CHARACTERIZATION OF STARFISH YOLK AND CORTICAL GRANULE PROTEINS, AND OF A NOVEL EXTRACELLULAR PROTEOGLYCAN IMPLICATED IN DIGESTIVE TRACT MORPHOGENESIS

By

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ABSTRACT

Extracellular matrix (ECM) is thought to play a major role in morphogenesis by influencing processes such as cell migration and differentiation, although the specific mechanisms involved are poorly understood. This study examined the ECM and egg storage granules of starfish (Pisaster ochraceus) embryos, and attempted to identify components important for digestive tract morphogenesis. Three monoclonal antibodies were developed with specificities for the ECM, yolk and cortical granules in Pisaster eggs and embryos. These antibodies were then used to localize, isolate and characterize the antigens through early development, using immunohistochemistry, immunocytochemistry, immunochemical and biochemical techniques. The first antibody, PM1, binds to a large extracellular proteoglycan, which appears in the blastocoel matrix at mid-gastrulation, and is synthesized only by endodermally-derived tissues. The use of PM1 antibody as a function blocking agent in live embryo cultures suggested that it plays an important role in digestive tract morphogenesis. A second antibody recognizes a protein localized in cortical granules of unfertilized eggs. The majority of these granules are located in the peripheral egg cytoplasm and are released at fertilization. However, a second morphologically identical population of granules remain dispersed throughout the egg cytoplasm, and appear to contribute to ECMs of the developing embryo, including the blastocoel ECM, basement membranes, and the hyaline layer. The function of this protein is currently unknown; however, it has a different storage and secretion profile from the PM1 proteoglycan, suggesting its role in the blastocoel matrix may be different. A third antibody recognizes proteins stored in yolk granules located throughout the egg and cells of the developing embryo, which do not appear to contribute to ECMs during embryogenesis. Partial biochemical characterizations using the anti-yolk antibody revealed that there are several molecular species of yolk proteins present in the oocyte, and that their molecular composition changes during embryogenesis. Depletion of the yolk proteins is not significant until the larval stage, suggesting that they do not play a major role until later in development.
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<tr>
<td>B-xyloside</td>
<td>8-D-xylopyranoside</td>
</tr>
<tr>
<td>BL</td>
<td>basal lamina</td>
</tr>
<tr>
<td>BM</td>
<td>basement membrane</td>
</tr>
<tr>
<td>CAPS</td>
<td>3-(Cyclohexylamino)-1-propanesulfonic acid</td>
</tr>
<tr>
<td>Con A</td>
<td>Concanavalin A</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's modified Eagles medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
</tr>
<tr>
<td>ECM</td>
<td>extracellular matrix</td>
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<tr>
<td>EDTA</td>
<td>ethylenediamine tetraacetic acid</td>
</tr>
<tr>
<td>EGTA</td>
<td>[ethyleneglycol-bis-(β-amino ethyl ether) N,N,N',N'-tetraacetic acid</td>
</tr>
<tr>
<td>Endo F</td>
<td>endoglycosidase F</td>
</tr>
<tr>
<td>FITC</td>
<td>fluorescein isothiocyanate</td>
</tr>
<tr>
<td>GAGs</td>
<td>glycosaminoglycans</td>
</tr>
<tr>
<td>g</td>
<td>gravity</td>
</tr>
<tr>
<td>HAT</td>
<td>hypoxanthine aminopterin thymidine</td>
</tr>
<tr>
<td>HL</td>
<td>hyaline layer</td>
</tr>
<tr>
<td>IgG</td>
<td>immunoglobulin G</td>
</tr>
<tr>
<td>IgM</td>
<td>immunoglobulin M</td>
</tr>
<tr>
<td>Mr</td>
<td>relative mobility</td>
</tr>
<tr>
<td>N-linked</td>
<td>asparagine-linked</td>
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<tr>
<td>O-linked</td>
<td>serine/threonine-linked</td>
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<td>PAGE</td>
<td>polyacrylamide gel electrophoresis</td>
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<tr>
<td>PAS</td>
<td>periodic acid Schiff</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PC3H2</td>
<td><em>Pisaster</em> cortical 3H2</td>
</tr>
<tr>
<td>PEG</td>
<td>polyethylene glycol</td>
</tr>
<tr>
<td>PC3H2</td>
<td><em>Pisaster</em> cortical 3H2</td>
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<tr>
<td>Abbreviation</td>
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<tr>
<td>PH3C8</td>
<td><em>Pisaster</em> hyaline 3C8</td>
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<td><em>Pisaster</em> matrix-1</td>
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<td>PY4F8</td>
<td><em>Pisaster</em> yolk 4F8</td>
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<tr>
<td>PMSF</td>
<td>phenylmethyldisulfonyl fluoride</td>
</tr>
<tr>
<td>PVDF</td>
<td>polyvinylidene difluoride</td>
</tr>
<tr>
<td>PY4F8</td>
<td><em>Pisaster</em> yolk 4F8</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
</tr>
<tr>
<td>TBST</td>
<td>Tris buffered saline, tween</td>
</tr>
<tr>
<td>TEM</td>
<td>transmission electron microscopy</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris buffered saline</td>
</tr>
<tr>
<td>VBS</td>
<td>veronal buffered saline</td>
</tr>
<tr>
<td>Vtg</td>
<td>vitellogenin</td>
</tr>
<tr>
<td>WGA</td>
<td>wheat germ agglutinin</td>
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I would like to gratefully acknowledge the continued support of my supervisor, Dr. Bruce Crawford. His guidance, encouragement and demand for excellence in the lab and at the desk has made my graduate education second to none. I feel very privileged to have had him as a mentor and friend over the years. I would also like to thank the members of my thesis supervisory committee, Drs. Wayne Vogl, Nelly Auersperg and Ravi Shah, for their commitment to help me through my Ph. D., offering guidance, critical evaluation of my work, and encouragement. The department of Anatomy at UBC has been a home to me for several years, and I would like to thank all its members, past and present, for making it a truly remarkable place to be. I am especially thankful for the leadership of Dr. Charles Slonecker, who was department head for most of my years there. His support of the graduate student program and enthusiasm in teaching will not be forgotten. Special thanks also go to people close to me without whose support as friends and colleagues, I most certainly would not have survived the Ph. D. experience, particularly to Zeid Mohamedali, Dave Woods, and Steve Cumming. Finally, I am indebted to my parents, for their love and encouragement over the years, and for believing in the path I have chosen to follow. I could not have done it without them.
I. INTRODUCTION

General Overview

Morphogenesis is the process whereby cells and tissues are organized into highly ordered three dimensional structures that make up an organism. Although it is easy to observe the structural changes that occur as development proceeds, the underlying mechanisms that are responsible for these changes are not readily apparent. During morphogenesis, cells participate in interactions as they organize themselves into the complex patterns characteristic of organs. These interactions which simply put involve cell-cell contact or cell-extracellular matrix (ECM) contact, are necessary for the development and maintenance of proper tissue architecture. Together, these interactions are responsible for directing cell behavior during morphogenesis, which includes adhesion, migration, growth and differentiation, ultimately resulting in the emergence of structure in the embryo. The ECM is a major component in embryonic systems, occupying the vast spaces and cavities that are formed from the blastula stage onwards. ECM provides an environment through which cells can migrate, and a substratum for their adhesion and guidance. In addition, it can affect growth and cell differentiation (reviewed by Hay, 1991; Adams and Watt, 1993; Lin and Bissell, 1993). When investigating the role of ECM in morphogenesis, the developmentally regulated appearance of specific components in the ECM is often taken as an indication that these components have a specific role in the morphogenetic events that are occurring at that time. The object of many studies has therefore been to study components of ECM during development by investigating when they are synthesized, where they are secreted, and what morphogenetic events are occurring during this time. In some cases, components appearing in embryonic ECMs are not synthesized de novo, but are derived from maternal stores of the oocyte. Questions then arise as to when, where, and how these components are stored, and how their deployment to the ECM regulated. In many cases, it is unclear whether ECM components that are stored in yolk and other granules of the oocyte play a different role than ECM components that are synthesized during early development (Alliegro et al., 1992).
Investigations into the details of the mechanisms by which ECM influences cells to form complex tissues are no simple task. In most cases, these processes occur inside opaque embryos so that it is very difficult to observe the events directly. Most research in this field has therefore focused on describing the stages of organogenesis using fixed tissue, or alternatively by showing how the behaviour of particular cells and the molecules they make change under different conditions in tissue culture. While tremendous advances have been made in reconstituting the natural cell-ECM environments using tissue culture, these model systems fall short of the in vivo situation, as the complete repertoire of ECM proteins and the factors they bind to are still not known (Passaniti, 1992). In this respect, there has been more and more attention devoted to studying the mechanisms of cell-ECM interactions in invertebrate organisms, since they contain many of the ECM elements found in vertebrates (reviewed in Har-El and Tanzer, 1993), and since these elements are sometimes easier to study in morphologically simpler organisms, which are often translucent and thus permit the visualization of morphogenesis in vivo. In the present study, efforts were made to investigate cell-ECM interactions involved in early morphogenesis of the starfish *Pisaster ochraceus*. As little is known about the characterization of the ECM components in this organism, I choose to approach these studies by developing monoclonal antibodies against components of the embryonic ECM, which in some cases were also localized in egg storage granules. In this way, these components could be identified, characterized and their tissue distributions mapped through embryogenesis. There are two main areas of work which resulted from the investigations, and they are presented in distinct sections throughout the thesis. Part A documents the work resulting from a monoclonal antibody against an extracellular proteoglycan of the embryo and early larvae, while Part B represents the work resulting from 2 antibodies generated against components of the embryo that were also localized in storage granules of the oocyte.
PART A: The PM1 proteoglycan in starfish gut morphogenesis

1. Overview

During embryonic development, epithelial rearrangements result in the formation of cavities, which allow space for the migrations of mesenchyme cells, as well as for movements and foldings of epithelial sheets. Extensive work on many different species has shown that these cavities are filled with an extracellular matrix (ECM), composed mainly of collagens (types I and II fibrillar, and type IV basement membrane), non-collagenous glycoproteins (fibronectin, laminin, and tenascin) and proteoglycans (heparin sulfate, chondroitin sulfate). Additional proteins include nidogen, and thrombospondin (reviewed by Hay, 1991). These components have binding sites for each other and are capable of forming an interlacing network or web of molecules (reviewed by Hardingham and Fosang, 1992; Adams and Watt, 1993). That the ECM plays a key role in interactions which occur during morphogenesis, such as providing a mechanical substratum upon which epithelial and mesenchymal cells can adhere and migrate, has been suggested by several investigators (Grobstein, 1954; Pierce, 1966; Hay, 1981; Thiery et al., 1983; Ekblom et al., 1986). However, the mechanisms involved in cell-ECM interactions have remained largely a mystery until recently, when cell-binding sites within individual ECM components and specific cell surface receptors have been identified (reviewed by Hynes, 1992). Of all the ECM components, proteoglycans are among the most poorly understood in their capacity to affect cell behavior during morphogenesis. Because of their structural complexity, the functional domains of individual proteoglycans have been fairly difficult to study and characterize, and therefore much more has been learned of the role of ECM glycoproteins such as fibronectin and laminin in morphogenesis. However, it is clear that proteoglycans play an important role in morphogenesis, and with the recent advent of molecular biological techniques and domain-specific antibodies, this field is rapidly progressing. In this section, proteoglycans will be introduced primarily as macromolecules of the extracellular environment, and I will discuss what is known of their role in developmental processes. In addition, the starfish model system that was used to study proteoglycan function during development will be introduced.
2. Proteoglycans

(a) Structure of proteoglycans

Proteoglycans are present in phylogenetically diverse species, being abundant in sponges, the most ancestral of the known metazoan animals (Misevic and Burger, 1990), as well as in sea cucumbers (Vieira and Mourao, 1988; Kariya et al., 1990), fruit flies (Brower et al., 1987) and cockroaches (Carbonetto et al., 1983), in addition to vertebrates. They are structurally very complex, in that each contains a core protein with one or more covalently bound glycosaminoglycan (GAG) chains. GAGs are linear polymers of repeating disaccharides that contain one hexosamine and a carboxylate and/or a sulfate ester. There are four classes of GAGs, and their chemical structures are summarized in Fig. 1 (Wight et al., 1991). These include hyaluronic acid, chondroitin sulfate and its epimerized homologue, dermatan sulfate, keratan sulfate, and heparan sulfate/heparin. Except for hyaluronic acid, all GAGs are synthesized covalently bound to a core protein. Their attachment to the core protein occurs through a linkage region in a sequence which consists of a xylose, galactose, galactose and uronic acid residue, followed by the repeating disaccharide units that make up the GAG chain proper.

The core proteins of about 20 proteoglycans have been sequenced and they have been given names which often reflect their biological activities (e.g. aggrecan, which facilitates aggregation of cartilage matrix components) (reviewed by Kjellén and Lindahl, 1991). These proteoglycans are distributed at various locations in tissue, some occurring in intracellular locations, and others in cell surface or extracellular matrices. Those secreted and deposited into the ECM include aggrecan, versican, decorin, biglycan, fibromodulin, and in general are rich in chondroitin and/or dermatan sulfate residues. The basement membrane proteoglycans, also a secreted ECM, are characteristically rich in heparan sulfate. A second class include cell surface proteoglycans, which may be anchored to the plasma membrane through a hydrophobic peptide domain (fibroglycan, syndecan, betaglycan, thrombomodulin, and CD44), by a lipid anchor (glypican) or by association with other membrane proteins (invariant chain). These cell surface proteoglycans are also rich in heparan sulfate residues. A third class resides intracellularly in secretory
Fig. 1. Chemical structures of glycosaminoglycans

In this figure, the repeating disaccharide backbone structure of the four classes of GAGs are shown (from Wight et al., 1991).

(A) Hyaluronic acid (HA), also known as hyaluronan or hyaluronate, has the simplest GAG structure, consisting of an alternating polymer of N-acetylglucosamine and glucuronic acid. A single molecule can have a molecular weight of up to 10 million, which corresponds to approximately 25,000 repeat disaccharides. HA is the only GAG which is not synthesized covalently bound to a protein core, nor is it sulfated.

(B) Chondroitin sulfate (CS)/Dermatan Sulfate (DS). CS has the same basic backbone structure as HA but with N-acetylgalactosamine replacing the N-acetylglucosamine. Individual chains are seldom more than 100 kDa, corresponding to approximately 250 repeat disaccharides. The most common sites of sulfation are the 4 and 6 positions of the N-acetylgalactosamine residue (Fig. 1B, dotted and solid arrows). Dermatan sulfate, the epimerized form of CS, in which the D-glucuronic acid is converted to L-iduronic acid, can be sulfated at position 2 (Fig. 1B, asterisk).

(C) Keratan sulfate (KS) consists of a backbone structure with alternating N-acetylglucosamine and galactose residues. Individual chains are seldom more than 40 kDa, which corresponds to approximately 80 disaccharide repeats.

(D) Heparan sulfate (HS) and Heparin have the same backbone structure, that of repeating N-acetylglucosamine and glucuronic acid, which differ from the other GAGs in their α1,4 linkage. Individual chains are usually below 50 kDa. Less than 50% of the N-acetyl groups are converted to N-sulfates in HS whereas usually 70% or more are converted in heparin.
A. Hyaluronic Acid
-1,4-glcUA-β-1,3-glcNAc-β-

B. Chondroitin/Dermatan Sulfate
-1,4-glcUA-β
-1,4-idoUA-α→1,3-galNAc-β-

C. Keratan Sulfate
-1,3-gal-β-1,4-glcNAc-β-

D. Heparan Sulfate/Heparin
-1,4-glcUA-β
-1,4-idoUA-α→1,4-glcNAc-α-
granules (serglycin, chromogranin A proteoglycan) (Kjellén and Lindahl, 1991; Gallagher, 1989; Esko, 1991). Although many other proteoglycans exist, cDNA or genomic clones of the core proteins have not yet been reported, and they are therefore referred to on the basis of their GAG content.

(b) Functions of proteoglycans

The structural complexity of proteoglycans arises from the fact that these macromolecules have different core proteins and different numbers and lengths of individual GAG chains. Some also contain more than one type of GAG chain; for example, syndecan, aggrecan and serglycin. Yet other contain asparagine (N)-linked oligosaccharides typical of glycoproteins, and serine/threonine (O)-linked glycans found in mucins, in addition to their extensive GAG chains (reviewed by Jackson et al., 1991). These structural complexities enable proteoglycans to take part in a wide variety of biological functions, only a few of which are understood. Some of these include:

(1) Providing resilience to tissues. GAG chains, with their highly charged sulfate and carboxylate groups dominate the physical properties of the protein to which they are attached. Proteoglycans in the ECM thus function physically as creators of a water-filled compartment; their high fixed negative charge attracts counter ions, and the osmotic imbalance caused by a local high concentration of ions draws water from the surrounding areas (Hardingham and Bayliss, 1990).

