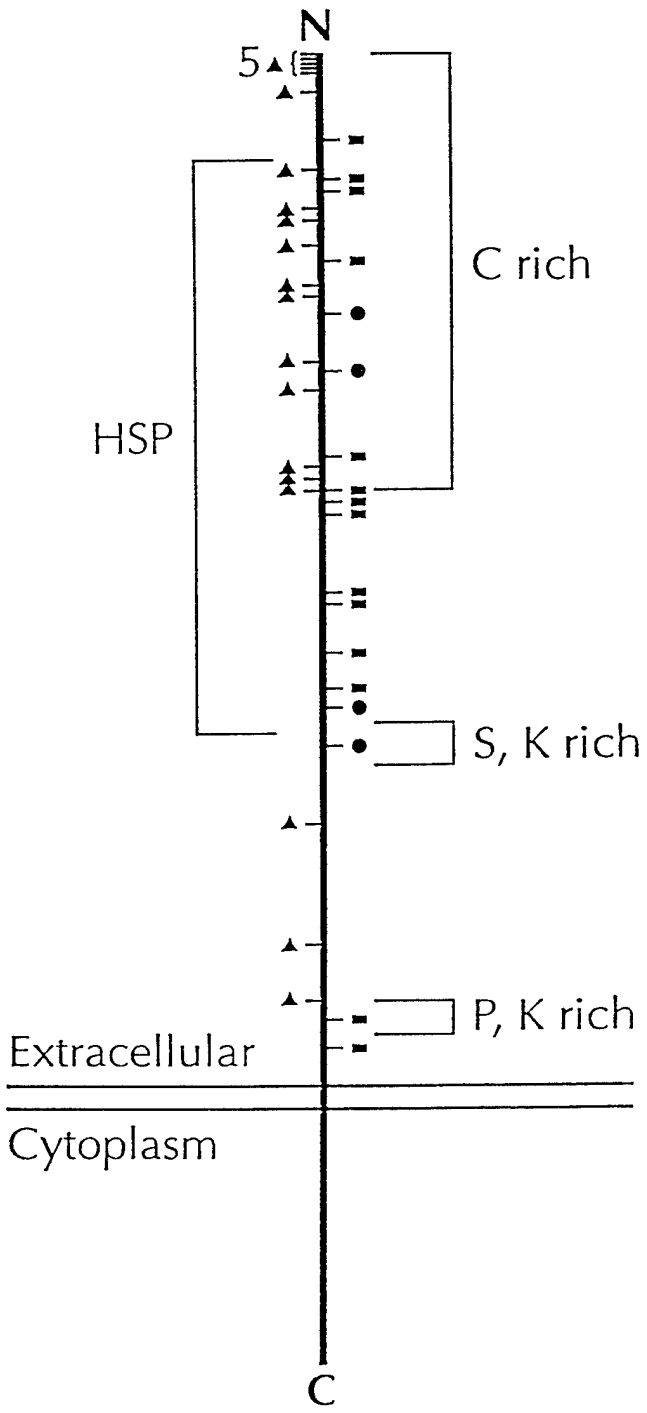

Figure 1 Diagram of the structure of the egg receptor for sperm. Cysteine (C) residues are denoted by triangles, potential *O*-glycosylation sites by rectangles and *N*-glycosylation sites by circles. The region with HSP homology is indicated.

In subsequent studies the availability of antibody

and the finding that the receptor binds to wheat germ agglutinin (WGA) facilitated purification of the intact receptor to homogeneity (Ohlendieck *et al.*, 1993). The intact receptor was found to have an apparent molecular weight of 350 and to contain sulphated saccharides that are typical components of *N*- and *O*-linked oligosaccharide chains. Consistent with the deduction from the cDNA that the N-terminus is rich in cysteine residues, the receptor has been shown to exist as a disulphide-bonded homo-tetramer (Ohlendieck *et al.*, 1994).

The 70 kDa extracellular domain and the recombinant fragment of the extracellular domain, and the intact receptor were found to inhibit fertilisation species-specifically and prevented sperm adhesion to beads coated with these molecules (Foltz *et al.*, 1993; Ohlendieck *et al.*, 1993). The inhibition of fertilisation by the intact receptor was observed even after its removal from the membrane and separation from other protein components. Moreover, the pure receptor retains the biologically relevant property of being adhesive, i.e. of binding to acrosome-reacted sperm. Interestingly, it appears that the carbohydrate chains may not play a dominant role in the binding process, at least when this process is studied *in vitro*, because on a molar basis the unglycosylated recombinant protein encoding for the extracellular domain inhibits fertilisation as effectively as does the intact glycoprotein. In agreement with this finding, the combined oligosaccharide chains released from the receptor by hydrazinolysis, although inhibitory, were found to be much less active on a molar basis than the polypeptide chain (S.T. Dhume & W.J. Lennarz, unpublished data). However, this finding is provisional since the molarity of the pure, biological oligosaccharide in the mixture is not yet known. Moreover, the modest inhibition of the oligosaccharide should not be interpreted to mean that the oligosaccharide chains play no role in the gamete interaction process, because the inhibition studies measure only an endpoint, not the kinetics of the binding process.

Outstanding questions

Although bindin and its receptor on the surface of the egg have now been characterised, the detailed mechanism of their interaction remains to be elucidated. Is the binding process a multi-step event in which the first slow, low-affinity step involves the carbohydrate chains followed by rapid tight binding of the bindin to the polypeptide chain? Where on the carbohydrate chains and on the polypeptide chains does binding occur? Preliminary evidence with deletion constructs of the recombinant extracellular domain of the receptor suggests that a relatively small region of the polypeptide is involved (Stears & Lennarz, 1993). But it remains to be determined what level and type of binding forces are necessary to bring about the attachment of a motile sperm to a cell surface molecule. By taking an average of the value of the swimming force generated by starfish (W.H. Wright, G.J. Sonek & M.W. Berns, personal communication) and by mammalian sperm (Baltz *et al.*, 1988) and applying it to *S. purpuratus* it can be calculated that binding of a motile sperm to between 1 to 10 receptor molecules would be sufficient to retain it on the surface of the egg (S. McLaughlin, personal communication). Interestingly, this range of values, which seems reasonable given that the receptor exists as a tetramer, agrees well with the estimate that only a few bonds are required to bind a mass sperm to the zona pellucida. However, the types of forces involved in these binding processes remain to be determined.