(2) Storage sites for growth factors. Several proteoglycans have been shown to bind growth factors, including those with chondroitin sulphate chains, which bind to Platelet factor 4 (Périn et al., 1988), as well as betaglycan and decorin, which bind TGF-β (Andres et al., 1989; Yamaguchi et al., 1990). This suggests that proteoglycans are indirectly able to modulate the activity of growth factors, and may provide local tissue-bound reservoirs of growth factors. For example, in Chinese hamster ovary cells, the binding of TGF-β by decorin directly neutralizes the activity of the growth factor (Ruoslahti and Yamaguchi, 1991).
(3) **Matrix organizers.** It is likely that proteoglycans play a role in the assembly of other ECM components, and there are several indications that proteoglycan-collagen interactions are important in the regulation of collagen fibrillogenesis and matrix assembly (Hascall and Hascall, 1981; Hardingham and Fosang, 1992). Studies have shown that when corneal dermatan sulfate proteoglycan is biochemically altered, the organization of corneal stroma was disrupted, including focal alterations in collagen fibril packing and a disruption of lamellar organization (Hahn and Birk, 1992). Proteoglycans also link other matrix proteins together, and are often found as a component of ECM networks. The large cartilage proteoglycan aggrecan exists as huge multimolecular aggregates comprising numerous proteoglycan monomers which are non-covalently bound to hyaluronan and are stabilized by a glycoprotein (link protein) (Hardingham and Bayliss, 1990).

(4) **Substratum for migrating cells.** Several studies indicate that extracellular and cell surface proteoglycans are important for cell migration. This is supported by the findings that migrating endothelial cells exhibit increased chondroitin sulfate and dermatan sulfate proteoglycan synthesis as compared to sessile cells *in vitro* (Kinsella and Wight, 1986). Another proteoglycan Perlecan, which is associated with laminin in the basement membrane, has been shown to promote neurite outgrowth (Hantaz-Ambroise *et al.*, 1987). In addition, the use of mutant Chinese hamster ovary cells that are defective in GAG synthesis have provided insight into the mechanisms of cell surface GAG chains in adhesion. Studies have shown that cell surface heparin sulfate proteoglycans can mediate attachment to type V collagen, thrombospondin and fibronectin (reviewed by Esko, 1991). Several cell surface vertebrate proteoglycans have also been implicated with cell migration, including those of the syndecan family, thrombomodulin, and CD44 (Hardingham and Fossang, 1992). In addition, primary mesenchyme cell migration in sea urchin embryos is blocked when proteoglycan synthesis is disrupted (Akasaka *et al.*, 1980; Solursh *et al.*, 1986). This could be due to a disruption of a chondroitin sulfate/dermatan sulfate proteoglycan present on sea urchin primary mesenchyme cells, which has been implicated with cell migration (Lane and Solursh, 1991).
(5) **Growth factor activity.** Epidermal growth factor-like motifs in some proteoglycans (i.e. aggrecan) may function in modulating the proliferative and metabolic activities of chondrocytes and fibroblast (Hardingham and Fosang, 1992), as similar functions have been attributed to glycoproteins such as laminin and tenascin bearing these motifs (Engel, 1991).

(c) **Proteoglycans in development**

Proteoglycans as a class of macromolecules have been identified and studied in a variety of developmental systems, although clear evidence for the functions of specific proteoglycans in developmental processes remains sparse. Several experimental approaches have been used to determine the roles that proteoglycans play in development. The most common of these involves the examination of temporal and spatial changes in proteoglycan composition and/or distribution during development. Several different systems have been investigated, including human bone formation, in which the distribution of biglycan and decorin were studied using antibodies and cDNA probes (Bianco et al., 1990); chick neural crest development, in which antibodies were used to study the distributions of cytotactin and its chondroitin sulfate proteoglycan ligand (Tan et al., 1987; Hoffman et al., 1988; Perris et al., 1991); and rat skin formation, in which biochemical quantifications of chondroitin sulfate proteoglycan revealed a striking decrease in concentrations during the transition from the fetus to the newborn (Habuchi et al., 1986).

Other studies aimed at understanding the role of proteoglycans in development have involved physically changing their properties and then examining the developmental consequences of these alterations. The most common of these have involved the use of the chemical β-D xyloside, which inhibits normal synthesis of those proteoglycans with O-linked xylose-mediated GAG chains (all but keratin sulfate proteoglycans). The β-xyloside competes with core protein xylosides for GAG chain attachment, thereby creating a free xyloside-GAG molecule (Okayama et al., 1973; Galligani et al., 1975). Numerous studies have examined the effects of β-xyloside on developmental processes, including chick feather formation.
(Goetinck and Carlone, 1988), murine renal development (Platt et al., 1987; Lelongt et al., 1988), sea urchin mesenchymal migration (Solursh et al., 1986; Lane and Solursh, 1991) and avian corneal stroma (Hahn and Birk, 1992). Although there is no typical morphogenetic response to this treatment, development and/or matrix structure is affected in all cases, which suggests that proteoglycans play an important role in development.

In echinoids, proteoglycan function has also been studied by culturing the embryos in sulfate-free sea water. Since all GAGs with the exception of hyaluronic acid contain sulfate groups, this treatment is thought to alter the normal synthesis of proteoglycans. Sulfate deprivation results in the reduction in a 15-30 nm diameter granular component present in the blastocoel and basal lamina of sea urchin embryos (Katow and Solursh, 1979). Scanning electron microscopical investigations of sulfate-deprived embryos have also revealed an inhibition of pseudopodia formation by mesenchyme cells and abnormal arrangements of cells in the blastocoel (Akasaka et al., 1980), suggesting that some sulfated glycoprotein or proteoglycan conjugates are responsible for normal gastrulation and mesenchyme cell migration in sea urchin embryos (Katow and Solursh, 1981; Akasaka and Terayama, 1983; Venkatasubramanian and Solursh, 1984). Although these experiments provide information about sulfated glycoconjugates during development, they do not provide information about specific proteoglycans. One way in which single proteoglycans can be targeted is by using monospecific antibodies, which can be used to block the function of proteoglycans in vivo. This approach has been used often to study the functions of glycoproteins in development. For example, in early amphibian embryos, fibronectin appears as a component of loose fibrillar matrix which underlies the blastocoel roof during early gastrulation. This matrix network has been shown to be the substratum for migrating involuting mesodermal cells, and if the cells are deprived of this contact with antibodies directed against fibronectin, the embryos respond with a collapse and wrinkling of the blastocoel roof, which results from an inhibition of mesoderm migration (Lee et al., 1984; Boucaut et al., 1984). Studies of this nature have also been carried out in sea urchin embryos to examine the function of the major protein of the external ECM, hyalin. After incubation in sea water
containing a monoclonal antibody against this protein, embryos fail to gastrulate, and are inhibited in arm rudiment formation (Adelson and Hymphreys, 1988).

A third approach that has been used to study proteoglycans in development processes has made use of mutations that have occurred in organisms. One of the more extensively studied proteoglycans in this regard is aggrecan, for which several mutants have been described at the level of the core protein, its glycosylation, and sulfation in the chicken, mouse and turkey. These mutants characteristically have cartilaginous rudiments that are reduced in size, with the more severe affects being present in mutations occurring at the level of the core protein or its glycosylation (reviewed by Goetinck, 1985). It thus appears that aggrecan plays an important structural role in the development of cartilage. While studies focused at the level of the gene are quite obviously very powerful, such studies are far less accessible than the more traditional methods. Additionally, the groundwork of identifying and characterizing ECM components has yet to be accomplished in several organisms that are commonly used for studies in development. One of these is the starfish embryo.

3. Starfish embryos: a model system for studying cell-ECM interactions during morphogenesis

Starfish embryos and early larvae offer advantages over many other organisms for the study of cell-ECM interactions during morphogenesis. During most of their early development they exhibit a relatively simple morphology consisting of an ectoderm and endoderm, which are separated by a blastocoel containing mesenchyme cells and an extensive gel-like ECM (Strathmann, 1989; Crawford, 1990). This ECM is rich in alcianophilic fibers (Crawford, 1989, 1990), similar to those described in vertebrate and sea urchin embryos (Endo and Noda, 1977; Katow and Solursh, 1979; Kawabe et al., 1981). When combined with their optical transparency and the fact that, unlike sea urchin embryos, they do not form spicules, the events of morphogenesis are easily visualized in this organism. In addition, they can be raised in large
synchronous cultures to obtain the amount of material required for many biochemical studies. Despite the many advantages of starfish for developmental studies we know relatively little about their ECM components. Lectin labelling studies at the light and transmission electron microscope (TEM) level have shown that this material is rich in carbohydrate moieties, specifically, Con A (Concanavalin A) and Wheat germ agglutinin binding sites (Reimer and Crawford, 1991; Reimer et al., 1992). In addition, observations by Crawford and Crawford (1992) have demonstrated that starfish ECM contains very large (Sepharose CL-2B-excluded) sulfated glycoproteins which do not contain known GAGs typical of vertebrate (reviewed by Hardingham and Fosang, 1992) or other invertebrate (Lane and Solursh, 1991; Kariya et al., 1990) proteoglycans. Although such elements have been identified, neither isolation of individual components nor a functional analysis of the effect of these components on the different morphogenetic events in early starfish development has yet been accomplished.

4 Digestive tract morphogenesis in *Pisaster ochraceus*

Digestive tract morphogenesis in embryos of the starfish, *P. ochraceus*, has been described previously (Crawford and Abed, 1983; Abed and Crawford, 1986), and is summarized in Fig. 2. Briefly, it begins with the onset of gastrulation when ectodermal cells at the vegetal region of the embryo begin to ingress into the blastocoel, forming the archenteron or primitive gut endoderm. As development proceeds, the archenteron elongates and gives rise to the mesenchyme cells, which undergo an epithelial-mesenchymal transition and migrate off the expanded archenteron tip. These mesenchyme cells migrate through the ECM-rich blastocoel and are believed to re-organize as well as synthesize some elements of the matrix (Crawford and Reimer, unpublished observations). As the archenteron continues to elongate, a blister of basal lamina (BL) is formed at its tip, which then extends over to and fuses with the BL of the presumptive stomodeal ectoderm. The newly formed tube of BL acts as a conduit along which endoderm and stomodeal cells appear to migrate, leading to
Fig. 2. The early development of *Pisaster ochraceus*

(A) Embryos form a hollow blastula that is enveloped in a fertilization membrane at 24 hours post-fertilization, when grown at 12° C.

(B) Gastrulation begins in the hatched blastula, with an invagination of cells at the vegetal region that form the archenteron (a) or primitive gut endoderm.

(C) At 3 days post-fertilization, mesenchyme cells (mc) form off the expanded tip of the archenteron and migrate through the blastocoel (b).

(D) The archenteron continues to elongate, and a blister of basement membrane (bm) forms at its tip; although the basement membrane is not visible, its position can be inferred from the presence of scattered cells on its endodermal surface (D, E-arrows). Concurrently, the archenteron begins to form coelomic pouches (c) at its ends from which a second population of mesenchyme cells emerge.

(E) At a slightly later stage in this side view, while the bm cannot be seen, its position is marked by the flattened surfaces of cells located within it, and extends over to the presumptive stomodeal ectoderm.

(F) The embryo in "E" turned 90° shows both coelomic pouches (c) which are bulging off the side of the archenteron, as well as the well developed blister of bm involved in mouth formation.

(G) Further development involves segmentation of the gut into the esophageal (e) and stomach (s) regions. Some mesenchyme cells (arrows) have settled on the esophagus where they will develop into smooth muscle cells with processes wrapping around the esophagus.

(H) A similar stage as (G), but turned 90°. Note the stomach (s), esophagus (e), and coeloms (c), as well as the mesenchyme cells which are migrating through the blastocoel with processes extended (arrows).

(I) This 8 day-old feeding bipinnaria larvae has a well-developed segmented digestive tract, including a mouth (m), esophagus (e), stomach (s) and intestine (i). Bar = 50 μm.
the formation of a mouth. At the same time, the archenteron forms two pouches at its end, the coeloms, and soon after a second population of mesenchyme cells emerge from this region. These mesenchyme cells appear to represent a distinct population, which migrate through the blastocoel and settle on the esophageal region of the developing gut tube, where they attach to the BL and differentiate into smooth muscle (Crawford, 1990). Further development involves segmentation of the gut into the esophageal, stomach and intestinal regions, and with the formation of the mouth, the feeding bipinnaria larvae is formed. The coelomic pouches detach from the gut and remain suspended in the matrix adjacent to the midline.

5. Statement of the Problem (Part A)

Although digestive tract morphogenesis of starfish development presents an interesting system in which to study cell-ECM interactions for the reasons mentioned above, ECM components of this organism remain uncharacterized. The organization of the matrix does, however, appear morphologically similar to other well characterized matrices, and since several other invertebrates contain ECM components common to many species, it is likely that these components are also present in the starfish embryo. In studying cell-ECM interactions that occur during starfish development, information pertaining to ECM mechanisms in general development can thus be obtained. The primary objective of this work was therefore to isolate and characterized a component or components of the starfish ECM that were specifically involved with digestive tract morphogenesis to attempt to understand the mechanisms by which ECM influences cell behavior during development. As previous attempts at identifying starfish ECM in situ with antibodies directed against vertebrate ECM components failed, it was necessary to use a different approach in identifying and studying starfish ECM. This involved generating starfish-specific monoclonal antibodies against a pool of proteins extracted from embryo homogenates, and then using immunohistochemistry to screen for a specific component of the ECM in the blastocoel. The antibody was then used to describe the synthesis and secretion patterns of the antigen during early starfish embryo
morphogenesis. A further objective was to use the antibody in various biochemical techniques to both isolate and characterize the antigen. And finally, to address the question of whether the ECM component was morphogenetically active during this period in development, the antibody was introduced into living cultures of starfish embryos as a function-blocking perturbation agent.

Part B: Yolk and cortical granules in starfish eggs

1. Overview

The unfertilized oocyte contains various granules that serve as storage sites for proteins, the majority of which are yolk granules or platelets. These granules contain the yolk proteins, which are derivatives of the large precursor, vitellogenin. Vitellogenins are closely related, both functionally and structurally in widely divergent species, including worms, sea urchins, fish, frogs and chickens (reviewed by Byrne et al. 1989).

Although yolk proteins of many different species have been studied and characterized, and their ontogeny, transport and uptake mechanisms into the egg, including receptor characterization is known, the details of how yolk is processed and utilized in embryos during development remain obscure. The function traditionally ascribed to yolk is that it serves as a store of nutrients for embryogenesis by providing raw materials (amino acids, carbohydrates, and lipids) for use by the developing embryo (reviewed by Williams, 1967). However, there is little direct evidence for this proposed function (Byrne et al., 1989). Another population of storage granules in the egg are the cortical granules which, as their name implies, are located in the cortical egg cytoplasm. In many species, these granules undergo exocytosis at fertilization, and the contents are thought to provide a block to polyspermy (reviewed by Cran and Esper, 1990). Other components are poorly characterized and therefore many functions of the cortical granules remain unknown. While proteins are stored in distinct granules of the unfertilized oocyte, in many cases it is unclear what the significance of this discrete packaging is, and several types of storage granules have yet to be investigated thoroughly and compared through phylogeny. There have been reports that
protein stored in yolk and cortical granules contribute to ECMs of the early embryo (Outenreath et al., 1988; Gratwohl et al., 1991), although literature is sparse and in some cases contradictory. In the present study, an attempt was made to identify yolk and cortical granules components in the starfish and to study their course in early development in order to try to come to a better understanding of their functions.

2. Yolk proteins

Yolk proteins are abundant in the unfertilized oocyte, and are stored in yolk platelets or granules which often comprise the bulk volume of an egg. During embryogenesis in oviparous species, it is thought that these proteins eventually undergo catabolism to provide raw materials, such as amino acids, carbohydrates and lipids, for use by the developing embryo, although very little is known about the utilization of yolk material (Schechtman, 1956; Deuchar, 1963; Mancuso, 1964). The majority of proteins stored in the yolk are phosphoglycoproteins, and are derived from the large precursor vitellogenin (Pan et al., 1969; Wahli, 1988). In oviparous animals, these proteins are thought to function much like the milk nutrient proteins in mammals or the storage proteins of plant seeds (Anderson, 1974). The amount of yolk present in eggs varies depending on the development strategies or organisms. For example, in many invertebrates and lower chordates (tunicates) the eggs are smaller and contain a moderate proportion of yolk, (approximately 30% of the total egg volume) (Harvey, 1956). These animals rely on other sources of nutrition in the early stages of development, and are observed to form a larval stage which can feed itself fairly rapidly. Development then continues from this mobile self-feeding form (Postlethwait and Giorgi, 1985; Strathmann, 1987). Other organisms such as mammals form a placenta which supplies food and oxygen for the embryo during its long gestation, and therefore their yolk requirement is small. Amphibian eggs contain much more yolk than do eggs of Amphioxus or the echinoderms, however these embryos still develop a feeding larval stage which is efficient in obtaining food (Karasaki, 1963). On the other extreme are the eggs of fishes, reptiles, birds, and most smaller starfish, in which the majority of the egg volume is yolk. As these animals develop either without a feeding
larval stage or placental attachment, they require a sufficient amount of yolk for nourishment throughout development.

3. Vitellogenesis

Vitellogenesis refers to the synthesis and accumulation of yolk proteins in the growing oocytes (Wahli et al., 1981). As mentioned earlier, vitellogenins are closely related, both functionally and structurally in widely divergent species, including worms, sea urchins, fish, frogs and chickens (reviewed by Byrne et al. 1989). In all species, vitellogenin synthesis occurs in specific tissues, although this varies among species. Vitellogenin is expressed in the intestine of the nematodes (Kimble and Sharrock, 1983), the intestine and gonads of echinoderms (Shyu et al., 1986)\(^1\), the fat-body of female insects (Bownes, 1986)\(^2\), and the liver of vertebrates (Bergink and Wallace 1974; Wang and Williams, 1982; Wahli et al., 1981). Common to all these major sites of synthesis are their endodermal origin.

Vitellogenin expression occurs as a specific response to hormones, specifically estrogen in vertebrates, juvenile hormone in most insects, or ecdysone in some dipterans (Wang and Williams, 1982; Wahli and Ryffel, 1985). Often, a complex cascade of hormonal interactions is involved in the pathway; for example, in the amphibian during mating season, the hypothalamus secretes gonadotrophic releasing hormone, which acts on the pituitary to secrete gonadotrophic hormones. These stimulate follicle cells to secrete estrogen, which then act on the liver to activate the synthesis and secretion of vitellogenin. The estrogen induces vitellogenin at both the transcriptional and translational levels, and stabilizes the vitellogenin mRNA, increasing its half-life from 16 hours to 3 weeks (reviewed by Maller, 1985).

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\(^1\)Echinoderms appear to be unique in that at least in the sea urchin, Vtg is synthesized by both sexes and not only in the intestine but also in the gonads.

\(^2\)Drosophila yolk proteins are synthesized in the ovary in addition to the fat body.
Following synthesis, the vitellogenins are secreted into the body fluid, blood, hemolymph or coelomic fluid, depending on the organism, and are then transported to the ovary where they are taken up by the developing oocyte. This involves the clustering of proteins to specific receptors on the egg plasma membrane to form coated pits, which are then endocytosed to become coated vesicles (Goldstein et al., 1979). In the bird, amphibian, fish and insect, it has been shown that the coated vesicles deliver the vitellogenins to yolk granules where they are stored for utilization later on in embryonic development (reviewed by Byrne et al., 1989).

After synthesis in the rough endoplasmic reticulum, vitellogenins generally undergo various post-translational modifications (e.g. glycosylation, phosphorylation, proteolytic cleavage) prior to their secretion, during their transit to the oocyte, and/or following their selective uptake (Wang et al., 1983; Sharrock 1984). Once in the oocyte they are cleaved, usually via acid hydrolases such as β-cathepsins, to form the mature yolk proteins of the egg (Medina et al., 1988). The proteolytic products vary among some species in both number and size. In vertebrates, vitellogenin is split into two smaller proteins: the heavily phosphorylated phovitin (30-35 kDa) and the lipoprotein lipovitellin (120 kDa) (Wiley and Wallace, 1981). The former has a highly unusual composition, in that more than half of its residues are serine residues, most of which are phosphorylated. These two proteins are packaged together into membrane-bounded yolk platelets (reviewed by Byrne et al., 1989). Invertebrate mature yolk proteins generally do not contain these phovitins (Tirumalai and Subramoniam, 1992; Martinez and Wheeler, 1991; Scott and Lennarz, 1989), and thus there is an interesting evolutionary pattern which exists and because of it, vitellogenin has been used extensively for the study of the molecular processes of evolution.
4. Yolk proteins of echinoids

Information on the protein composition of yolk or of its utilization is non-existent in the starfish. However, yolk proteins of the sea urchin, a closely related species have been studied rather extensively over the past 10 years. In the sea urchin Strongylocentrotus purpuratus, a vitellogenin of 155 kDa is synthesized and post-translationally modified to a 195 kDa form in cells of the adult intestine (Shyu et al., 1986) and coelomocytes (Harrington and Ozaki, 1986); it is then secreted into the coelomic fluid, and taken up by the ovary and the oocytes. In the oocyte, the vitellogenin is further modified to a mature 160-180 kDa yolk glycoprotein, where it has been localized to yolk platelets using immunogold electron microscopy (Harrington and Easton, 1982; Shyu et al., 1986; Scott and Lennarz 1989). Scott and Lennarz (1989) used a monoclonal antibody raised against the 90 kDa protein from S. Purpuratus to test for cross-reactivity to other species, including those from the Asteroids, Ophiuroids, and Holothuroids as well as widely divergent species such as chicken, Xenopus laevis, and Drosophila. While the antibody cross-reacted with all sea urchin species studied, in no case did the polyclonal antibody recognize any glycoproteins of the other species. This indicates that although yolk proteins are found in many different species throughout the animal kingdom, they contain epitopes which are highly specific among members of a phylogenetic class.

5. Fate of the yolk proteins in sea urchins

In many different sea urchins examined, the general pattern which has been observed is a disappearance of the major 160-180 kDa yolk protein of the egg during embryogenesis with the concomitant appearance of glycoproteins having lower molecular masses. In S. purpuratus, these glycoproteins have molecular masses of 115, 108, 90, 83 and 68 kDa. In addition, a homologous set of yolk glycoproteins with similar molecular masses in the embryos of the sea urchins Arbacia punctulata, L. pictus, Hemicentrotus pulcherrimus, Anthocidaris crassispina, and the sand dollar Dendraster excentricus
were identified (Ozaki, 1980; Yokota and Kato 1988; Scott and Lennarz, 1989). Studies have found that these lower molecular mass proteins are in fact derived from the major vitellogenin by a process involving limited proteolysis (Kari and Rottmann, 1985). The initiation of this step-wise proteolysis occurs at different stages of development in the sea urchin, for example proteolysis is initiated at the early blastula stage (12 hours) in *S. purpuratus*, whereas in *L. pictus*, it is initiated at the gastrula stage (48 hours) (Scott and Lennarz, 1989).

Several observations have indicated that the step-wise proteolysis of sea urchin vitellogenin occurs via the actions of a cathepsin B-like enzyme, which is activated by acidic conditions (Yokota and Kato, 1988; Mallya et al., 1992). There is a transient acidification of the yolk platelets, which is sufficient to activate the cathepsin-B enzyme in both *S. purpuratus* and *L. pictus* (Mallya et al., 92). In addition, inhibitors of cathepsin B activity have been shown to block the degradation of the major yolk protein in the sea urchin *S. purpuratus* (Mallya et al., 1992). And finally, a partial purification of a cathepsin B-like enzyme from sea urchin eggs has been reported (Okada and Yokota, 1990).

6. Functions of the major yolk proteins in the sea urchin

(a) *Nutrient store*

As with other organisms, the precise function of sea urchin yolk proteins in embryos during early development is unknown, although they are believed to provide an energy source for use during early development. A puzzling observation is that despite changes in molecular masses of yolk proteins, no decrease in the amount of yolk protein in the yolk granules is detected over the course of development. Furthermore, the chemical composition of yolk platelets in terms of phospholipid, triglyceride, hexose, sialic acid, and RNA, remains unchanged from the zygote to the pluteus larva (Armant et al., 1986). For example, the embryo makes the 84 and 65 kDa proteins from the 180 kDa protein without using them for nutrition during its non-feeding (pre-larval) stages (Armant et al., 1986). Thus, while the constituents of
the yolk granules are proteolytically processed but not subsequently degraded to free amino acids to replenish the cellular pool, their possible function remains unclear. A similar situation has been observed in frogs, where the yolk platelets remain in much the same condition until their disappearance at the larval stage (Karasaki, 1963). Additionally, in some insects, yolk proteins are not depleted until just before hatching (McGregor and Loughton, 1974).

To explain these observations, it has been proposed (Armant et al., 1986) that in the sea urchin, these proteins may be used as emergency stores of nutrients for the feeding pluteus larva. The proposition, however, was challenged by (Scott et al., 1990), who showed that starved larvae exhibited only a slight reduction in the amount of yolk glycoproteins and were stunted as compared with larvae given an external food source, which developed normally and showed a significant decrease in the level of yolk glycoproteins. Thus, it appears that the yolk may not be serving as a reserve store for larvae, but rather its utilization appears to be dependent on conditions of growth.

(b) Extracellular functions

There is a growing body of evidence to suggest that yolk proteins become incorporated into membranes and other extracellular structures of early embryos. One such protein which has been identified recently is the sea urchin glycoprotein toposome (Noll et al., 1985; Mantranga et al., 1986). It is one of the major components of yolk granules in the sea urchin, and is present as a 22S glycoprotein complex, consisting of 6-160 kDa subunits which are proteolytically processed during development into fragments ranging from 70-155 kDa. In the egg, toposome is stored in yolk granules and in the central electron-dense compartment of the cortical granules, and is also present in the plasma membranes. At fertilization, the protein stored in the cortical granules is exocytosed and becomes part of a double layer enveloping the hatched blastula on the outside of the hyaline layer, i.e. the apical lamina. The toposomes in the yolk granules are processed by partial proteolysis and secreted in this form to all the external surfaces of newly formed cells (Gratwohl et al., 1991). In the blastula, the unprocessed 160 kDa form is
found on the outside of the hyaline layer, outlining the two borders of the apical lamina as well associated with the microvilli. All plasma membranes of the developing embryo are covered with toposomes originating from yolk granules. By contrast, hyalin, another ECM protein that contributes to the hyaline layer, is stored exclusively in the homogeneous part of the cortical granules in the oocyte. In the hatched blastula nearly all hyalin is seen on the inside of the apical lamina, but it does not appear associated with plasma membranes. Cervello and Matranga (1989) have shown that toposome displays the characteristics of a cell adhesion molecule. They propose that vitellogenin is the primordial form of a cell-adhesion molecule, and that the biologically active form, toposome, is a cleavage product of it.

Reports of yolk proteins appearing in extracellular sites have also been observed in other organisms. Sanders et al. (1990) has used antibodies to identify endogenous lectins in chick embryos and has shown that these proteins, which are stored in the yolk platelets of unfertilized oocytes, are secreted into the ECM at the epiblast stage. Hamazaki et al. (1989) has shown in the Japanese rice patty fish (Oryzias latipes) that a glycoprotein present only in yolk granules of spawning female (SF substance) becomes localized to the inner layer of the ovarian egg envelope of the growing oocyte. Furthermore, this protein shares many characteristics of vitellogenin, in that they are both synthesized by the liver and not the oocyte, and are transported there through the circulatory system. And finally, Outenreath et al. (1988) have found that an endogenous lectin, XL-43 (from X. laevis) is present in cortical granules and in vesicles in the unfertilized oocyte, and becomes localized to the ECM during development, where it may function as a substrate for cell migration.

The results of these studies seem to indicate that the nutritional role traditionally attributed to what has been loosely termed "yolk" must be questioned. The new concept emerging is that much of what has been regarded as yolk may be material stored for the assembly of membranes during periods of rapid embryonic and larval growth, or for extracellular matrices. However, several reports have failed to show that proteins stored in yolk granules are secreted in the above manner, and in the case of sea urchins, the major yolk glycoproteins remain within these organelles even after proteolytic processing, and are not
translocated to other organelles such as lysosomes or the cell surface as suggested by Gratwohl et al., (1991). It appears that the material stored in yolk platelets is perhaps more heterogeneous than previously thought. What is also evident, is that there still remain many unanswered questions on the subject of yolk protein function and utilization during embryogenesis.

7. Cortical granules

(a) General

Another type of granule found in many oocytes including those of mammals, amphibians and invertebrates are the cortical granules, which, as their name implies, underlie the plasma membrane in the cortical cytoplasm of unfertilized eggs. These are not as numerous as yolk, and generally are very short-lived once development begins. In many organisms, such as sea urchins, frogs and hamsters, their function appears to be restricted to the events occurring at fertilization, where they undergo a triggered exocytosis and help to create a block to polyspermy. In other species, such as some polychaetes, molluscs, mussels and clams, they are not released upon egg activation, and so perform a different function (reviewed by Anderson, 1974; Gulyas, 1980). Several aspects of their function still remain unknown, largely because the composition of these granules is poorly characterized. They have been shown to contain fucosyl and sialyl-rich glycoconjugates in the mouse (Lee et al., 1988) as well as certain proteases and ovoperoxide in the mouse and sea urchin (reviewed by Cran and Esper, 1990). However, the physiological role of these components has yet to be defined. Perhaps the best studied organism in this area has been the sea urchin, in which the elevation of the fertilization membrane, a direct consequence of cortical granule release, is very pronounced. This phenomenon, which also occurs in starfish (Fig. 3) is unlike the response of the mammalian oocyte, in which case the zona pellucida becomes refractory to further sperm penetration but does not result in any overt structural change.
Fig. 3. Formation of the fertilization membrane as viewed with Nomarski phase contrast microscopy.

This figure shows the dramatic appearance of the fertilization membrane or envelop after fertilization in the starfish, *P. ochraceus*.

(A) Immature oocyte, showing an intact germinal vesicle (arrows) which surrounds the nucleus (n).

(B) A mature oocyte 5 minutes after fertilization. Note that the germinal vesicle has broken down, and that a fertilization membrane is becoming apparent (arrows).

(C) One hour after fertilization, the fertilization membrane (fm) is well raised off the surface of the egg.

(D) Eight hours after fertilization at the first cleavage stage, the appearance of 2 polar bodies (pb) is noted, as well as a prominent fertilization membrane (fm).

(E, F) At the 4 cell stage through to the blastula, the embryo continues to develop within the confines of the fertilization membrane. Shortly after, the blastula "hatches" out of this membrane and begins to swim freely. b = blastocoel. Bar = 50 μm.
(b) Cortical granules of echinoderms

The contents of sea urchin cortical granules are among the best characterized. Some of the components become part of the fertilization membrane (Baginski et al., 1982), while others become a part of the hyaline layer, the ECM associated with the apical ectoderm of the embryos. One of these proteins is hyalin, which is stored both in the cortical granules as well as in low density granules dispersed throughout the egg (Hylander and Summers, 1982; Gratwohl et al., 1991), and which is the major component of the embryonic hyaline layer. Another protein stored in both the cortical and yolk granules is toposome, which is deployed to the hyaline layer shortly after fertilization, and which is also associated with plasma membranes of the egg and early embryo (Gratwohl et al., 1991). Starfish cortical granules have been identified morphologically, and are similar to those of sea urchins, in that they have a very electron dense component and a varied morphology. Several histochemical studies have been carried out on cortical granules in starfish (Crawford and Abed, 1986, Sousa and Azevedo, 1989) which have shown that they contain acid mucopolysaccharides and acid phosphatase components that are exocytosed upon fertilization. However, information about their protein constituents and how this relates to other species is sparse.