A number of other issues also must be resolved. One central question is: Are these two surface components of the gametes sufficient to accomplish both the binding process and the fusion event that may trigger egg activation? On one hand, it is clear that when the isolated ligand – bindin – in the form of aggregates is added to eggs it causes species-preferential aggregation. Thus, isolated bindin acts *somewhat* like acrosome-reacted sperm. However, given the facts that (1) the aggregation is not as species-specific as fertilisation and (2) the aggregation is not followed by egg activation, it is clear that the ligand alone does not have the properties of acrosome-reacted sperm. Is this because isolated bindin does not have the same physical properties as bindin coating the tip of the acrosomal process? Or is bindin alone not sufficient to activate eggs? That is, is another surface component of the sperm required for high-fidelity binding and for induction of fusion? If another sperm component (presumably on the plasma membrane) is involved, does it act via interaction with the receptor? Or is there another egg protein to which it binds? With respect to the fusion event, virtually nothing is known in terms of the molecules that may be involved. Evidence that a metalloendoprotease is involved has been reported (Roe *et al.*, 1988). However, neither the site nor the identity of the enzyme, nor of its substrate, have been identified.

Not only must the questions outlined above be resolved, but the molecular basis of the species specificity must be precisely defined. This clearly will require a detailed structural comparison of the receptors and ligands in sea urchin gametes that both do and do not cross-fertilise. Finally, it is not known whether similar or related receptor–ligand pairs involved in fertilisation exist in higher organisms. Considering the rapid progress in application of contemporary tools of cell and molecular biology to problems in develop-

mental biology, it is to be hoped that answers to these questions about the molecular basis of fertilisation will be forthcoming in the next few years.

Acknowledgements

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Spiculogenesis in the sea urchin embryo: Studies on the SM30 spicule matrix protein

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When proteins isolated from spicules of *Strongylocentrotus purpuratus* embryos were examined by western blot analysis, a major protein of approximately 43 kDa was observed to react with the monoclonal antibody, mAb 1223. Previous studies have established that this antibody recognizes an asparagine-linked, anionic carbohydrate epitope on the cell surface glycoprotein, msp130. This protein has been shown to be specifically associated with the primary mesenchyme cells involved in assembly of the spicule. Moreover, several lines of evidence have implicated the carbohydrate epitope in Ca^{2+} deposition into the growing spicule. The 43 kDa, spicule matrix protein detected with mAb 1223 also reacted with a polyclonal antibody to a known spicule matrix protein, SM30. Further characterization experiments, including deglycosylation using PNGaseF, two-dimensional electrophoresis, and immunoprecipitation, verified that the 43 kDa spicule matrix protein had a pI of approximately 4.0, contained the carbohydrate epitope recognized by monoclonal antibody mAb 1223 and reacted with anti-SM30. Electron microscopy confirmed the presence of proteins within the demineralized spicule that reacted with mAb 1223 and anti-SM30. We conclude that the spicule matrix protein, SM30, is a glycoprotein containing carbohydrate chains similar or identical to those on the primary mesenchyme cell membrane glycoprotein, msp130.

Key words: biomineralization, sea urchin, spiculogenesis.

Introduction

The role of proteins that participate in the formation of mineralized tissue is poorly understood, primarily because of the complex nature of the tissues studied. The sea urchin embryo provides a simple system with which to study the cellular and molecular aspects of this morphogenetic process. The endoskeletal spicules synthesized by sea urchin larvae are calcitic assemblages of calcium and magnesium carbonate that are believed to contain several intracrystalline proteins, termed spicule matrix proteins (Wilt & Benson 1988; Benson & Wilt 1992). This calcareous skeleton is produced by the primary mesenchyme (PM) cells, a group of approximately 32 cells that ingress into the blastocoel cavity and migrate to the edge of the blastocoel where they form a ring-like distribution. At the edge of the blastocoel they subsequently fuse to form syncytical structures and initiate the deposition of calcite. This assembly process can be studied using isolated PM cells cultured *in vitro* (Okazaki 1975).

Studies have shown that the synthesis of N-linked glycoproteins is necessary for spiculogenesis in intact embryos (Schneider *et al.* 1978; Heifatz & Lennarz 1979), as well as PM cells cultured *in vitro* (Mintz *et al.* 1981). Subsequent work has identified a 130 kD PM cell-specific glycoprotein, msp130 (Anstron *et al.* 1987). Earlier this protein had been shown independently to play an essential role in spicule formation because antibody, mAb 1223 to a carbohydrate epitope prevented the deposition of $CaCO_3$ *in vitro* (Carson *et al.* 1985). Further characterization of the immunoreactive proteins has localized the epitope to an anionic, asparagine-linked oligosaccharide chain that has been shown subsequently to bind divalent cations including Ca^{2+} (Farach-Carson *et al.* 1989). This epitope has been shown to be present on a number of glycoproteins in PM cells of several species (Shimizu *et al.* 1988; Shimizu-Nishikawa *et al.* 1990; Kabakoff *et al.* 1992). In every case it appears the glycoproteins contain sulfated N-linked oligosaccharide chains. Consistent with an essential role for this carbohydrate chain in biomineralization, it has been shown that inhibition of processing of the oligosaccharide chain to its mature form results in a block in spiculogenesis (Kabakoff & Lennarz 1990).

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In the current study we have investigated the occurrence of this mAb 1223 epitope in the organic matrix of the spicule from the sea urchin, *Strongylocentrotus purpuratus*. Using western blot analysis, we determined that the major mAb 1223-reactive protein in the spicule matrix is a 43 kDa molecule. On the basis of deglycosylation experiments, two-dimensional (2-D) gel electrophoresis, immunoprecipitation and immunoblotting, this 1223-reactive protein species has been identified as the previously cloned and sequenced SM30 spicule matrix protein (George *et al.* 1991). Thus, one of the major proteins of the spicule bears the same immunoreactive carbohydrate chains first found on the GPI-anchored cell surface protein, msp130, specific to PM cells. Using electron microscopy, we also provide evidence that intracrystalline components of the spicule can be labeled with mAb 1223 and with antibody to SM30.