8. Statement of the problem (Part B)

Yolk proteins are highly conserved through phylogeny, which suggests that they have an essential function to play during development and morphogenesis, yet the mechanisms of yolk utilization still remain a mystery. Some reports indicate that yolk is progressively degraded through embryonic development, while others indicate that depletion occurs later in development when the organism has reached a feeding larval stage. Yet there are other reports that some of the proteins stored in yolk are not catabolized for nutrition, but are secreted into ECMs of the developing embryo. The primary objectives of this part of the study were: (1) to investigate the yolk proteins of the starfish P. ochraceus, and determine whether they are catabolized for nutrition during early development; and (2) to investigate whether there
is any evidence that ECM proteins of the early embryo are derived from maternal stores in the oocyte, and if so, determine whether they are stored in the yolk granules and/or other storage granules. The approach used was similar to that in Part A. This involved generating antibodies against proteins of the gastrula-stage embryo, and then using immunohistochemistry to localize components of yolk granules and other storage granules of the embryo and oocyte. The antibodies were also used to carefully trace the pattern of distribution of these antigens throughout development using both immunofluorescence and immunogold TEM. A final objective involved the use of the antibodies to characterize the antigens, so that they could be compared with known yolk and other components of oocyte storage granules.
II. MATERIALS AND METHODS

1. Embryo preparation

Ripe adult starfish (*Pisaster ochraceus*) collected from the intertidal zone near Sidney, B.C. were maintained in sea water tanks (12°C) in the Department of Zoology, University of British Columbia. The adults were kept under conditions of constant light in order to prevent spawning. Embryo cultures were prepared and maintained as previously described (Crawford and Abed, 1983). Sea water used for culturing was collected from the Department of Fisheries and Oceans in West Vancouver, and was filtered through a Whatman #1 filter and aerated prior to use. Ovaries removed from the adult female by excising 1 arm were placed in 0.1 mg/ml 1-methyl adenine in sea water at 12°C so as to induce the oocytes to complete meiosis so they would be ready for fertilization. After approximately 70 minutes, the breakdown of germinal vesicles was complete, indicating that the oocytes had reached full maturity, and they were washed in aerated filtered sea water. Testes isolated from adult male starfish, also by excising 1 arm, were placed in a plastic petri dish and kept dry until the eggs were ready for fertilization. A few drops of sperm were then place in 20 ml sea water to make a cloudy suspension, and after a few minutes, the sperm were checked for motility by microscopic observation. Sufficient eggs to cover 1/2-3/4 of the bottom of a 1 liter plastic beaker were fertilized with 0.5 ml dilute sperm solution in 400 ml sea water. Following hatching at approximately 24 hours, the swimming embryos were poured into new beakers to separate them from the debris on the bottom of the beakers. Embryos at various developmental stages were harvested by gentle centrifugation (125 x g for 3 minutes), the majority of sea water was removed, and the embryos were either stored at -20°C for further use in biochemical studies, or fixed by freeze-substitution for immunohistochemical studies.
2. Fixation and embedding of oocytes and embryos

It was crucial for the success of this study to combine methods that maintained maximum tissue structure and immunoreactivity. For this reason, material was fixed by freeze-substitution according to the method of Campbell et al. (1991), which is detailed in Appendix 1. Embryos of various developmental stages were cryoprotected for 30 minutes in 15% 2,3-butanediol (Sigma) in sea water, and then approximately 1 μl of thick suspension was placed on 50 mesh nickel TEM grids. In the case of immature and mature oocytes, the cryoprotectant was omitted as it caused excessive tissue collapse. Residual liquid was removed from the embryos with filter paper, and the grid together with the embryos was quickly plunged into liquid propane that had been pre-cooled to -196°C with liquid nitrogen. The frozen embryos were then placed in either absolute ethanol or into Alcian blue (Marivac)-saturated ethanol which had been pre-cooled to -90°C with a dry ice-acetone bath. They were maintained at low temperature (below -85°C) for a period of 5 days with daily additions of liquid nitrogen, during which time the ethanol substituted for the water. Subsequently, they were slowly warmed to room temperature and embedded into a plastic resin, either JB4 (Polysciences) or LR White (JBS), as per manufacturers instructions. Material embedded in JB4 was sectioned at 1-1.5 μm and stained for immunofluorescence as described below. Material in LR White was sectioned for TEM at 50-60 nm and stained using the colloidal gold technique, also as described below.

3. Monoclonal antibody production

In the following section, the steps required for immunogen preparation and monoclonal antibody production and purification are detailed (summarized in Fig. 4). This involved: 1) obtaining a detergent extract of proteins from starfish embryos; 2) selecting for an ECM-rich fraction of the extract which was used to immunize mice for monoclonal antibody production; 3) screening the hybridoma supernatant using thin section immunohistochemistry.
Fig. 4. Protocol for monoclonal antibody production against starfish ECM and yolk proteins

Embryos at the late gastrula stage were homogenized in a detergent buffer, and the extracted proteins were passed over a Con A-Sepharose affinity column to obtain a glycoconjugate-enriched fraction from the embryo homogenate. The Con A-binding fraction was released from the column using the specific competitor α-methyl mannoside. The fraction was then used to immunize mice for monoclonal antibody production. Tail bleeds and hybridoma supernatants were tested for antibodies directed to the ECM and egg proteins using immunofluorescence on thin plastic sections of fixed embryos.
1. Detergent Extraction of Embryos

2. Con A-Sepharose Fractionation

Peak 1: Non-binding fraction
Peak 2: Con A-binding fraction

3. Immunization

4. Hybridoma Production

5. Screening with immunofluorescence
(a) Preparation of the immunogen

i) Detergent homogenization of embryos

All steps of the embryo homogenization and extraction were performed on ice to retard the activity of protease action. Starfish gastrulae which had been reared, concentrated and stored at -20°C, as described above, were thawed quickly and solubilized with an equal volume of the extraction buffer consisting of 20 mM Tris, 0.5 M NaCl, pH 7.4 with 1% Brij 56 [polyoxyethylene 10 cetylether (Sigma)]. The embryos were homogenized first using a Dounce tissue homogenizer (10 plunges), followed by a brief sonication with a Fisher probe sonic dismembrator (15 seconds at 45%). The homogenate was then extracted for 30 minutes on ice, centrifuged at 35,000 rpm (100,000 g) for 1 hour at 4°C, and the supernatant was collected and either stored at -70°C or used immediately.

Brij 56, a non-ionic detergent, was chosen to aid membrane breakup during the homogenization. Although other detergents such as Triton X, Nonidet P-40 and the Tween series, are often used as mild reagents in homogenization buffers, all of these detergents have a high absorbance at 280 nm due to the presence of phenol rings. These detergents are therefore incompatible with chromatographic procedures in which protein monitoring off the column uses UV absorbance at a 280. Brij 56, although a mild non-ionic detergent, does not contain phenol rings and therefore does not interfere with protein monitoring if this method of detection is used.

ii) Protease inhibitors

Since the release of intracellular proteases is unavoidable after ultrasonic treatment, protease inhibitors were an essential addition to all buffers which were used for embryo homogenization and extraction. The protease inhibitor cocktail used in most of the studies included PMSF (phenylmethylsulfonylfuoride), active against serine proteases at concentrations of 1 mM, pepstatin A, active against acid proteases at concentrations of 1 μg/ml, iodoacetamide, active against covalent thiol proteases at concentrations of 10 mM, and the potent metalloproteinase inhibitors EDTA (ethylenediamine tetraacetic acid) and EGTA [ethyleneglycol-bis-(β-amino ethyl ether) N,N',N'-tetraacetic acid] active at concentrations of 1-5 mM.
(see Table 1 for substrate specificities of protease inhibitors). However, homogenization, extraction, and running buffers that were used for Con A-affinity chromatography did not include the metalloproteinase inhibitors EDTA or EGTA, as Con A requires calcium ions for binding activity.

iii) Con A-affinity chromatography

A large number of ECM components contain complex carbohydrate chains which have a high content of mannose residues at their terminal end. Examples include glycoproteins such as laminin, fibronectin, several collagens, as well as proteoglycans, many of which contain N and O-linked oligosaccharide chains in addition to GAG chains. As Concanavalin A (Con A) has a very strong affinity for mannose residues, it is often used as a general marker of such carbohydrate groups. To determine whether starfish embryonic ECM contained Con A-binding sites, sections of late gastrula-stage embryos were stained with FITC-Con A (Fig. 5). Positive staining was observed in large granules of all cell types, as well as throughout the ECM-rich blastocoel and over basement membranes. For this reason, it was chosen to obtain an ECM-enriched fraction from the embryo extract using solid phase affinity chromatography. The details of this procedure are listed in Appendix 2. A 10 ml Con A-Sepharose 4B (Sigma) column was pre-equilibrated with 10 column volumes of washing buffer (20 mM Tris-HCl, 0.5 M NaCl, pH 7.4), and pre-washed with 3 column volumes of the elution buffer (0.5 M α-methylmannoside in the washing buffer). For chromatography, the embryo extract prepared as described above [section 3(a)i] was first dialyzed against 20 mM Tris, 0.5 M NaCl, pH 7.4, and then 1 ml was applied to the lectin column. The sample was recycled through the column 2 times, and then washed through with 10 column volumes of washing buffer. The bound fraction was released from the Con A-Sepharose with the addition of 3 ml elution buffer, and the peak was monitored with an a 280 ultraviolet detector (Pharmacia). The fractions under the peak were pooled and concentrated in dialysis tubing (MW cutoff 12-14,000; Spectrum Medical) that was placed over polyethylene glycol (PEG) beads (16,000-20,000 MW; Sigma). After a 20-fold reduction in volume was achieved, the approximate protein concentration of the solution was determined using UV absorption at 280 nm (see Appendix 3 for details), and the sample was analyzed with SDS-PAGE (Fig. 6).
<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Protease target</th>
<th>Effective Concentration</th>
<th>Stock</th>
<th>Comments</th>
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<td>EDTA/EGTA</td>
<td>Metalloproteinases (divalent cation-dependent proteases)</td>
<td>1-5 mM</td>
<td>0.5 M in H2O, pH 8.0</td>
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<td>Pepstatin A</td>
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<td>1 µg/ml</td>
<td>1 mg/ml DMSO</td>
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<tr>
<td>PMSF</td>
<td>Serine proteases (some thiol proteases and carboxypeptidases)</td>
<td>1 mM</td>
<td>5 mg/ml Acetone</td>
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<tr>
<td>Iodoacetamide</td>
<td>Triosephosphate dehydrogenase (covalent thiol proteases)</td>
<td>10 mM</td>
<td>10 mg/ml PBS</td>
<td>Add fresh at each step</td>
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Fig. 5. Con A-FITC staining of the late gastrula embryo (*P. ochraceus*).

A section of a gastrula stage embryo (*P. ochraceus*) that has been fixed by freeze substitution in ethanol, embedded in JB4 plastic resin, and sectioned at 1.5 µm. Staining was performed using biotin-tagged Con A followed with streptavidin-FITC.

(A) Labelling of the large intracellular granules in cells of the ectoderm (ec), endoderm (en) and mesenchyme cells (mc) is evident. Con A also labels the matrix of the blastocoel (b). Bar = 36 µm.

(B) A higher magnification of an embryo prepared as in A, showing a region of the lower gut endoderm and the ectoderm which are separated by matrix in the blastocoel. Labelling of the basement membranes (bm) underlying the epithelium is observed. In addition, the hyaline layer (hl), which surrounds the embryo on the apical surface of the epithelium, shows binding by Con A. Bar = 10 µm.
iv) Immunogen preparation and immunization

For immunogen preparation, the Con A-bound fraction was dialyzed against PBS (no azide) and the protein concentration of the solution was adjusted to 4 mg/ml PBS. Equal parts were then mixed with Freund's incomplete adjuvant (Sigma) to form an emulsion. This was achieved using two, 1 ml glass syringes that were fitted with a 20 gauge copper connector. In one syringe, 0.5 ml of the protein solution were loaded, while 0.5 ml of the adjuvant were loaded in the other syringe, and the mixture was displaced back and forth through the connector until a white stiff emulsion was produced. The immunogen was then introduced subcutaneously into the backs of 4 week old BALB/c mice (100 µl immunogen per mouse, corresponding to approximately 200 µg protein per mouse). One month following the initial immunization, the mice were boosted by intraperitoneal injection with 100 µg of concentrated protein solution containing no adjuvant. Five days following the boost, a test bleed was taken from the tail. The blood was suspended in 2% blotto at a concentration of 1:10 and 1:100, and tested on thin sections of JB4-embedded freeze substituted embryos (section 4). Mice were boosted by intraperitoneal injection of pure protein solution at 1 month intervals until a positive tail bleed was obtained (Fig. 7). The spleen was then harvested for hybridoma production 5 days following the final boost. Details of the immunization protocol are summarized in Appendix 4.

b) Hybridoma production

Monoclonal antibodies were prepared according to the protocol summarized in Kannangara et al. (1989), and are detailed in Appendix 5. Confluent cultures (5-6 petri dishes, 9 mm) of mouse myeloma cells were prepared and washed in DMEM. The spleen from one mouse was harvested, the cells were isolated, and mixed together with the myeloma cells. The fusogen was added and the cell mixture was washed and resuspended in HAT media with 20% FCS. Thymocytes, to be used as a feeder cell population, were harvested and mixed with the fused cell mixture, which was then plated into 24-well tissue culture plates (Linbro). After 3-4 days, 100 µl HT media were added to each of the wells, and 7 days
Fig. 6. Electrophoretic analysis of the Con A-affinity fraction from embryo homogenates.

The figure shows proteins separated by electrophoresis on 3-10% gradient gels. Lane 1 shows the crude detergent extract from starfish gastrulae, while lane 2 shows the Con A affinity-purified fraction from the crude extract. In all figure of this thesis, where appropriate, molecular mass standards indicated on the left are in kilodaltons (kDa).
Fig. 7. Immunohistochemical analysis of mouse tail bleed

(A) A mid-saggital section of a plastic-embedded gastrula fixed by freeze-substitution and stained with serum isolated from a mouse that had been immunized with a Con A-enriched fraction of embryo homogenates. Antibody binding to several ECM structures is apparent, including the diffuse matrix of the blastocoel (b) and the hyaline layer (hl), a specialized ECM found at the apex of the epithelium surrounding the embryo and lining the digestive tract lumen. Bar = 40 μm.

(B) A higher magnification of an embryo prepared as in A, showing part of the ectoderm and gut endoderm. Staining of the basement membranes (arrowheads), the hyaline layer (hl) as well as light staining of large granules (g) in the epithelial cells are apparent. Bar = 15 μm.
post-fusion, the cells were replenished with fresh media. Screening of the hybridoma supernatant was started about 10 days post-fusion, and was done on embryo sections as described in section 4. Desirable clones were selected based on immunolocalization studies in gastrula stage embryos. These were then subcloned and expanded for further production of hybridoma supernatant (Appendices 6, 7). The clones were assayed for their isotype using the Serotec mouse monoclonal isotyping kit. Details of this assay are outlined in Appendix 8. The following monoclonals were used for this study: PM1 (Pisaster matrix 1) of the IgM isotype, PY4F8 (Pisaster yolk 4F8), PC3H2 (Pisaster cortical 3H2), and Pisaster 212, all of the IgG1 isotype, and PH3C8 (Pisaster hyaline 3C8) of the IgG2a isotype.

(c) Ascites production

For large scale antibody production, the PM1 hybridomas were grown in culture to densities of roughly 1 x 10^6 cells per ml and the cells were collected and washed in PBS (no azide) for ascites production. Ascites tumors were induced in pristane-primed BALB/c mice by the intraperitoneal injection of 5-10 x 10^6 cloned hybridoma cells. Approximately 7 - 10 days after injection, ascites fluid was drained from the peritoneal cavities of the infected mice using 20 gauge sterile needles and gentle belly massage. The mice were put under mild sedation with Halothane for this procedure. The ascites fluid was clarified by centrifugation (10 minutes, 10,000 x g), and defatted as follows: To 5 ml ascitic fluid were added 5 ml VBS (veronal-buffered saline; 4 mM barbitone, 0.15 M NaCl, 0.8 mM Mg²⁺, 0.3 mM Ca²⁺), pH 7.2, and 150 mg silicon dioxide powder. The mixture was incubated at room temperature for 30 minutes with occasional shaking, and then centrifuged 2000 x g for 20 minutes (Neoh et al., 1986).

(d) Purification of PM1 antibody

After the PM1 isotype was determined to be IgM, further purification of the ascites was carried out using Sephacryl S-300 gel filtration chromatography. Initially, conventional size separation chromatography was performed, in which tris-buffered saline was the equilibrating and washing buffer. This, however, gave
inadequate peak separation from α-2 macroglobulin, which also eluted in the first peak with IgM. In addition, there was some overlap with peak 2, IgG. Subsequently, the low ionic strength procedure of Bouvet et al. (1984) was used with superior results. In this procedure, a 2.5 x 100 cm column (Pharmacia) was prepared at room temperature and equilibrated with a low ionic strength buffer (5 mM sodium phosphate, pH 7.5; no azide). Five ml of purified ascites were loaded onto the column, followed immediately by 50 ml high ionic strength buffer (50 mM sodium phosphate, 2.0 M NaCl, 20 mM sodium azide, pH 7.5). The proteins were then eluted with the equilibrating buffer (low ionic strength), and fractions of 5.0 ml were collected into tubes containing 250 µl high ionic strength buffer. Because of the insolubility of IgM in the very low ionic strength buffer, it was retained in the column, and therefore it eluted in the final peak, and was well separated from the other proteins in the ascites fluid. The IgM was then concentrated in dialysis bags placed over PEG beads, or alternatively via ultrafiltration (Centricon-50; Amicon).