Materials and Methods

Materials

Strongylocentrotus purpuratus sea urchins were purchased from Marinus (Long Beach, CA, USA). Artificial sea water (Instant Ocean) was obtained from Aquarium Systems (Mentor, OH). Peroxidase conjugated goat anti-mouse and goat anti-rabbit IgG and alkaline phosphatase conjugated secondary antibodies and recombinant PNGaseF were supplied by Boehringer Mannheim (Indianapolis, IN, USA). BCIP and NBT were purchased from Promega Corporation, (WI, USA). Nitrocellulose (0.2 µm) was obtained from Schleicher and Schuell, Inc. (NH, USA). Enzyme chemiluminescence (ECL) kits were purchased from Amersham Life Science, (IL, USA). Protein A Sepharose CL 4B was purchased from Pharmacia, NJ. Prestained high molecular weight standards were obtained from Gibco BRL, MD. All other chemicals were of the highest grade available.

Preparation of embryo protein extracts

Gametes were collected, eggs were fertilized and embryos were cultured as described previously (Heifetz & Lennarz 1979). The embryos were cultured as 1% suspensions in 0.45 µmol/L filtered artificial sea water (FSW) at 14°C. At various stages in their development 200 mL aliquots of embryos were removed and collected by centrifugation at 3000 *g* for 3 min. After washing once in FSW, protein extracts were prepared according to the method of Kabakoff and Lennarz (1979).

Isolation of spicule matrix proteins

Spicule matrix protein was prepared using a modification of the procedure according to Benson *et al.* (1986). Essentially, pluteus stage larvae were collected by centrifugation, washed once in ice-cold calcium and magnesium free sea water (CMFSW) and then resuspended in a Dounce homogenizer in five volumes of ice cold 10 mmol/L Tris-Cl buffer, pH 7.4, containing a mixture of protease inhibitors (leupeptin 1 µg/mL, antipain 2 µg/mL, benzamidine 10 µg/mL and aprotinin 2 µg/mL). After incubating on ice for 15 min the homogenate was centrifuged at 4000 *g* for 3 min and the supernatant discarded. This step was repeated. The pellet was resuspended in five volumes of ice cold 20 mmol/L Tris-Cl buffer, pH 7.4, containing 2% Triton X-100 (w/v) and 4% sodium deoxycholate (w/v). The homogenate was centrifuged at 4000 *g* for 3 min and the supernatant discarded. This step was repeated until the pellet was off-white in appearance. To free the spicules of any membranous material, the pellet was then homogenized for less than 1 min in five volumes of 20 mmol/L Tris buffer, pH 8.0, containing 2% sodium hypochlorite. After centrifugation the pellet was washed five times with distilled water. Typically, 0.2 mL of spicule pellet was demineralized in 1.5 mL 50 mmol/L EDTA, pH 8.0 at 4°C overnight. After centrifugation to remove insoluble components, the supernatant was dialyzed against 6 L of distilled water at 4°C overnight and then lyophilized.

Gel electrophoresis

Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS PAGE) was carried out using a BioRad Mini Protean II apparatus (Biorad, USA) in accordance with the method of Laemmli (1970). Immediately following electrophoresis, the proteins were electroblotted onto nitrocellulose membrane (0.2 µm) according to the method of Towbin *et al.* (1979). Western blotting was then carried out according to Maciewicz and Knight (1988) using the monoclonal antibodies mAb 1223 (Carson *et al.* 1985), mAb A3 (Leaf *et al.* 1987) and the polyclonal anti-SM30 IgG prepared as described below. Two-dimensional electrophoresis was performed using a 2.5–10 pH gradient and a BioRad 2-D gel apparatus. Electrophoresis in the first dimension was carried out at 200, 300, 400, and 500 V for 10 min each and 750 V for 2 h. An 8% SDS PAGE gel was used for the second dimension.

Immunoprecipitation

Total spicule matrix protein was subjected to immunoprecipitation using monoclonal antibody mAb 1223. After an overnight incubation in TBS at 4°C, the IgG complex was removed by incubating the sample with Protein A Sepharose beads. After removing the supernatant the beads were washed 10 times with distilled

water. The immunoprecipitated protein(s) were then released by boiling the beads with Laemmli buffer. Samples were then analyzed by SDS PAGE and western blotting.

Preparation of anti-SM30

The antiserum raised against *S. purpuratus* SM30 protein was prepared by injecting a preparation of a maltose binding protein/SM30 fusion protein into a rabbit. The fusion protein was constructed by subcloning the SM30 cDNA, pNG7 (George *et al.* 1991), into the expression vector pMat cR1 (New England Biolabs).

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Distribution of SM30

Small aliquots (150–200 µL) of embryos at different stages in development were treated with 10 mmol/L Tris-Cl, pH 8.0, containing 2% cholic acid overnight at 4°C. After centrifugation the pellet was washed 10 times with distilled water and then further extracted with 50 mmol/L EDTA, pH 8.0, overnight at 4°C. The supernatants from both of these extractions were dialyzed against distilled water for 2 days at 4°C and then lyophilized. The samples were then reconstituted in equal volumes of distilled water and examined by SDS PAGE and western blotting. The relative distribution of SM30 at each stage of development was estimated by scanning with an optical densitometer.

Electron microscopy

Isolated, bleached spicules (Benson *et al.* 1986) were lyophilized and stored at −20°C in a tightly closed tube until use. A portion of the lyophilized spicules were prepared for scanning electron microscopy (SEM) by sprinkling the dried spicules onto a sticky specimen stub and lightly coating with carbon or gold. Scanning electron microscopy was performed at 5 kV on a JEOL 5300 scanning electron microscope. For transmission electron microscopy (EM), dried, bleached spicules were fixed in 2.5% paraformaldehyde with 0.1% glutaraldehyde for 30 min. They were then washed three times in distilled water and demineralized in 4% uranyl acetate, pH 4.8 for 3 min. After centrifugation, the pellet was washed again in distilled water, dehydrated through graded ethanol solutions and embedded in LR white resin (Polysciences). Prism stage embryos were similarly fixed in 2.5% paraformaldehyde containing 0.1% glutaraldehyde, then demineralized in 50 mmol/L EDTA, pH 8.0. Embryos were examined under polarized light until all or almost all spicules had disappeared. The samples were then washed, dehydrated and processed into LR white resin. Thin sections (70 nm) were cut on an Ultracut Ultramicrotome and picked up on formvar and carbon coated 200 mesh nickel grids. The sections were blocked with PBS/10% fetal calf serum for 30 min and then floated on drops of primary antibody for 60 min at room temperature. Primary antibodies were mAb 1223 at 1:100 dilution and SM30 diluted 1:10. The grids were washed three times over 15 min in buffer and incubated on the appropriate 5 or 10 nm second antibody gold diluted 1:20 for 60 min at room temperature. The labeled grids were washed three times with the same buffer for 15 min, followed by two washes with PBS for 10 min and extensive washing in distilled water. Sections were stained for 3 minutes in 2% aqueous uranyl acetate, washed in distilled water and air dried. Transmission electron microscopy was performed in a JEOL 1200EX Analytical Electron Microscope operated at 80 kV.

Results and Discussion

SDS PAGE and western blot analysis

When total spicule matrix protein from *S. purpuratus* pluteus stage embryos was separated by SDS PAGE

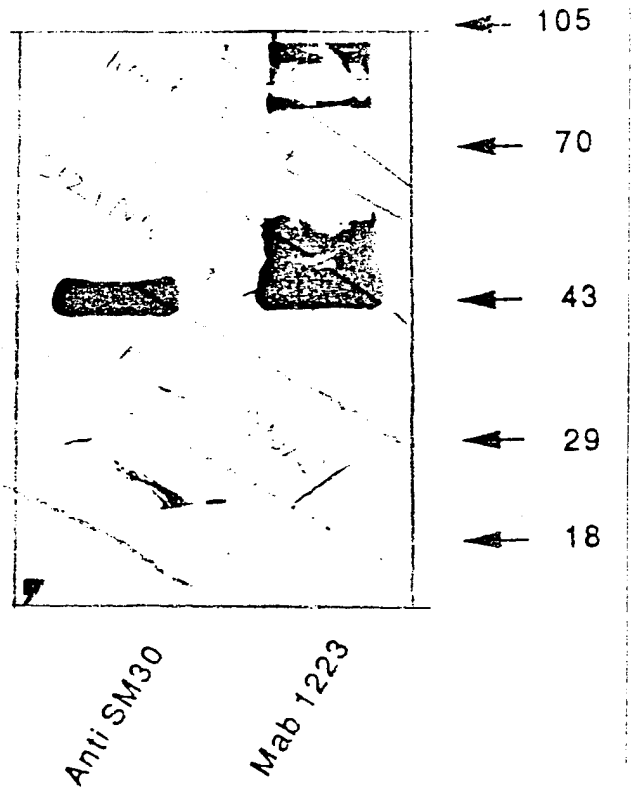

Fig. 1. Comparison of anti-SM30 and mAb 1223-reactive proteins in the matrix of *S. purpuratus* spicules. Total spicule matrix protein (10 µg), prepared from bleach treated spicules as described in Materials and Methods, was subjected to SDS-PAGE followed by western blot analysis using anti-SM30 (1:1000) or mAb 1223 (1:2000) and alkaline phosphatase-conjugated secondary antibody (1:1000).

and then examined by Western blot analysis using anti-SM30 antisera, a protein with an apparent mass of 43 kDa was detected (Fig. 1). When the same samples were analyzed using the monoclonal antibody, mAb 1223, an antibody directed against the oligosaccharide chains on the PM cell-specific protein msp130, a protein with an identical M_r of 43 kDa was also detected. Earlier studies had, in fact, detected cross-reactivity with a 43 kDa protein (Shimizu *et al.* 1988; Shimizu-Nisikawa *et al.* 1990). Although SM30 has a predicted M_r of 30.6 kDa based on the gene sequence (George *et al.* 1991), we have demonstrated that the protein migrates at approximately 43 kDa. In a similar context, we have also demonstrated that when SM30 is expressed in *Xenopus*, a protein with a comparable molecular weight of 46 kDa is synthesized (unpubl. data).

Two dimensional electrophoresis

To confirm the finding that the SM30 protein contained the mAb 1223-reactive epitope, samples of total spicule matrix proteins were subjected to pH gradient electrophoresis in the first dimension and then SDS PAGE in the second dimension followed by western blot analysis using anti-SM30 or mAb 1223. This experiment identified a single protein with a molecular mass of 43 kDa and an approximate pI of 4.0 that reacted with both antibodies (Fig. 2). A number of other proteins which reacted only with mAb 1223, were also detected in the spicule matrix; these probably correspond to the higher molecular weight immunoreactive species detected in the one-dimensional analysis of spicule proteins (Fig. 1).

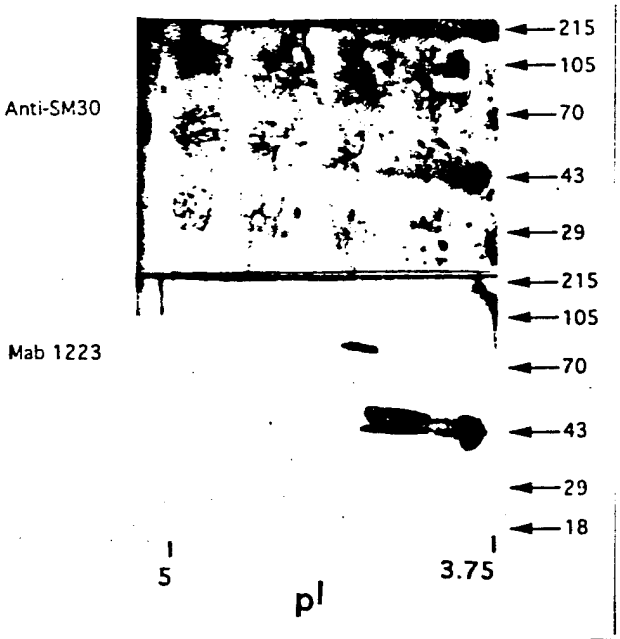

Fig. 2. Two-dimensional electrophoresis of total spicule matrix proteins from *S. purpuratus*. Total spicule matrix protein (10 µg) was subjected to 2-D gel electrophoresis. After probing with anti-SM30 antibody (1:1000), the blot was 'stripped' in 0.0625 mol/L Tris-HCl, pH 6.7, containing 0.1 mol/L 2-mercaptoethanol and 2% SDS at 60°C for 30 min after which ECL substrate was reapplied to ensure complete removal of both primary and secondary antibody. The blot was then reprobed with mAb 1223 antibody (1:2000). Horse radish peroxidase-conjugated secondary antibody (1:1000) was used in both cases.