(e) IgM fragmentation

PM1 antibody was digested into smaller IgG-like fragments with an estimated Mr of 180 kDa, using the low temperature pepsin proteolysis method of Pascula and Clem (1992). Under these conditions, reasonably homogeneous proteolytic fragments can be generated with a single step digestion. To achieve this, 1 ml of a 10 mg/ml purified IgM solution was dialyzed against 20 mM sodium acetate, 0.15 M NaCl, pH 4. Crystalline pepsin dissolved in the same buffer was added at a ratio of 1:15 w/w enzyme to antibody, and the IgM was digested at 4°C for 24 hours. The digestion was stopped by increasing the pH of the solution to inactivate the pepsin with the addition 0.1 ml of 2 M Tris-HCl, pH 8.6, and the fragments were separated using gel filtration on a Sephacryl S300 column as above. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) was carried out under non-reducing conditions to confirm the digestion of IgM (900 kDa) to smaller fragments with Mr of 130 kDa (see Fig. 8).
Fig. 8. Electrophoretic analysis of PM1 pepsin fragmentation.

The figure shows PM1 antibody, an IgM class monoclonal with a molecular mass of 900 kDa, which has been purified from ascites fluid using gel chromatography, and analyzed by SDS polyacrylamide electrophoresis under non-reducing conditions on 3-10% gradient gels before and after proteolysis with pepsin. Lane 1, undigested PM1, showing a strong band at 900 kDa. Lane 2 shows the separation of PM1 fragments after 24 hour digestion with pepsin (4°C). In addition to some undigested material at the top of the gel, the predominant band is seen at 130 kDa. Lane 3 shows a 130 kDa enriched fraction (arrow) of the digest isolated by gel filtration chromatography; only a small amount of undigested IgM is present in this fraction.
4. Immunohistochemistry

(a) Immunofluorescence staining

Thin sections (1-1.5 μm) of JB4-embedded freeze substituted material were stained with the monoclonal antibodies PM1, P212 or PY4F8. The sections were first pre-incubated with 0.05% rabbit IgG (Sigma) in PBS containing 0.2% Carnation non-fat milk powder (PBS/blotto) for 30 minutes. This step ensured that sites prone to non-specific binding were blocked, a technique which reduced the background staining substantially. Next, undiluted hybridoma supernatant was applied to the sections for 1 hour. After 2 washes of 15 minutes each in PBS/blotto, the sections were incubated for 1 hour in FITC-conjugated secondary antibodies that were prepared in rabbits against either mouse IgM or mouse IgG, depending on the isotype of the primary antibody. The sections were washed as above, mounted with 16% gelvatol, 0.4% DABCO (1.4-diazabicyclo [2.2.2]octane; Aldrich) 30% glycerol in PBS, pH 7.2 (Taylor and Helmer, 1974; Johnson et al., 1982), and photographed with Fugicolor 1600 film or Kodak TMAX 3200 on a Zeiss Axiophot Photomicroscope equipped with epifluorescence optics (see Appendix 9 for details). Control sections were stained as above with normal pooled mouse IgM or IgG replacing the primary antibody.

(b) PM1 and P212/PH3C8 double immunofluorescence staining

Multiple immunofluorescence labelling was carried out in some cases to compare the localization of two different monoclonals on the same tissue section. This involved the PM1 antibody together with one of two different monoclonal antibodies that were developed in this lab: P3C8 which binds to the hyaline layer, and P212 which binds to a plasma membrane-associated antigen. The sections were first labelled with the hybridoma supernatants P3C8 or P212 (both IgG class antibodies) for 1 hour and washed as above, after which biotin-labelled goat anti-mouse secondary antibodies were applied to the sections for 1 hour. The sections were washed as above, and incubated with a streptavidin-texas red fluorochrome (Molecular Probes) for 1 hour. Next, the sections were washed and PM1 immunostaining was done as
described above. Sections were washed, mounted and photographed by double exposure on a Zeiss Axiophot Photomicroscope as described above. Controls were stained as above with mouse IgM and IgG replacing the primary antibodies.

(c) **Double immunofluorescence with PC3H2 and PY4F8**

For double label immunofluorescence using 2 monoclonals of the IgG class, the sections were labelled with PY4F8 hybridoma supernatant for 1 hour and washed as above, after which a biotin-labelled goat anti-mouse secondary antibody was applied to the section for 1 hour. Sections were washed and probed with a streptavidin-texas red fluorochrome (Molecular Probes) for 1 hour. Next, the sections were washed and stained with PC3H2 hybridoma supernatant as described above, washed, mounted and photographed by double exposure on a Zeiss Axiophot Photomicroscope. Controls were stained as above with mouse IgG replacing the primary antibodies.

(d) **Lectin histochemistry**

Sections were preincubated with 2% blotto for 30 minutes and then were stained for 1 hour with FITC-conjugates of the following lectins (Sigma): Con A (Concanavalin A), WGA (wheat germ agglutinin), RCA120 (*ricinus communis* agglutinin-120), UEA-I (*ulex europeus* agglutinin) and SBA (soybean agglutinin). The lectins were diluted in 2% blotto to a final concentration of 200 μg/ml. Sections were then washed in 2% blotto for 15 minutes, and prepared for photography as described above. For controls, the lectins were first incubated for 1 hour with 1 M concentrations of their target sugars: These were α-methyl mannoside for Con A, N-acetyl glucosamine for WGA, galactose for RCA120, L-Fucose for UEA-I, and N-acetyl galactosamine for SBA. The lectins were then applied to tissue sections as described above.
(e) **Immunogold electron microscopy**

Ultrathin sections (50-60 nm) of LR White-embedded material were picked up on parlodion/carbon-coated 100 mesh nickel grids and stained as follows. The grids were floated on drops of the pre-incubation buffer consisting of 10% normal serum of the secondary host (either goat or rabbit serum; Pierce) in PBS/blotto for 1 hour, followed by a 90 minute incubation in undiluted hybridoma supernatant. The grids were then washed 2 times over 30 minutes with PBS/blotto, and floated on a goat anti-mouse IgG/M-colloidal gold conjugate that was prepared in the lab (see Appendix 10 for details). This involved first preparing gold particles with a mean particle diameter of 25 nm (Au25) after the method of Frens, (1973), in which chloroauric acid is reduced with sodium citrate to form a heterodisperse gold sol. The gold particles were then coupled to goat anti-mouse IgG/M after the methods of Slot and Geuze (1985), and is detailed in Appendices 11 and 12. The grids were washed as above, rinsed in distilled water, and stained with saturated aqueous uranyl acetate (10 minutes) and lead citrate (5 minutes) (Reynolds, 1963). Electron microscopy was performed on a Philips 301 TEM. Control grids were stained as above, with normal mouse IgG or IgM replacing the primary antibody.

5. **SDS PAGE and Western blotting**

(a) **Gels and blotting membranes**

Gradient acrylamide gels (3-10%) were used for the majority of the experiments, because they facilitated the separation of large antigens better than the low percentage conventional acrylamide gels (detailed in Appendix 13). These gels also offered several advantages for Western blotting. While low percentage acrylamide gels were often prone to adhere to transfer membranes during electro-elution, the gradient gels were less likely to adhere to the membranes. There were still some problems with nitrocellulose, because it adhered to the low percentage regions of the gradient gels. However, for most of the experiments, the Biorad PVDF (polyvinylidene difluoride) membrane was used and proved superior to
nitrocellulose, offering higher binding affinity of large proteins, higher retention of proteins during elution, and lower background for immunostaining.

b) Developmental Western blot analysis

i) Sample preparation and SDS-PAGE

Oocytes and embryos at the following stages were used for the developmental Western blot analysis: Unfertilized immature oocytes, blastulae (2 days post-fertilization), gastrulae (4 days post-fertilization), and bipinnaria larvae (9 days post-fertilization). One ml of packed eggs or embryos were suspended in an equal volume of ice-cold extraction buffer. For PM1 Western analysis, the extraction buffer used was a high ionic strength buffer designed to solubilize extracellular proteoglycans, and contained 4 M guanidine hydrochloride, 50 mM sodium acetate, 10 mM EDTA, 1 mM PMSF, 1 mM iodoacetamide, and 1 μg/ml pepstatin A. For the PY4F8 Western analysis, the extraction buffer was designed to solubilize intracellular proteins and those contained within storage granules; it consisted of 10 mM Tris, 50 mM NaCl, 1% Triton X-100 (Sigma), 0.5% sodium deoxycholate (Sigma), 0.1% SDS (BDH), pH 7.3, with the protease inhibitors mentioned above. Tissue homogenization was performed on ice, first using a Dounce tissue homogenizer (10 plunges), followed by a brief sonication with a Fisher probe sonic dismembrator (15 seconds at 45%). The homogenate was then extracted overnight at 4°C with constant rotation and centrifuged at 35,000 rpm (100,000 g) for 1 hour in at 4°C in a Sorvall SS65 rotor. The supernatant was collected, and prepared for electrophoresis by boiling in SDS-PAGE sample reducing buffer at a ratio of 1:10. Analysis of the antigens was performed on 3-10% gradient gels in a Biorad vertical mini-slab system utilizing the buffer system described by Laemmli (1970). For total protein visualization and for PM1 Western blot analysis, lanes were loaded with approximately 10 μg/ml protein/lane as determined by the Biorad DC Protein Assay. For PY4F8 immunoblots, lanes were loaded with approximately 5 μg/lane of reduced sample, or with 2 μg/lane non-reduced sample.
ii) Western blots

Gels were equilibrated for 30 minutes in transfer buffer [10 mM CAPS (3-[Cyclohexylamino]-1-propanesulfonic acid; Sigma), pH 11.0], and proteins were electroeluted onto PVDF membrane (Biorad) for 1 hour at 100 v. Membranes were rinsed 15 minutes in distilled water, and then either stained for total protein with 0.025% Coomassie Brilliant Blue R-250 (Sigma) in 40% methanol, or processed for immunoblotting. Immunoreactive bands were visualized as follows: Membranes were blocked for 1 hour at 37°C in TBST (10 mM Tris, 0.15 M NaCl, 0.2% Tween 20; BDH) with 5% milk powder and 0.5% normal goat serum. Following this, the membranes were rinsed in washing buffer (TBS with 0.1% milk powder), and were incubated overnight at room temperature with a 1:1 dilution of primary hybridoma supernatant in TBST, with 1% milk powder and 1% BSA. The membranes were then washed 3 times for 15 minutes in the TBS washing buffer, and incubated for 90 minutes in goat anti-mouse IgG/M-biotin (Pierce) diluted in TBST with 1% milk powder. The membranes were washed as above, and incubated in streptavidin-HRP (Pierce) diluted in TBST with 1% milk powder for a further 90 minutes. After a final wash, immunoreactive bands were visualized by incubating the strips with 4-chloronaphthol (Sigma; 0.03% 4-chloronaphthol in 50 mM Tris, pH 7.6 with 10 μl of 30% hydrogen peroxide) at room temperature with agitation for 10-30 minutes. Apparent molecular masses (Mr) for SDS-PAGE and Western blots were estimated by comparison with Sigma high molecular weight standards and mouse Engelbreth-Holm-Swarm (EHS) laminin (Sigma), the latter of which has an estimated Mr of 900 kDa (non-reducing) and 420/220 kDa (reducing).

(c) Digestive tract and coelomic fluid analysis

Digestive tract material was isolated from adult female starfish and suspended in an equal volume of extraction buffer. Extraction was performed with a tissue homogenizer and with mild ultrasonic treatment as described in section 3(a), and the supernatant was collected, and prepared for electrophoresis by boiling in SDS-PAGE sample buffer at a ratio of 1:10. Coelomic fluid isolated from adult female starfish after the removal of 1 arm was concentrated 20-fold via ultrafiltration (Centricon-50) prior to suspension in
SDS-PAGE sample buffer. Analysis of the antigens was performed on 3-10% gradient gels in a BioRad vertical mini-slab system utilizing the buffer system described by Laemmli (1970).

6. Isolation and characterization of antigens

(a) Immunoaffinity purification of the PM1 antigen

For further purification of the PM1 antigen, a PM1 immunoaffinity column was prepared following a protocol described in Appendix 14. Purified PM1 antibody from 2 ml ascites fluid was coupled to 1 ml Affi-Gel 10 (BioRad) according to manufacturers directions. The antibody was first dialyzed against coupling buffer (0.25 M sodium bicarbonate, pH 8.7) at 4°C for 24 hours. Affi-gel 10 was then activated by washing with 10 volumes of ice-cold distilled water; the dialyzed antibody was added quickly to 1 ml of the activated Affi-gel 10, and the mixture was rotated end over end for 4 hours at 4°C. Following coupling, 0.1 ml of 1 M ethanolamine HCL (pH 8) were added to block any remaining active esters, and the gel was rotated a further 1 hour at room temperature. The coupled gel was transferred to a 3 ml column made from an empty syringe barrel, and equilibrated with TBS, pH 7.5. Affinity chromatography was carried out at 4°C as follows: 1 ml of guanidine hydrochloride embryo extract was dialyzed against TBS overnight, and then applied to the affinity column at a flow rate of 5 ml/hour. After extensive washing with TBS, the PM1 antigen was eluted off the column using 2 ml 0.1 M triethylamine, pH 11.0. The peak was detected using UV (a 280) , and was collected in 1 ml fractions into tubes containing 100 μl 1 M Tris, pH 6.0; fractions were pooled, concentrated via ultrafiltration (Centricon-50), and stored at -70°C. The protease inhibitors PMSF and pepstatin A were routinely used throughout the procedure, and PMSF was supplemented every 18 hours.
(b) Immunoprecipitations with PY4F8

Embryo extracts were immunoprecipitated with PY4F8 (or P212 as a control) hybridoma supernatant using Protein-A Sepharose 4B (Sigma) and goat anti-mouse IgG (Pierce) as a linker (Appendix 15). All steps were performed at 4°C. Briefly, 100 µl settled Protein A-beads were washed twice for 10 minutes each with embryo extraction buffer (10 mM Tris, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, pH 7.3) (pre-wash) and then washed with the immunoprecipitation buffer, consisting of 20 mM Tris, 150 mM NaCl, 0.1% Triton X-100, 25 mM EDTA, 1 mM iodoacetamide, 1 mM PMSF, 1 µg/ml pepstatin A, and 1 mM EGTA, pH 7.8. Following this, 50 µl of rabbit anti-mouse IgG linker were added to the beads at a concentration of 50 µg/ml, and the mixture was rotated end over end for 1 hour. The beads were then washed for 30 minutes with 3 changes of buffer, after which 100 µl hybridoma supernatant were added to them. They were rotated for 1 hour, and then washed for 30 minutes as above. Oocyte or embryo detergent extract (100 µl) was added along with a fresh dose of PMSF (10 µl of a 1 mg/ml stock of PMSF in acetone), and the beads were rotated for 1 hour. The final wash was extensive to ensure all unbound proteins were removed from the bead suspension (4 changes of buffer over 30 minutes). The antigen was separated from the beads by resuspending the bead suspension in 200 µl of reducing SDS-PAGE sample buffer, and heating them to 85°C for 15 minutes. To collect the sample, the beads were centrifuged for 1 minute at 125 x g, the supernatant was collected, and stored at -20°C until analysis by SDS-PAGE was required.

(c) Biochemical stains

Aliquots of affinity-purified PM1 antigen were subject to SDS-PAGE on 3-10 % gradient gels, and were stained with various dyes (see below) for biochemical characterization. All gels were photographed on a light box using Kodak high contrast copy film, and film was developed with D19 full strength for 6 minutes.
i) Coomassie Blue

For total protein detection, gels were placed in Coomassie blue (0.25% in 50% methanol, 10% acetic acid) for 30 minutes, and then destained with several changes of 10% methanol, 10% acetic acid.

ii) Alcian Blue

For glycosaminoglycan (GAG) detection, Alcian blue staining using the critical electrolyte method of Wall and Gyi (1988) was used as follows: after SDS-PAGE, the gel was washed exhaustively to remove all traces of SDS, first with 50% methanol, 7% acetic acid for 1 hour, then with distilled water for 1 hour. These steps were repeated once before the gel was finally transferred to a solution of 0.2% Alcian blue 8GX (Marivac), 50 mM MgCl₂, in 3% acetic acid overnight. The gel was then destained with several changes of 50 mM MgCl₂, in 3% acetic acid. To increase the contrast of the light blue bands, a yellow filter was used during photography.

iii) Periodic acid/Schiff (PAS)

PAS stain (Lillie, 1951) was used to detect glycoproteins as follows: After SDS-PAGE, the gel was fixed overnight in 40% methanol, 7% acetic acid, rinsed in distilled water, and placed in 1% periodic acid in 3% acetic acid for 50 minutes. The gel was washed in several changes of distilled water, and placed in Schiff's reagent in the dark for 50 minutes. Following this, the gel was washed with 3 changes of 0.5% sulfite wash (0.5 g potassium metabisulphite, 5% N HCl), and then rinsed in distilled water. To increase the contrast of the pink bands, a green filter was used for photography.