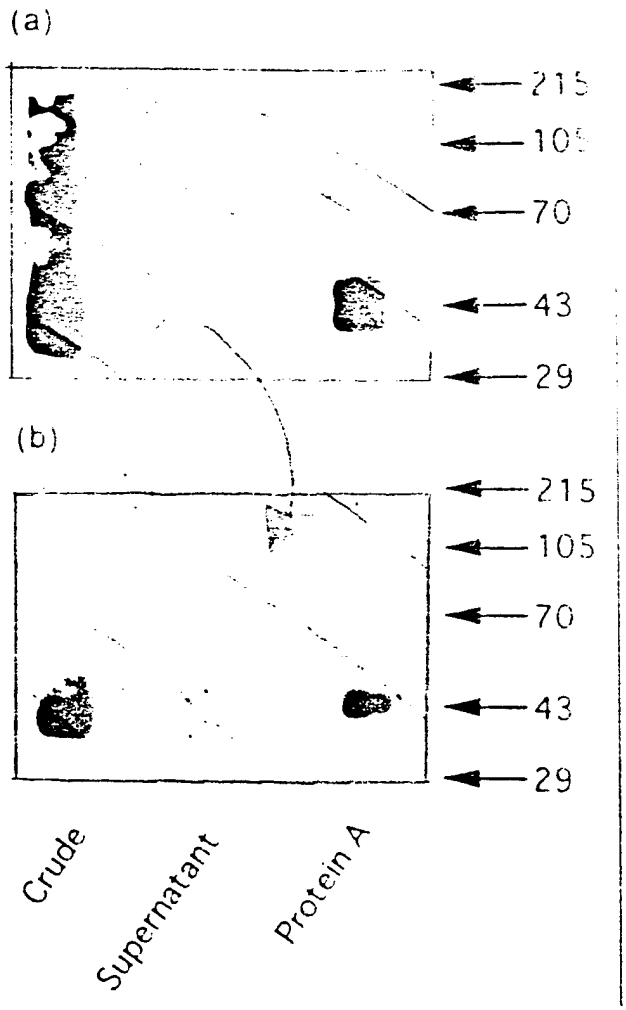

Fig. 3. Immunoprecipitation of SM30 from *S. purpuratus*. Crude total spicule matrix proteins (20 µg, Crude), was reacted with mAb 1223 antibody in TBS overnight at 4°C. The mixture was then incubated with Protein A Sepharose beads for 1 h at 4°C. After removing the supernatant (Supernatant), the beads were washed ten times with TBS and then the immunoprecipitated proteins were eluted from the beads (Protein A) by boiling with Laemmli buffer and subjected to SDS PAGE and Western blot analysis using (a) mAb 1223 (1:1000) and (b) anti-SM30 (1:500) antibodies. Horse radish peroxidase-conjugated secondary antibody (1:1000) was used to visualize the protein bands.

Immunoprecipitation

Immunoprecipitation was carried out as an additional method to verify that the SM30 protein contained the mAb 1223 carbohydrate epitope. The singular advantage of this method was that it was possible to use one antibody, mAb 1223, to precipitate all proteins possessing this carbohydrate epitope, and to use a second antibody, anti SM30, to determine if any of the precipitated proteins contained the SM30 epitope. As shown in Fig. 3, the major 43 kDa protein was removed from the total spicule matrix protein extract by treatment with mAb 1223 followed by Protein A Sepharose. When the bound mixture was eluted from the Sepharose beads and analyzed by western blot, it was shown to react with both mAb 1223 and anti-SM30. This result corroborates the initial finding that the SM30 contains the same or a similar carbohydrate epitope first detected on msp130.

Deglycosylation studies

Having demonstrated that SM30 possessed oligosaccharide side chains similar or identical to those present on the PM cell specific protein, msp130, a sample of total spicule matrix protein was treated with PNGaseF and then subjected to SDS PAGE and western blot analysis. When the treated proteins were probed with anti-SM30 and mAb 1223 antibodies, an identical pattern of immunoreactivity was observed (Fig. 4). A molecular weight shift of approximately 3 kDa occurred after treatment with PNGaseF. This shift was in good agreement with the estimated molecular weight of the mAb 1223-reactive carbohydrate chains of msp130-related proteins (Farach-Carson *et al.* 1989). The slight retention of reactivity to mAb 1223 might be due to incomplete deglycosylation or to the presence of PNGaseF-insensitive immunoreactive carbohydrate chains.

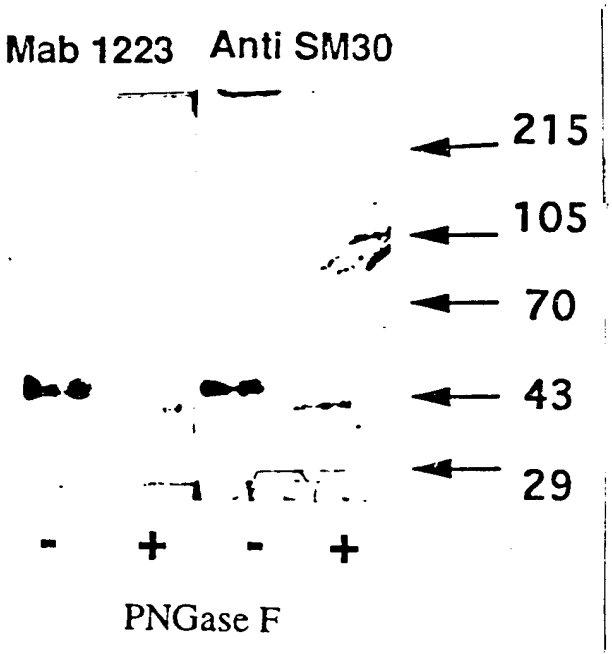

Fig. 4. Deglycosylation of spicule matrix proteins from *S. purpuratus*. Total spicule matrix protein (10 μg) was treated with PNGaseF according to the manufacturers instructions. After SDS-PAGE and western blotting, the proteins were visualized using anti-SM30 (1:1000) or mAb 1223 (1:2000) and alkaline phosphatase-conjugated secondary antibody (1:1000).

Developmental expression of mAb 1223 and anti-SM30 immunoreactivity

When cholate soluble protein extracts of *S. purpuratus* embryos at various stages of development were separated by SDS PAGE and then examined by western blot analysis using the monoclonal antibody mAb 1223, a major band at 43 kDa, as well as other immunoreactive bands, were detected. As shown in Fig. 5a, the 43 kDa protein appeared to be developmentally regulated, being first detectable at approximately 48 h after fertilization (late gastrula, day 2) and reaching a maximum level at the 96 h pluteus stage (day 4). When the same samples were analyzed using the anti-SM30 antiserum, a similar pattern was observed (Fig. 5b). However, the finding of a band in the day 1 preparation (mesenchyme blastula) was surprising because George *et al.* (1991) had previously reported that SM30 transcripts were first detectable at late mesenchyme blastula stage, just a few hours before the onset of gastrulation. It was apparent, however, that the band present in day 1 was slightly but distinctly slower in mobility than that found in later stages. This observation, together with the fact that there was no mAb 1223 immunoreactivity associated with this band in the day 1 preparation (Fig. 5a), indicated that the band detected using anti-SM30 may represent a non-specific, cross reacting protein. For the purposes of comparison a mixture of spicule matrix proteins which was analyzed and found to contain the same anti-SM30 and mAb 1223 immunoreactive protein at 43 kDa is shown in lane S (Fig. 5a,b). A number of lower molecular weight proteins were also found to react with anti-SM30; this probably is the result of limited proteolytic degradation.

Given the interesting possibility that there may be a family of msp130-like proteins associated with the spicule matrix, the embryo extracts were probed with the monoclonal antibody, mAb A3, which is directed against the polypeptide backbone of msp130 (Leaf *et al.* 1987). As shown in Fig. 5c, a 43 kDa protein was not detected by mAb A3 in either the embryo extracts (lanes 0–4) or in the spicule matrix (lane S); the antibody only recognized msp130 (day 2–4). Therefore, it

was clear that although the carbohydrate chain of msp130 was present in the spicule matrix, the polypeptide chain was not.

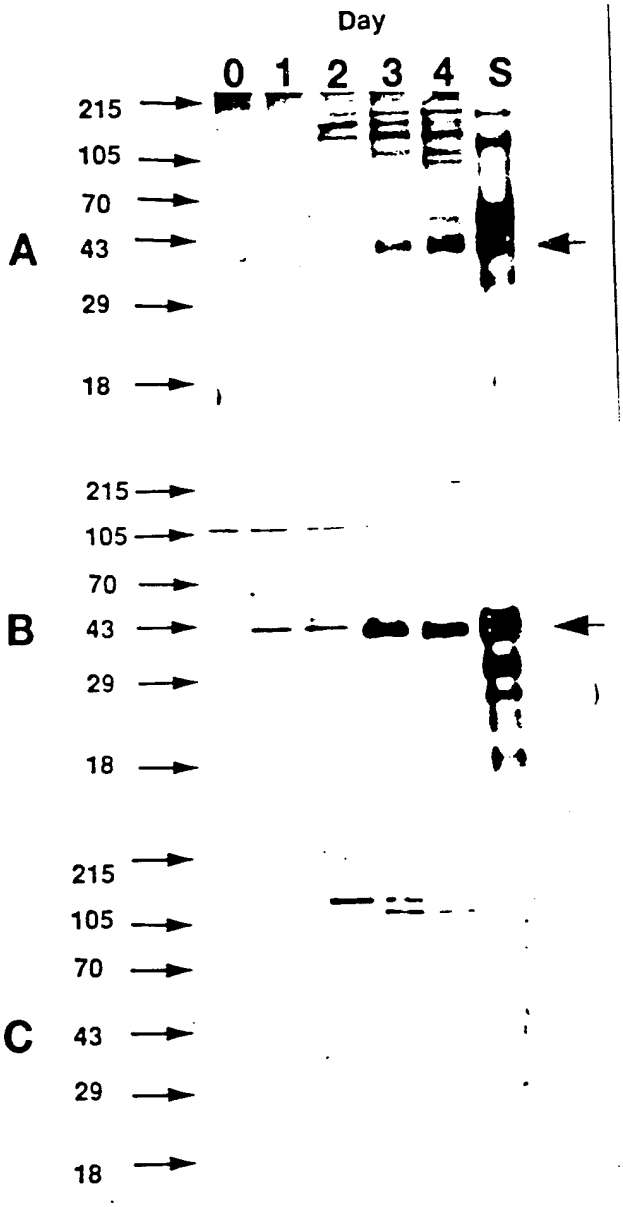

Fig. 5. Developmental expression of mAb 1223-reactive proteins (A), SM30 protein (B) and msp130 (C). Embryo protein extracts (20 µg) were examined by SDS-PAGE and western blot analysis using anti-SM30 (1:1000), mAb 1223 (1:2000) or mAb A3 (1:1000) and alkaline phosphatase conjugated secondary antibody (1:1000). Total spicule protein (10 µg, lane S) was prepared as described in Materials and Methods. Negative controls were carried out using pre-immune serum or omitting the primary antibody. Lane 0, unfertilized egg; lane 1 = 24 h (mesenchyme blastula); lane 2 = 48 h (late gastrula); lane 3 = 72 h, (prism); lane 4 = 90 h, (pluteus).