(d) Sodium periodate treatment

Strips of PVDF containing affinity-purified PM1 antigen were pre-incubated in TBS with 5% milk powder and 0.5% normal goat serum for 1 hour at 37°C. For the conventional periodate treatment, the method of Woodward et al. (1985) was used, in which blots were rinsed in 50 mM sodium acetate, pH 4.5, and then incubated in 50 mM periodic acid (Sigma) in 50 mM sodium acetate, pH 4.5, for 1 hour at room temperature.
in the dark. Control strips were incubated in buffer alone as above. Both experimental and control blots were then rinsed with acetate buffer for 3 changes of 10 minutes each, and exposed to 50 mM sodium borohydride (Sigma) in PBS for 30 minutes at room temperature. Alternatively, some strips were treated with periodate in a high ionic strength buffer after the method of Scott and Harbinson (1968) as follows: the strips were blocked as above, then rinsed in 50 mM sodium acetate, 0.2 M sodium perchlorate (BDH), pH 3.0, and incubated in 50 mM periodic acid in 50 mM sodium acetate, 0.2 M sodium perchlorate, pH 3.0 for 24 hours at 37°C in the dark. Control blots were incubated in buffer alone under the same conditions of time and temperature. Both the experimental and control blots were then rinsed in the high ionic strength buffer for 3 changes of 10 minutes each, and placed in 50 mM sodium borohydride in PBS for 30 minutes at room temperature. Following this, blots from both treatments were rinsed with TBS for 3 changes of 10 minutes, and were processed altogether for immunoblotting as above.

(e) Lectin labelling of Western blots

Strips of PVDF containing PY4F8 immunoprecipitations were pre-incubated for 1 hour in a blocking solution to reduce non-specific binding of the lectins. For Con A blots, the block was TBS with 5% milk powder, however milk powder was not compatible with WGA staining, and therefore 3% BSA was used to block the blots in this case. After blocking, the blots were rinsed briefly in buffer without blocking agent, and incubated overnight at room temperature in a 1:500 dilution of Con A-biotin (Pierce) in TBST with 1% milk powder, or WGA-biotin (Sigma) in (PBST) PBS with 0.2% Tween 20. Following rinsing for 3 x 10 minutes in TBS or PBS respectively, the blots were treated for 1 hour with a 1:500 dilution of streptavidin-HRP in TBST with 1% milk powder for Con A-probed blots, or in PBST for WGA-probed blots. After a final washing step, the blots were processed for detection using 4 chloronaphthol as described in section 5 above.
7. Analysis of antigens by enzymatic digestion

Prior to all digestions, aliquots of pure PM1 antigen were dialyzed in semi-micro dialysis tubing (MW cutoff 12-14,000; Spectrum Medical) against the appropriate digestion buffer, to ensure that the correct buffer and pH requirements of the enzymes were satisfied. Protease inhibitors (1 mM PMSF, and 1 µg/ml mM pepstatin A) were included in each digestion buffer.

a) Sugar-degrading enzymes

Pure PM1 antigen was digested with the following enzymes (for a list of enzymes, their sources and their substrate specificities, see Table 2): chondroitinase AC and ABC (0.2 U/ml in 0.1 M Tris, 30 mM sodium acetate, pH 7.3 with 10 mM EDTA), heparitinase (0.15 U/ml in 0.1 M sodium acetate, 1 mM calcium acetate, pH 7.0), testicular hyaluronidase (20 mg/ml in 0.1 M Tris, 30 mM sodium acetate, pH 5.6), and bovine kidney α-L fucosidase (0.2 U/ml in 0.1 M citrate-phosphate, pH 5.5) (Appendix 16). Following fucosidase digestion, some samples were dialyzed in 0.1 M Tris, 30 mM sodium acetate, pH 7.0 with 10 mM EDTA and further digested with chondroitinase ABC or AC as above. All digestions were carried out at 37°C for 24 hours, except for the heparitinase digestion, which was carried out at 43°C for the same time period. The reactions were stopped by boiling in reducing or non-reducing SDS-PAGE sample buffer for 5 minutes.

The following substrates were used to check the activity of the GAG-degrading enzymes (Sigma): chondroitin sulfates A and B, heparitin sulfate (bovine intestinal mucosa), and hyaluronic acid (human umbilical cord). Solutions of 0.1 mg/ml were digested with the enzymes listed above using the same enzyme concentrations, pH, time and temperature. Following the digestions, 10 µl aliquots were spotted onto Whatman #1 filter paper, and allowed to dry for 30 minutes. The filter paper was then stained for 15 minutes with 1% Alcian blue in 3% acetic acid, and destained with 3% acetic acid. Control digests received no enzyme treatment but were incubated as above.
Table 2: Enzymes and chemicals used in the structural analysis of glycoconjugates

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Source $^1$</th>
<th>Substrates</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Testicular hyaluronidase</td>
<td>Bovine testes</td>
<td>Hyaluronate, chondroitin 4 and 6 sulfates dermatan sulfate (in part)</td>
<td>Meyer, 1970</td>
</tr>
<tr>
<td>Chondroitinase ABC</td>
<td>Proteus vulgaris</td>
<td>Chondroitin 4 and 6 sulfates Dermatan sulfate and hyaluronate (slowly)</td>
<td>Yamagata et al., 1968</td>
</tr>
<tr>
<td>(chondroitin ABC lyase)</td>
<td></td>
<td>Chondroitin 4 and 6 sulfates hyaluronate (slowly)</td>
<td></td>
</tr>
<tr>
<td>Chondroitinase AC</td>
<td>Arthrobacter aurescens</td>
<td>Heparin, heparan sulfate</td>
<td>Suzuki, 1972</td>
</tr>
<tr>
<td>(chondroitin AC lyase)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heparinase III</td>
<td>Flavobacterium heparinum</td>
<td></td>
<td>Linker and Hovingh, 1972</td>
</tr>
<tr>
<td>(heparitinase I)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>α-L-Fucosidase</td>
<td>Bovine kidney</td>
<td>p-nitrophenyl α-L-fucoside</td>
<td>Iijima et al., 1971</td>
</tr>
<tr>
<td>Endoglycosidase F</td>
<td>Flavobacterium meningosepticum</td>
<td>N-linked high mannose and complex oligos</td>
<td>Elder and Alexander, 1982</td>
</tr>
<tr>
<td>8-D nitropyranoxyloside</td>
<td></td>
<td>Inhibits O-linked oligo assembly on proteoglycans $^2$</td>
<td>Roden, 1980</td>
</tr>
<tr>
<td>(8-xyloside)</td>
<td></td>
<td>Inhibits assembly of N-linked oligosaccharides</td>
<td>Elbein, 1987</td>
</tr>
</tbody>
</table>

$^1$ All enzymes and chemicals listed obtained from Sigma

$^2$ An exception to this is corneal keratan sulfate, which is N-linked.
(b) Endoglycosidase F digestion

For endoglycosidase F (endo F) digestion, aliquots of pure antigen or extracts prepared from gastrula-stage embryos were first dialyzed overnight in 0.1 M sodium phosphate, 50 mM EDTA, 0.5% Nonidet P-40, 0.1% SDS, pH 6.1 with protease inhibitors. To 5 µl of the antigen or homogenate was then added 20 U/ml endo F, and the digestion was carried out for 18 hours at 37°C (Elder and Alexander, 1982). Samples, including controls which were incubated in digestion buffer without enzymes, were subject to SDS-PAGE on 3-10% gradient gels under reducing conditions, and were transferred to PVDF membrane and probed with PM1 or PY4F8 antibodies as described in Section 5.

(c) Trypsin

PM1 antigen was also digested with trypsin 1:250 (Difco) at a concentration of 1 mg trypsin/pure antigen. The digestion was allowed to proceed for 3 hours at 37°C. Control digests included PM1 alone with protease inhibitors under the same conditions of time and temperature.

8. In vivo perturbation studies

(a) PM1 antibody perturbations

The in vivo effects of PM1 antibody on the development of blastulae, early gastrulae and post-mesenchyme-stage gastrulae were examined. The embryos were placed into 24-well tissue culture plates (Linbro) containing freshly airated Millipore-filtered (0.22 µm) sea water supplemented with 0.06 mg/ml gentamycin sulfate (Sigma), and containing purified PM1 antibody (whole molecule or fragmented) at concentrations ranging from 0 µg/ml to 100 µg/ml. Control embryos were grown in sea water containing pooled mouse IgM or IgG at the same concentrations as was used for the experimentals. The embryos were grown at 12°C, and the development of both experimentals and controls was monitored with Nomarski differential interference microscopy. Some of the embryos were removed from the treated sea
water 4 hours after the addition of antibody, fixed by freeze-substitution in 100% ethanol, and embedded in JB4. These embryos were sectioned (1-1.5 μm) and were immunolabelled with a secondary antibody (rabbit anti-mouse FITC) to determine if the PM1 antibody had entered the blastocoel. The embryos were photographed at the end of the experimental period using DIC optics as follows: immobilization of the swimming embryos was achieved with a brief fixation in 0.5% glutaraldehyde in 80% sea water, after which they were were placed on glass slides with coverglass held off the slide at a distance of 0.5 mm with plasticine. Photographs were taken on Fugicolor 100 film with a 16 x objective, using a Zeiss photomicroscope II fitted with a DIC slider and color prism. At the end of the experimental period, as determined when the controls reached mouth formation stage (5 1/2 days), experimental and control embryos were fixed by freeze-substitution and embedded into JB4 or LR White for morphological investigations at both the light and electron microscopic level. LM sections were stained with Richardson's cationic dye (0.5% methylene blue, 0.5% Azure II, 0.5% borax).

(b) β-D-Xyloside

Nitrophenyl β-D-xylopyrannoside (β-xyloside; Sigma) was solubilized in 95% ethanol and mixed with 2 volumes of PBS, pH 7.4, for a final stock concentration of 20 mg/ml. Embryos at the pre-mesenchyme early gastrulae stage (3 days) were transferred into sea water containing 5 mM and 0.5 mM concentrations of β-xyloside, and development was monitored. Controls consisted of embryos incubated in seawater containing 5 mM and 0.5 mM Nitrophenyl β-D-galactopyrannoside (Sigma) prepared in the same manner. The embryos were collected daily and transferred into sea water with freshly supplemented β-xyloside. At 5 1/2 days of development, embryos from the various treatments were photographed with DIC optics as above, and fixed by freeze substitution, embedded into JB4, and sectioned for PM1 immunostaining.
(c) Tunicamycin

Three day embryos were transferred into sea water containing 0.2 and 2 μg/ml tunicamycin (Sigma), prepared from a 1 mg/ml stock solution in DMSO (controls included DMSO alone). The embryos were collected and transferred into fresh incubation media daily until the controls reached mouth formation stage (5 1/2 days). At this time, embryos were photographed using DIC optics as described above, or fixed by freeze-substitution, embedded into JB4 and sectioned for PM1 immunofluorescence staining, also as described above.
III. RESULTS

PART A: The PM1 proteoglycan in starfish gut morphogenesis

1. Immunolocalization of the PM1 antigen

(a) Immunofluorescence

Immunofluorescence staining with the PM1 antibody revealed that the PM1 antigen was present throughout the blastocoel ECM of early bipinnaria stage larvae (Figs 9A, 9B; green fluorochrome). The matrix was very strongly labelled at this late stage in development, and had a granular appearance. The PM1 label was also present in the digestive tract (endoderm) as well as in the digestive tract lumen where labelling was strongest in the esophageal region (Fig. 9A). Coelomic pouches (not shown), which are also derived from the endoderm, showed a similar pattern of labelling. The PM1 antibody did not bind to the hyaline layer, the other major extracellular component of the embryo which is located on the apical side of the ectodermal epithelium surrounding the entire embryo (Fig. 9A-red), although label was present over regions of the ECM lining the gut, which is continuous with the hyaline layer and which has been described as morphologically similar to the hyaline layer (Crawford and Abed, 1986). This area of specialized ECM (hereafter referred to as the gut ECM) displays at least 2 epitopes that are not present in the hyaline layer, including the PM1 antigen (Fig. 9A) and the PG5F9 antigen (data not shown), indicating that although it appears similar morphologically, it is not identical to the hyaline layer. Labelling of basement membranes (BM) was not obvious, because it was difficult to distinguish BM labelling from labelling of the adjacent ECM at the light microscopic level. In addition to extracellular localizations in the blastocoel and digestive tract lumen, the PM1 antigen was also detected intracellularly in the endodermal cells of the digestive tract, suggesting that synthesis of the antigen might be occurring in these cells (Fig. 9B). PM1 labelling in these cells was characterized by brightly stained granules, which were often more towards the apex of the cells. Labelled granules were also sometimes aligned at the cell-cell interface, suggesting that the material was between the epithelial cells. Mesenchyme cells actively migrating through the blastocoel displayed filopodial extensions and were closely associated with the PM1 antigen-
Fig. 9. Immunofluorescence localization of PM1 antibody in the early larvae.

(A) A 1 1/2 μm median sagittal section through an early bipinnaria larva, which has been fixed by freeze-substitution into ethanol and embedded in plastic (JB4). The double-labelled section shows all elements of the digestive tract, including the mouth (m), esophagus (e), stomach (s) and intestine (i). PM1 antibody (green) binds to the matrix throughout the blastocoel (b) and is also found in the lumen of the digestive tract, with an especially high concentration of label in the esophageal region. No staining of the hyaline layer (hl) is observed with the PM1 antibody, which in this section has been stained with an anti-hyaline layer antibody (red). Bar = 40 μm.

(B) A higher magnification view showing the gut endoderm of a late gastrula embryo prepared as above, and stained with PM1 antibody. The endodermal cells (en) are strongly labelled and show numerous bright granules in the cell apices. Other PM1 positive granules are aligned in rows (arrowheads). Simultaneous observations with phase contrast microscopy (not shown) suggests that they are following the lateral cellular borders. Labelling is also observed over the gut ECM, which is associated with the apical surfaces of endodermal cells, and is especially pronounced in the esophageal (e) region. Bar = 15 μm.

(C) A section through a late gastrula embryo prepared as above, which has been stained with both PM1 antibody (green), and with the P212 antibody (red). The latter antibody binds to an antigen associated with membranes of both mesenchyme and epithelial cells. Mesenchyme cells are actively migrating through the PM1-rich matrix, and also contain intracellular PM1-positive granules (arrow). Bar = 10 μm.
rich matrix, as were the basal surfaces of the epithelial cells (Fig. 9C). Some mesenchyme cells also showed labelling of intracellular granules, indicating that these cells may be synthesizing the antigen as well (Fig. 9C).

(b) Immunogold

Immunogold staining with the PM1 antibody revealed several aspects of localization, synthesis and trafficking routes that were not obvious with immunofluorescence. Gold particles were typically not found over the major strands of blastocoel matrix, but rather on amorphous material that was often associated with them. Only a few gold particles were associated with the basal lamina (BL) suggesting that little of the antigen was localized here (Fig. 10A, B). Golgi complexes of endodermal cells were typically located in the apex of cells close to the gut lumen. Gold particles were found almost exclusively over the trans-Golgi region, as well as in Golgi-associated vesicles (Fig. 10C, D), indicating that the PM1 antibody might recognize a carbohydrate epitope, as carbohydrates are known to be added and modified here (Kornfeld and Kornfeld, 1985). The PM1 antigen was also detected in the gut ECM, which borders the apical surface of endodermal cells (Fig. 10D). However it was not present in the morphologically similar hyaline layer, which borders the apical surfaces of ectodermal cells. Gold particles were also present intercellularly between the lateral borders of adjacent cells (Fig. 11A), suggesting that the antigen may reach the blastocoel by exocytosis at the lateral cell borders below the junctional complexes, followed by migration between the cells and through the BL. The Golgi complexes of mesenchymal cells were also decorated with immunogold (Fig. 11B), indicating that synthesis may also have been occurring in these cells. Control sections stained as above with mouse IgM replacing PM1 antibody showed no gold labelling (data not shown).
Fig. 10. Immunogold localization of the PM1 antigen in the ECM and Golgi of late gastrula embryos.

In this figure and the one following (Fig. 11), embryos were fixed by freeze-substitution in ethanol and embedded in LR White resin. As osmium has been eliminated from the fixation protocol to maintain maximum antigenicity, the visualization of membranes is poor.

(A) A TEM showing the basal region of 2 endoderm cells, their accompanying basal lamina (bl), and the matrix-filled blastocoel cavity. Although the ECM is heavily labelled with gold particles, very few gold particles are observed over the bl. Bar = 0.6 μm.

(B) A higher magnification of the ECM shown in Figure A, demonstrating that the labelling with PM1 is over amorphous regions which are associated with the fibrous meshwork of matrix, rather than directly on the major fibers. Bar = 0.2 μm.

(C) This figure shows the Golgi apparatus located in the apical region of an endodermal cell. Note that PM1 labelling in the Golgi appears to be restricted to the trans-Golgi region (arrows), as well as to Golgi-associated vesicles (arrowheads), suggesting that the antibody may be recognizing a carbohydrate epitope. Bar = 0.3 μm.

(D) A section through the apical region of an esophageal endoderm cell of an early bipinnaria larva, showing the gut ECM which borders the apical surfaces of the endoderm cells, extending into the lumen of the digestive tract. Gold particles are observed in the Golgi complex (gc) as well as in associated vesicles, indicating that the PM1 antigen is secreted in vesicles. Some of the PM1 antigen is directed towards the lumen of the digestive tract, where it is found over the gut ECM (straight arrows). In addition, isolated aggregates of gold labelled material are also located between the Golgi apparatus and the base of the cell (curved arrows) suggesting that this material may be moving towards the blastocoel. Bar = 1.0 μm
Fig. 11. Immunogold localization of the PM1 antigen in mesenchyme and endoderm cells.

(A) A TEM of the basal region of 2 gut epithelial cells of a gastrula stage embryo, demonstrating that material labelled with PM1 antibody is found in the space between the 2 cells. This suggests that transport of the antigen from the Golgi complex to the blastocoel may occur via an intercellular route, with material that is secreted at the lateral cell borders basal to the cell junctions passing between the cells and through the basal lamina (bl) to enter the blastocoel. Bar = 1.0 μm.

(B) A TEM of a mesenchyme cell from a gastrula showing gold particles that are aligned along the Golgi (arrows), suggesting that the PM1 antigen may also be synthesized by these cells. Bar = 2.0 μm.
2. Developmental distribution of PM1 Immunoreactivity

PM1 immunoreactivity was first detected in the vegetal plate of the hatched blastula, where it was limited to a few brightly labelled granules in some of the vegetal plate cells, and some diffuse light staining throughout the blastocoel (Fig. 12A). Shortly after gastrulation, intracellular staining of the endodermal cells was more obvious, and was primarily found in the apices of these cells (Fig. 12B). As in the blastula stage, very little if any PM1 was detected in the blastocoel cavity of the early gastrula. By mid gastrula, as development of the mouth and digestive tract progressed, both PM1 staining and newly formed mesenchyme cells were observed in the blastocoel (Fig. 12C). PM1 staining of the blastocoel during the later stages of gastrulation was dramatically increased; strongly positive material was observed throughout the blastocoel, while some staining of the gut ECM was also apparent (Fig. 12D). As stated before, PM1 immunoreactivity was found throughout the blastocoel and gut of the early larvae. Throughout development, intracellular staining appeared to be restricted to endodermally-derived cells, including the digestive tract, coelomic pouches and mesenchyme cells. PM1-positive material was never observed in the ectoderm or stomodeal cells of the developing embryo/larva. No non-specific staining was observed in any of the normal mouse serum controls (data not shown).

3. Immunoblot analysis of the PM1 antigen in development

Analysis of the appearance of PM1 in development was also undertaken using Western blots. When examining material extracted from eggs through larval stages of development, a dramatic change in the apparent molecular masses of extractable material was seen over time, probably due to the depletion of the large yolk proteins, which are abundant in the early embryo (Fig. 13A) (see Part B). When the same 4 developmental stages, unfertilized oocyte, blastula, gastrula and early larva, were probed with PM1 antibody, staining was not present in the unfertilized oocyte. A faint band of immunoreactivity at about 600 kDa was
Fig. 12. Immunofluorescence localization of the PM1 antigen in early development.

The figure shows the localization of PM1 immunoreactivity during early development using 1 1/2 μm thick sagittal sections through embryos of different stages. Bar, 30 μm.

(A) A blastula-stage embryo in which labelling with PM1 is restricted to a few brightly stained granules in cells found in the vegetal plate region (arrowheads), and to some very lightly stained material in the blastocoel (b).

(B) An early gastrula-stage embryo, stained as above, in which endodermal cells have brightly labelled granules located primarily in the cell apices (arrowheads). There is still almost no labelled material in the blastocoel.

(C) A mid-gastrula embryo in which mesenchyme cell (mc) formation has begun. PM1 staining of ECM in the blastula has increased significantly at this stage. Note that intracellular staining is still restricted to the endoderm (arrowheads).

(D) A late gastrula embryo in which mouth formation is almost complete, and archenteron elongation and bending has occurred. The blastocoel is heavily labelled with PM1 antibody, and the endodermal cells have many labelled granules, suggesting that this is a very active period in PM1 synthesis.
detected at the blastula stage (Fig. 13B). By the gastrula stage, this band was stained strongly, and at the early larval stage, the intensity and size of the band increased further, indicating that there was a gradual increase in the amount of PM1 antigen present during gastrulation and early larval development. (Fig. 13B). The same amount of protein was loaded in each lane (10 μg) in order that the concentration of PM1 antigen in the egg and embryos could be compared through development. Because the amount of PM1 antigen in the embryo steadily increased during development, the material from gastrula and larval stages contained too much of the antigen for it to be separated optimally on the gel. This resulted in broadly stained, over-developed bands in lanes L and G (Fig. 13B), which none-the-less clearly indicated that the PM1 antigen steadily accumulated through development.

4. Perturbation of development with PM1 antibody

The function blocking experiments in which PM1 antibody was added directly to cultures of embryos initially involved the use of whole unfragmented antibody. Because of the large size of this antibody (IgM class, 900 kDa), the experiments were repeated with 130 kDa antibody fragments (see section 3e above); by using the smaller fragments, it was thought that the possibility of non-specific steric-related interactions occurring at the site of antibody binding would be diminished. The results of experiments in which whole and fragmented antibody were used were essentially the same, and the data reported in this section (Fig. 14) represents experiments in which fragmented PM1 antibody was used. Since the PM1 antibody was added directly to sea water in which early gastrula embryos were swimming, rather than administered directly into the blastocoel by injection, it was necessary to determine whether it had successfully entered the blastocoel. To achieve this, some embryos were removed from the incubation media 4 hours after the addition of antibody and stained with a secondary anti-PM1 antibody. These embryos showed positive staining in both the area of the blastocoel as well as in the endodermal cells (Fig. 14B), indicating that indeed the PM1 antibody had entered the embryo. No staining was apparent in embryos treated with normal mouse IgM in the incubation medium (Fig. 14C).
Fig. 13. Western blot analysis of the PM1 antigen in early development.

Crude embryonic extracts containing 10 μg/ml of protein/lane from either unfertilized oocytes (O), blastulae (BI), gastrulae (G) and early larvae (L) were separated by electrophoresis on 3-10% gradient gels. The material was then transferred onto PVDF membranes and stained either for total protein with Coomassie blue (A), or for PM1 immunoreactivity (B). PM1 does not appear to be present in the oocyte (O). It is first detected in the blastula stage where a faint band of staining with an apparent Mr greater than 600 kDa appears (BI, arrowhead). Following this, PM1 levels increase dramatically during gastrulation (G) and continue to increase during larval development (L). Overloading of the lanes contributes to the smeared bands in lanes G and L. The arrow marks the origin of the running gel.
Fig. 14. Effect of PM1 antibody on developing embryos *in vivo*.

This figure demonstrates the effect of PM1 antibody on the development of early gastrula embryos. The antibody was added directly to sea water containing swimming early gastrula embryos.

(A) A 1.5 μm mid sagittal section through a 3 day embryo fixed just prior to the addition of PM1 antibody.

(B) The embryo has been fixed by freeze substitution 4 hours after exposure to 10 μg/ml PM1 antibody, and probed with a FITC labelled anti-mouse IgM. The presence of label in the blastocoel demonstrates that the IgM penetrates into this region. The labelling seen in the endoderm is probably due to PM1 antibody which is bound to material located on the surface or between the endodermal cells.

(C) An embryo grown for 6 hours in the presence of non-specific mouse IgM (control) for comparison with Fig. B. The archenteron is more elongated than that of the experimental embryos. In addition, mesenchyme cells, which are not yet present in the experimentals (B), are beginning to be formed in the blastocoel of the controls.

(D) A median sagittal section through a control embryo (treated with mouse IgM) at 5 1/2 days of development. Mesenchyme cells are present in the blastocoel, the coeloms (c) are present, a mouth (m) has formed, the gut has become segmented, and an oral hood (oh) is forming.

(E) A median sagittal section through an embryo of the same age as that seen in Fig. D, following treatment with PM1 for 2 1/2 days. Although some mesenchyme cells have formed, the archenteron has elongated little beyond that seen in Fig. B (4 hours post-treatment) and shows no evidence of segmentation; the mouth and coeloms have also failed to form.

(F) This embryo is similar to that seen in Fig. E but has been photographed using DIC phase contrast optics; the mouth and coeloms have failed to differentiate and appear to be represented by a ball of disorganized cells at the tip of the archenteron (arrow). Bar = 40 μm.
Embryos from 3 different developmental stages were treated with PM1 antibody. These included the blastula, early gastrula and mesenchyme-cell gastrula stages. When blastula-stage embryos were grown in the presence of PM1 antibody, they continued to develop normally for 1 day, however shortly after archenteron formation, the embryos were affected by this treatment. These embryos resembled early gastrulæ that were grown under the same conditions (Fig. 14). By 5 1/2 days of development, the control embryo had formed mouth and coeloms similar to normal development (Fig. 14D). However, the morphology of embryos treated with 10 µg/ml antibody was very different from the controls. These embryos had a shortened unsegmented gut; in some cases the tip of the archenteron remained rounded (Fig. 14E), and in others it appeared to consist of a knot of irregular cells (Fig. 14F). No coelom or mouth formation was evident, and the blister of BM usually seen to extend off the tip of the archenteron was lacking. Although mesenchyme cells did form in these embryos, their arrangement in the blastocoel appeared random, and there was no evidence of mesenchyme cell differentiation. Gastrulæ at a slightly later stage of development, (mesenchyme cell-stage) were incubated with the PM1 antibody. These embryos were able to form a digestive tract, however, segmentation and size was altered somewhat, indicating that the effect of the antibody on development at this later stage was not as severe (data not shown).

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Aliquots of affinity-purified PM1 antigen were subject to SDS-PAGE and then stained with dyes specific for proteins and carbohydrates to determine the biochemical nature of this antigen. Although staining of the total embryo homogenate was evident in all cases, the pure antigen was stained only with Alcian blue; no Coomassie blue- or PAS-positive material was observed in lanes containing the PM1 antigen (Fig. 15). In addition, the PM1 antigen was not detected with silver staining (data not shown). The Alcian blue
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5. Biochemical characterization of the PM1 antigen

(a) Biochemical stains

Aliquots of affinity-purified PM1 antigen were subject to SDS-PAGE and then stained with dyes specific for proteins and carbohydrates to determine the biochemical nature of this antigen. Although staining of the total embryo homogenate was evident in all cases, the pure antigen was stained only with Alcian blue; no Coomassie blue- or PAS-positive material was observed in lanes containing the PM1 antigen (Fig. 15). In addition, the PM1 antigen was not detected with silver staining (data not shown). The Alcian blue
Guanidine extracts of bipinnaria larvae (E) and affinity-purified PM1 antigen (PM1) have been separated on SDS-PAGE and stained with Coomassie blue (A), PAS (B) and Alcian blue/magnesium chloride (C). In the case of PAS, EHS laminin (420/210 kDa) was added as a control (LN). Note that PM1 fails to stain with Coomassie blue and PAS (arrows), whereas both PM1 (arrow) and an equivalent band in the larval extract (E) stain with Alcian blue, suggesting that this molecule is a proteoglycan.
A. Coomassie Blue
(Proteins)

B. Periodic Acid-Schiff
(Glycoproteins)

C. Alcian Blue/MgCl₂
(Proteoglycans)
stained a single band migrating at 600 kDa, suggesting that the PM1 antigen contains many negatively charged alcianophilic groups characteristic of proteoglycans and GAGs (Fig. 15C).

(b) Subunit analysis

To determine the subunit nature of the PM1 antigen, it was subject to SDS-PAGE under both reducing and non-reducing conditions and then visualized by staining with Alcian blue, and by Western blot analysis. Fig. 16A shows that the apparent Mr of the PM1 antigen was the same under reducing (R) and non-reducing (NR) conditions when subject to SDS-PAGE. This would suggest that the PM1 antigen may consist of 1 large molecule rather than an aggregate of subunits. In addition, when the pure antigen was transferred to PVDF membrane and immunostained with PM1 antibody, the major bands present were again identical in their electrophoretic mobility (16B), indicating that the antibody is indeed binding to the affinity-pure antigen. The additional minor band migrating with at 900 kDa present under non-reducing conditions was also present in the control lane, and therefore represented some PM1 antibody (IgM subclass, 900 kDa) that was eluted off of the affinity column along with the PM1 antigen during chromatography.

(c) Enzymatic digestions

To further assay for the presence of GAG chains characteristic of proteoglycans, the PM1 antigen was subject to enzymatic degradation by hyaluronidase, chondroitinase ABC, chondroitinase AC and heparitinase, and was then analyzed for GAG content with SDS-PAGE with Alcian blue staining. Fig. 17 shows that in no cases was the staining intensity affected significantly by enzymatic treatment at concentrations which degrade known standards.
Fig. 16. Electrophoretic analysis of the PM1 antigen under reducing and non-reducing conditions.

Pure PM1 antigen was subject to SDS-PAGE under reducing and non-reducing conditions and then stained with Alcian blue (A) or transferred to PVDF and stained with PM1 antibody (B).

(A) The PM1 antigen has the same apparent Mr under reducing (R) and non-reducing (NR) conditions, indicating that it contain covalently-bound subunits.

(B) A Western blot showing a strong band of immunoreactivity at 600 kDa in both the reducing (R) and non-reducing (NR) lanes. An additional minor band apparent in the non-reducing lane (arrow) also present in the control lane (C) represents PM1 antibody (IgM subclass, 900 kDa), that has been eluted off the affinity column with the antigen. This blot demonstrates that the PM1 antibody does indeed recognize the affinity-purified antigen, and confirms that electrophoretic mobility is identical under both reducing and non-reducing conditions.
Fig. 17. Electrophoretic analysis of the PM1 antigen after treatment with GAG-degrading enzymes.

Aliquots of affinity-pure PM1 antigen were first digested with various GAG-degrading enzymes (37°C, 24 hours) and then analyzed by SDS-PAGE under reducing conditions. The gels were stained with Alcian blue in order to detect GAGs. Lane 1 shows the control digest in which the antigen was incubated without enzymes. Lane 2, chondroitinase ABC (ch. ABC)-digested material; lane 3, chondroitinase AC (ch. AC)-digested antigen; lane 4, antigen digested with heparitinase (hep.); and lane 5, hyaluronidase (hyal.)-digested antigen. The same amount of starting antigen was used in all the incubations. The band in lane 2 appears to be slightly weaker in intensity when compared to the control lane (1), indicating that there may be dermatan sulfate groups present on the PM1 antigen.
6. Epitope determination

(a) Periodate treatment

Antibody binding to the trans Golgi region of endodermal and mesenchyme cells suggested that the epitope was dependent on carbohydrate residues. To further investigate the nature of this epitope, sodium periodate treatment and enzymatic digestions of the antigen were performed (Fig. 18). When strips of PVDF membrane containing PM1 antigen were subject to sodium periodate oxidation under conventional conditions (20-50 mM periodate in 50 mM sodium acetate for 1 hour at room temperature) (Fig. 18A, lane 2), PM1 immunoreactivity did not appear to be different from the control (Fig. 18A, lane 1). When periodate oxidation was carried out in a high ionic strength buffer (0.2 M sodium perchlorate) the immunoreactivity was reduced significantly (Fig. 18, lane 4) when compared with the control blot (lane 3), suggesting that the PM1 antibody is directed towards a carbohydrate-dependent epitope that contains highly negatively charged groups.

(b) Endo F and trypsin treatments

Further investigations included enzymatic digestions of aliquots of the PM1 antigen, followed by Western blot analysis. After digestion with endo F, an enzyme which removes N-linked carbohydrate moieties, no difference in band profiles was observed when compared with the control digestion (Fig. 18B, lanes 1 and 2). This indicated that the PM1 antibody is not directed towards an N-linked carbohydrate epitope. In addition, the PM1 antigen did not appear to be sensitive to enzymatic degradation by this enzyme at concentrations that degraded control glycoproteins, since no shift in the apparent Mr of the band was evident after digestion. Trypsin treatment of the purified antigen resulted in the total loss of PM1 immunoreactivity, suggesting that the antigen may contain polypeptide components (Fig. 18B, lanes 3 and 4).
Fig. 18. Analysis of PM1 immunoreactivity after treatment with periodic acid, endoglycosidase F and trypsin.