Distribution of SM30 in PM cells and spicules

The relative amount of SM30 protein in the cholate-solubilized components of the embryos and in the isolated spicule after solubilization with EDTA was compared by utilizing both anti-SM30 and mAb 1223 antibodies. Densitometric scanning was carried out and the relative proportions of anti-SM30 (Fig. 6a) or mAb 1223 (Fig. 6b) reactivity were determined. From these results it was clear that there was little or no SM30 present in the embryo 24 h (day 1, mesenchyme blastula) after fertilization. However, at 48 h (day 2, late gastrula) SM30 was much more abundant, with the majority of it being present in the cholate-soluble cellular fraction. After 72 h (day 3, prism–pluteus), when the embryo contained a much larger endoskeleton, an increase in SM30 released from the isolated spicules

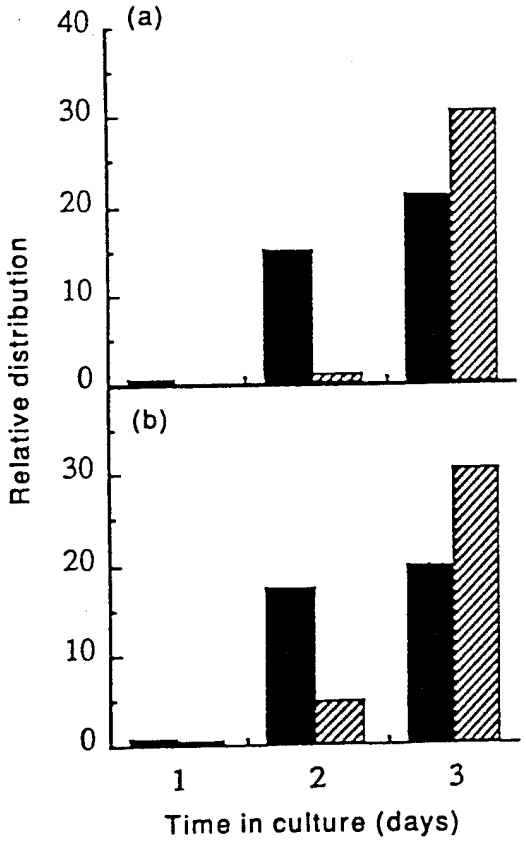

Fig. 6. Relative distribution of SM30 in embryos of *S. purpuratus*. Embryos were first solubilized with Tris-cholate buffer and the soluble proteins analyzed (■). After washing 10 times with distilled water the remaining insoluble material was extracted with EDTA and the EDTA soluble proteins analyzed (▨). Following SDS-PAGE and western blotting with either (a) anti-SM30 (1:500) or (b) mAb 1223 (1:2000) the relative proportion of the 43 kDa detected with each antibody was estimated by densitometry. Day 1 = 20 h, hatched blastula; day 2 = 42 h, mid gastrula; day 3 = 72 h, prism).

The arrows on the right indicate the position of SM30.

by EDTA was observed. These results indicated that SM30, was expected, was first synthesized by the PM cells and presumably then transferred to the growing spicule where it serves an as-yet unknown function in the matrix. The fact that the level of the SM30 in the cell fraction is high compared to its level in the spicule is not surprising, since in nature continuous spicule elongation occurs in the embryo over many weeks. It will be of interest in the future to directly determine the kinetics and path of its movement from the PM cell to the spicule by pulse-chase studies using metabolically labeled SM30.

Ultrastructural distribution of SM30 and 1223 epitopes

Okazaki (1960) showed that if isolated spicules were stripped of surface membranes and then demineralized, a fibrous matrix remained. This finding was confirmed by Benson *et al.* (1983), when they described a 'reticular or fibrous structure of irregular filaments' when isolated, SDS- and urea-treated spicules were demineralized *in situ* on an electron microscope grid. Furthermore, when the isolated spicules were demineralized and then embedded in resin and thin sectioned, Benson *et al.* (1983) observed concentric lamellae of irregular fibrillar material, which they concluded comprised the 'ground substance' of the spicule. We undertook a series of experiments based on these earlier findings.

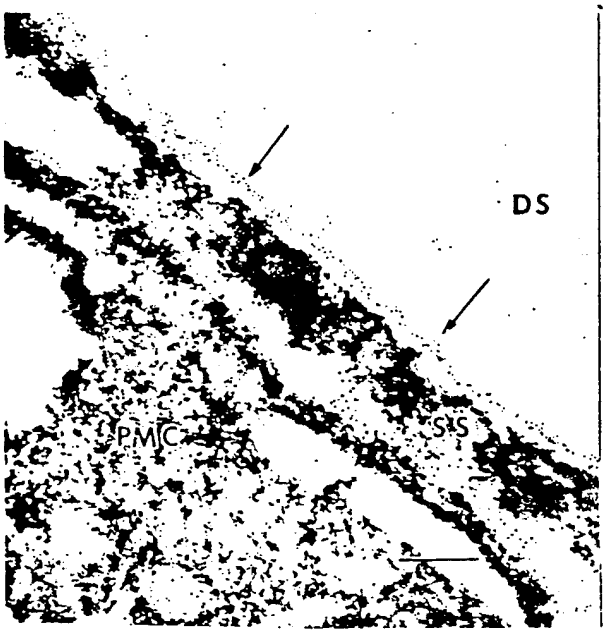

Fig. 7. A demineralized pluteus embryo labeled with mAb 1223 and second antibody gold illustrates heavy labeling of the PM cell (PMC) surface as well as the cytoplasm, the cytoplasmic spicule sheath (SS) and the outer fibrillar layer of the decalcified spicule (DS). Magnification bar = 200 nm.

Prism stage embryos were demineralized by treatment with EDTA and then examined by TEM. A dense meshwork of extracellular, fibrillar material was apparent within the coelomic cavity. Primary mesenchyme cells and their pseudopodia were easily recognizable, as was the cytoplasmic sheath of the demineralized spicules. A portion of a PM cell, the cytoplasmic sheath and the demineralized spicule compartment labeled with mAb 1223 is shown in Fig. 7. It is apparent that the PM cell and sheath surfaces exhibited strong immunoreactivity. Within the demineralized spicule compartment, the antibody gold particles, sometimes distributed as a double layer, clearly demarcated the edge of the spicule, separated by approximately 25 nm from the plasma membrane of the sheath. Scattered clumps of antibody gold appear deeper within the spicule compartment. Antibody to SM30 also labeled PM cell and demineralized spicules, although not as heavily as did mAb 1223 (results not shown).

In order to examine the presence of immunoreactive proteins within the spicule, spicules were isolated and treated with detergent and bleach to remove all surface organic material. The low magnification scanning electron micrograph in Fig. 8 confirmed that all cellular material had been removed from the bleach-treated spicules. As described by Okazaki and Inoue (1976), the spicules had a spongy-appearing granular surface, with occasional fenestra on the long rods. Such preparations were then fixed, demineralized, dehydrated and embedded in LR white resin.

Cross-sectioned 'ghosts' of organic material which remained following dissolution of the calcite, were found to be organized in concentric rings of fine electron-dense fibrillar material, as previously reported (Benson

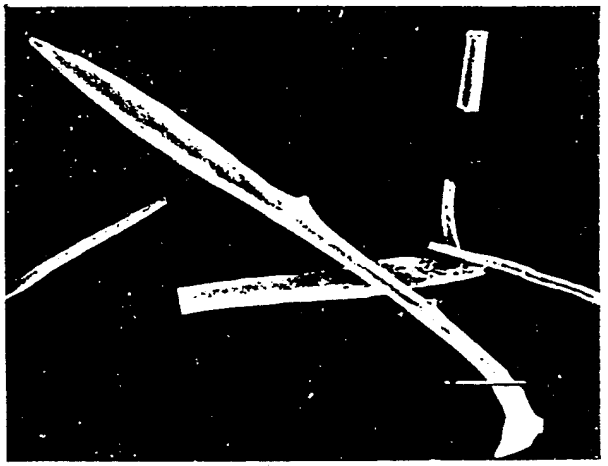

Fig. 8. A scanning electron micrograph clearly shows that no cytoplasmic or membrane components remain associated with the surface of the bleached spicules. Magnification bar = 10 μm.

Fig. 9. Spicules isolated and treated with bleach were fixed, demineralized and then the residual material embedded in resin. Following thin sectioning and immunolabeling, concentric rings that were labeled with mAb 1223, particularly on the double outer layer (arrow), were evident. Magnification bar = 200 nm.

Fig. 10. The fibrous appearing concentric rings of a cross-sectioned demineralized spicule label more heavily on the inner rings than the outer rings with antibody to SM30. Magnification bar = 200 nm.

et al. 1983). However, as shown in Fig. 9 at higher magnification the outer layer was seen to consist of a double layer, the two layers separated by about 20 nm. Membranes and cellular organelles were never seen, indicating that the cytoplasmic sheath and other cellular components had been completely removed by the detergent washing and bleach procedure. Some spicules were not completely demineralized, but retained a calcite core that was displaced during sectioning, leaving a hole in the plastic. As is apparent in Fig. 9, the double outer layer of the spicule labeled heavily with mAb 1223; the inner concentric rings, two to three in number, were also labeled, but to a lesser extent. Antibody to SM30 (Fig. 10) also labeled the outer layers of the spicule ghost, but not as heavily as it labeled the inner concentric rings. Control thin sections in which primary antibody was omitted were not labeled, as was the case using pre-immune anti-SM30 antisera.

Conclusion

These studies establish for the first time that one of the intracrystalline components of the sea urchin spicule is a 43 kDa glycoprotein that is recognized by both the monoclonal antibody, mAb 1223, an antibody generated against the carbohydrate epitope of msp130, and by a polyclonal antibody to the previously described spicule matrix protein, SM30. A variety of experimental procedures were used to validate this conclusion: one and two-dimensional gel electrophoresis, deglycosylation, immunoprecipitation, and immunocytochemistry. In the first three procedures the distribution of SM30 was assessed by western blot analysis with both of the antibodies. In ultrastructural studies antibody gold was used to study the distribution of the two epitopes in the spicule compartment. The monoclonal antibody mAb 1223 heavily labeled superficial lamellae of the cross-sectioned isolated demineralized structures, but anti-SM30 antibody labeled inner concentric lamellae more than the outer ones. The differential labeling observed with these two antibodies could be attributed to the fact that mAb 1223 labeled at least four proteins within the spicule as seen in Figs 1 and 2, whereas anti-SM30 labels only one protein.

Because SM30 was found to contain the mAb 1223 epitope, it was of interest to determine if the polypeptide chain of SM30 was in any way related to that of msp130. However, using another monoclonal antibody, mAb A3, generated to the polypeptide backbone of msp130 (Leaf *et al.* 1987), no immunoreactivity was observed in the spicule matrix sample. Further-

more, the developmental expression of msp130 was found to be very different from that of SM30; it was barely detectable at 24 h, peaked at 48 h and gradually declined by pluteus stage at 96 h (Kabakoff *et al.* 1992). In contrast, SM30 reached a maximum level at the pluteus stage of development. This developmental protein pattern is similar to the SM30 mRNA expression pattern seen by George *et al.* (1991). All of these results indicated that the only feature that SM30 and msp130 have in common is the oligosaccharide chains that react with the same monoclonal antibody, mAb 1223.

It has already been established that mAb 1223 blocks spicule formation (Carson *et al.* 1985), as well as deposition of radioactive calcium into growing spicules (Farach-Carson *et al.* 1985). Furthermore, inhibitors of oligosaccharide chain processing, an event prerequisite to formation of complex chains, block the formation of spicules by cultured primary mesenchyme cells (Kabakoff & Lennarz 1990). This finding, coupled with earlier studies on the effects of a more general inhibitor of glycosylation, tunicamycin, all point toward an essential role of the mAb 1223-reactive oligosaccharide chains in spiculogenesis. Although some preliminary experiments have shown that $^{45}Ca^{2+}$ binds to one or more of the intracrystalline spicule proteins (Benson *et al.* 1986), it is unknown which of the proteins is involved. The finding that SM30 in the spicule matrix carries negatively charged, sulfated oligosaccharide chains that react with mAb 1223 suggests that it may function in interacting with Ca^{2+}. Our finding that two other sea urchin species, *Lytechinus pictus* and *Lytechinus variegatus* (data not shown), contain a 43 kDa protein with the same characteristics as that described in this study on *S. purpuratus*, coupled with the fact that SM30 transcripts have been shown to be present in other mineralized tissues (George *et al.* 1991), suggest that this molecule warrants detailed studies with respect to its precise function in the mineralization process.

Acknowledgements

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2. The Defense Technical Information Center received the enclosed report (referenced below) which is not marked in accordance with the above reference.

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N00014-93-1-0229
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