The figure shows the effect of periodate oxidation (A) endoglycosidase F and tryptic digestions (B) on the PM1 epitope.

(A) Pure antigen was subject to SDS-PAGE under reducing conditions and transferred to PVDF. Strips of the membrane were then subject to periodate oxidation under conventional conditions (low ionic strength; lane 2) or with 0.2 M sodium perchlorate in the buffer (lane 4). Following periodate treatment, PM1 strips were reduced with sodium borohydrate and then immunostained with PM1 antibody. Controls strips were incubated in buffer (lane 1-low ionic strength, lane 3-high ionic strength) without periodate and then treated similarly to the experimentals. Only under conditions of high ionic strength was periodate effective in reducing PM1 immunoreactivity (lane 4, arrow), suggesting that the epitope contains highly charged anionic groups which are characteristic of GAGs.

(B) Aliquots of pure antigen were subject to enzymatic digestion (24 hours, 37°C) with endoglycosidase F (lane 2) or with trypsin (lane 4) prior to Western blot analysis with the PM1 antibody. After endo F digestion (lane 2), PM1 immunoreactivity is identical to that of the control digestion (lane 1). However, after digestion with trypsin, PM1 immunoreactivity is almost completely abolished (lane 4) when compared with the control digestion (lane 3), indicating that the PM1 antigen has a polypeptide component.
(c) Analysis with sugar-degrading enzymes

When aliquots of PM1 antigen were subject to the same GAG-degrading enzymes as used above and then transferred to PVDF, there was no noticeable difference in the intensities of PM1 immunoreactive bands or in the electrophoretic mobility of the antigen (Fig. 19, lanes 2-5), indicating as above that the PM1 antigen does not contain GAGs typical of those associated with vertebrate proteoglycans. Furthermore, when PM1 antigen was subject to α-fucosidase digestion prior to chondroitinase ABC or AC digestion (Fig. 19, lane 7, 8), there was no difference in PM1 immunoreactive band profiles when compared with the control (Fig. 19, lane 6), indicating that the PM1 epitope does not appear to be blocked from chondroitinase digestion by fucose residues as is the case in some sea cucumbers and sponges (Kariya, et al., 1990; Vieira and Mourao, 1988; Misevic and Burger, 1993).

7. Perturbation of development with tunicamycin and β-xyloside

(a) Morphological effects

Treatment with both tunicamycin and β-xyloside disrupted mouth and digestive tract morphogenesis in P. ochraceus embryos. Tunicamycin, which inhibits glycoprotein synthesis by preventing the assembly of N-linked oligosaccharide chains to proteins, was effective at a very small dose. At a time when control embryos had undergone mouth and coelom formation, embryos continually exposed to 0.2 μg/ml tunicamycin from early in gastrulation were characterized by a smaller rounded blastocoel with a very shortened archenteron displaying no evidence of segmentation. It was, however, bent over to one side of the ectoderm, as if attempting to establish contact with the presumptive stomodeal region for mouth formation (Fig. 20A). When embryos were grown for the same time period in the presence of 5 mM β-xyloside, which negatively competes for O-linked xylose-mediated GAG chain attachment to the protein core of proteoglycans, they were smaller than normal and the archenteron extended almost the entire length of the blastocoel. There was little or no segmentation or differentiation of the archenteron, and no apparent bending (Fig. 20C). In both tunicamycin- and β-xyloside-treated embryos, mesenchyme cell
Fig. 19. Western blot analysis of the PM1 antigen after treatment with GAG-degrading enzymes and fucosidase.

Aliquots of PM1 antigen were subject to enzymatic digestion with various GAG-degrading enzymes prior to Western blot analysis with the PM1 antibody. In addition to digestion with chondroitinase ABC (ch. ABC), chondroitinase AC (ch. AC), heparitinase (hep.) and hyaluronidase (hyal.) alone (lanes 2-5), aliquots of the antigen were also digested with α-fucosidase (fu) prior to digestion with ch. ABC and ch. AC (lanes 7, 8) to determine if fucose residues were blocking the activities of the chondroitinases. In all cases, immunoreactivity remains the same when compared with the controls (lanes 1, 6), indicating that the PM1 epitope is not altered by the above enzymes. Arrow indicates the start of the running gel.
formation occurred despite the fact that blastocoel expansion was drastically reduced. The β-xyloside-treated embryos strongly resembled those that were grown in the presence of the PM1 antibody.

(b) Effects on PM1 synthesis

Embryos were treated with tunicamycin as described above, fixed at 5 1/2 days growth, and processed for thin section immunofluorescence. These embryos showed strong PM1 labelling of material in the blastocoel (Fig. 20D) that was of a comparable staining intensity with that of the controls (Fig. 20E). This indicated that PM1 synthesis and/or secretion was occurring at normal levels, even though the embryos had experienced a developmental perturbation as a result of the tunicamycin treatment. However, embryos that were treated similarly with β-xyloside and examined at 5 1/2 days growth showed a significant reduction in the amount of PM1 staining in the blastocoel (Fig. 20F) when compared with the controls. This suggested that the synthesis and/or post translational modification of the PM1 antigen was disrupted by treatment with β-xyloside, and that the PM1 antigen shares characteristics of O-linked proteoglycans.
Fig. 20. Effects of tunicamycin and β-xyloside on *P. ochraceus* development and on PM1 synthesis.

This figure shows the effects of tunicamycin and β-xyloside on embryonic development (A-C) and on PM1 synthesis (D-F). Bar = 50 μm for A-F inclusive. (A-C) Embryos at the early gastrula stage were exposed to 0.2 μg/ml tunicamycin, 5 mM β-xyloside or control media, which was changed daily for 3 days. At 5 1/2 days, the tunicamycin-treated embryos (A) are smaller than controls (B), and have a rounded blastocoel with a shortened archenteron (a) which bends over to one side. The β-xyloside-treated embryos (C) are also smaller than the controls, and have an elongated essentially non-partitioned archenteron (a) that extends almost the length of the blastocoel (C). All controls are similar, having a normal tripartite differentiated digestive tract (B).

(D-F) Embryos corresponding to those in Figures A-C were fixed by freeze-substitution in ethanol and embedded in JB4. Mid-saggital sections were then immunostained with the PM1 antibody. Labelling of the matrix in the tunicamycin-treated embryo (D) was comparable in intensity to that of the control embryo (E), indicating that although tunicamycin disrupts normal development, it does not significantly alter the synthesis of the PM1 antigen. However PM1 labelling of β-xyloside embryos was significantly reduced (F) when compared to the control, indicating that this inhibitor of O-linked GAG assembly has a direct inhibitory effect on PM1 antigen synthesis and/or assembly (tun = tunicamycin; xyl = β-xyloside; cont = control). Bar = 50 μm.
1. Specificity of antibodies against starfish egg granules

When immature starfish (*P. ochraceus*) oocytes (those with germinal vesicles intact) were stained with the monoclonal antibodies PY4F8 and PC3H2, two distinct populations of granules were observed (Fig. 21). The granules labelled by the PY4F8 antibody clearly formed a major constituent of the oocyte volume; they were the larger of the 2 types, and were dispersed throughout the cytoplasm of the egg (Fig. 21A). This pattern of distribution is typical of yolk storage granules in other species, and suggests that the labelled granules seen in this starfish oocyte also contain yolk. The second granule type was labelled by the PC3H2 antibody; these granules were concentrated in the peripheral egg cytoplasm, and appeared to represent cortical granules. A few were also dispersed in a random fashion throughout the cytoplasm of the immature oocyte, although in lesser numbers (Fig. 21B). Double label immunofluorescence revealed that these 2 types of vesicles were distinct (Fig. 21C); the PY4F8-labelled granules reached diameters of 4 μm, and were absent from the cortical region of the egg. The PC3H2-labelled granules, roughly 1.5 μm in diameter, were primarily in the cortical cytoplasm but were also scattered throughout the cytoplasm interspersed between PY4F8-labelled granules, indicating that they were a distinct population.

2. Ultrastructural analysis of PY4F8 immunoreactivity

Ultrastructural observations of mature oocytes fixed by freeze-substitution in Alcian blue-saturated ethanol indicated that a large part of the egg cytoplasm was occupied by a homogeneous population of granules of intermediate staining density. These same granules were decorated with immunogold label after staining with PY4F8 antibody (Fig. 22A). Also present in the mature oocyte were the highly electron dense cortical granules located in the peripheral cytoplasm; these granules did not have PY4F8-gold label
Fig. 21. Immunofluorescence localization of yolk and cortical granule antigens.

Immature oocytes fixed by freeze substitution in ethanol and embedded in JB4 resin have been sectioned (1.5 μm) and immunolabelled with 2 antibodies against cytoplasmic granules.

(A) The PY4F8 monoclonal antibody labels ubiquitous granules dispersed throughout the egg cytoplasm that are characteristic of yolk granules or platelets. No staining is observed over the nucleus (n). Bar (A and B) = 40 μm.

(B) A serial section of the oocyte shown in Figure A but stained with PC3H2 monoclonal antibody shows labelling of a population of vesicles which are located primarily in the peripheral egg cytoplasm, but which are dispersed throughout the cytoplasm as well, although in lesser numbers. Staining of the nucleus (n) is again absent.

(C) A section through an immature oocyte prepared as above, which has been stained with both PY4F8 (red) and PC3H2 (green) identifies 2 different types of granules: the red PY4F8-labelled granules are larger, reaching diameters of 4 μm, and are absent from the cortical region of the egg, while the granules labelled with PC3H2 (green), are roughly 1 μm in diameter, and are found predominantly at the cortical cytoplasm just underlying the plasma membrane (pm). There are also some PC3H2-positive granules interspersed between the larger yolk granules throughout the cytoplasm (arrowheads). Bar = 15 μm.
Fig. 22. Immunogold localization of yolk antigens in the unfertilized oocyte.

TEMs of an unfertilized (mature) oocyte that has been prepared by freeze-substitution in Alcian blue-saturated ethanol, embedded in LR White resin, and stained with PY4F8 immunogold. Osmium has not been used in the fixative to maintain maximum antigenicity, and therefore the visualization of membranes is poor.

(A) At this low magnification, the oocyte cytoplasm shows many yolk vesicles (y) of intermediate staining density which are decorated with PY4F8-gold particles (see Fig. 22B), and the highly electron dense cortical granules (cg), which are much smaller and are located at the cell periphery. Also present are electron translucent vacuoles (v), some which contain amorphous material, but which have no gold label associated with them. Surrounding the oocyte adjacent to the egg pm is the vitelline membrane (vi), and next to that, the extensive jelly coat (jc). Bar = 3.0 μm.

(B) At high magnification, it can be observed that the yolk vesicles (y) are evenly decorated with PY4F8-gold label while the cortical granules (cg) have no label associated with them. In addition, another granule of intermediate staining density (g') is apparent in the cortex, but this granule has no gold label associated with it, suggesting that it may represents a distinct population. Bar = 1.0 μm.
associated with them (Fig. 22B). Other constituents of the egg cytoplasm included clear vacuoles, which were scattered throughout the egg cytoplasm, as well as a second population of granules, which were intermediate in density but fewer in number when compared with the yolk granules. These granules were not labelled with the immunogold PY4F8, indicating that there are at least 2 distinct populations of granules having intermediate staining density.

3. Ultrastructural localization of the PC3H2 antigen in the egg

Colloidal gold labelling of mature oocytes with PC3H2 (Fig. 23) confirmed that this antibody was directed to material in the structures previously identified as cortical granules (Crawford and Abed, 1986). These membrane bound granules were characterized by a highly electron dense periphery and a low density core, although patches of intermediate density were sometimes apparent. Labelling in this case was predominantly associated with the electron dense regions in the periphery (Fig. 23, inset). In addition, a few gold particles were found associated with the plasma membrane. No gold decoration of the yolk granules was observed.

4. Localization of the PC3H2 antigen in early development

As is the case in other echinoderms, cortical granules in starfish exhibit a cortical reaction which involves the release of their contents and elevation of the fertilization membrane (Holland, 1980). To observe the fate of the PC3H2 antigen during oocyte maturation and early development, immunofluorescence staining of sections of oocytes and early embryos were compared. Whereas in the unfertilized immature oocyte, granules were dispersed throughout the central and cortical cytoplasm, (Fig. 24A), the mature oocyte, observed just after fertilization, showed fewer granules in the central cytoplasm, and a higher concentration in the cortical cytoplasm (Fig. 24B). These activated eggs showed staining outside of the
Fig. 23. Immunogold localization of the PC3H2 antigen in the unfertilized oocyte.

A TEM of the cortical region of a mature oocyte stained with PC3H2 immunogold. Several gold decorated cortical granules (cg) are evident, and are located in the peripheral cytoplasm just under the egg plasma membrane. These membrane bound granules are diverse in their shapes, but typically have an electron dense component in the periphery, and an electron translucent region in the core. Patches of intermediate density are also apparent and are associated with the outer core. Gold particles are predominantly associated with the electron dense regions in the periphery (inset, bar = 0.3 μm). Some particles are also associated with the plasma membrane (arrows), however, no particles are associated with the yolk granules (y). vi = vitelline membrane. Bar = 1.5 μm.
Fig. 24. Immunofluorescence localization of the PC3H2 antigen in early development.

This figure compares the localization of PC3H2 immunoreactivity in 1.5 μm sections of the immature and just fertilized oocytes as well as in the 2 cell-stage and blastula stage embryo, using 1.5 μm thick sections. Bar, A-C = 10 μm; D = 40 μm.

(A) In this immature oocyte, labelling is present in both the cortical granules which are concentrated in the peripheral cytoplasm just beneath the plasma membrane (pm), and in granules scattered throughout the oocyte cytoplasm. Some light staining of the plasma membrane is also evident.

(B) A section through an oocyte which has been matured with the hormone 1-methyl adenine, fertilized, and fixed shortly after, showing that the majority of brightly stained granules are now concentrated in the cell periphery, with only a few scattered throughout the cytoplasm. A fertilization membrane (fm) has formed and is raised from the surface of the cell plasma membrane (pm). In addition, staining is evident on the outer side of the plasma membrane, indicating that some cortical granules have been activated and are releasing their contents into the space limited by the fertilization membrane.

(C) A section through a fertilized embryo after the first cleavage. The brightly stained cortical granules located at the periphery of the egg are no longer present at this stage. However, label is now concentrated on the outer side of the oocyte plasma membrane in the space limited by the fertilization membrane (fm), the perivitelline space (pvs). Several brightly stained granules still persist in the cell cytoplasm.

(D) A lower magnification view of an early blastula stage embryo, showing an ECM-filled blastocoel surrounded by blastomeres, some of which are still folded into the center of the blastocoel. The label is absent over the fertilization membrane (fm); however, brightly stained granules are still present in the blastomeres (arrowheads). The staining of the matrix in the blastocoel (b) is also evident.
cell plasma membrane in the space limited by the fertilization membrane (perivitelline space), suggesting that the contents of these granules was released into this space. By the second cleavage stage, there was a dramatic change in the PC3H2-staining pattern; brightly labelled granules were no longer present in the cortical cytoplasm, and a diffuse pattern of staining was present throughout the perivitelline space (Fig. 24C). Some cytoplasmic granules were labelled, however they were not located in the apical region of the cells. At 24 hours post-fertilization, the embryo had developed into a blastula, and PC3H2 staining was no longer present in the apical region, either in the perivitelline space nor associated with the fertilization membrane. However, some labelled granules were still present in the blastomeres, and in addition, labelling of the ECM of the blastocoel was evident (Fig. 24D).

Ultrastructural observations of blastula and gastrula stage embryos with PC3H2 immunogold revealed that these labelled granules had a morphology resembling the granules of the oocyte cortical cytoplasm, including an electron dense component over which gold particles were localized (Fig. 25A-C). In the gastrula, gold label was localized over ECM fibers of the blastocoel and the basal lamina (Fig. 25B ), as well as over one element of the hyaline layer (Fig. 25C). Further observations at this stage revealed that regions of the Golgi complexes were also labelled with a few gold particles, indicating that some new synthesis or post-translational modifications of the antigen was occurring. The cortical granules were often closely situated to the Golgi complex, suggesting that they may be providing a storage site for the newly synthesized antigen (Fig. 25C).

5. Distribution of PY4F8 Immunoreactivity during development

(a) Immunofluorescence

Further analysis was undertaken to study the fate of the PY4F8 immunoreactive yolk granules and their contents during development to determine whether they undergo depletion in a manner similar to known yolk proteins. Immunofluorescence on 1.5 μm sections of different staged embryos revealed that the
Fig. 25. Immunogold localization of the PC3H2 antigen in the early embryo

The figure shows TEMs of the unhatched blastula stage (A) and gastrula stage (B, C) embryos which have been fixed by freeze substitution in ethanol and processed for PC3H2 immunogold labelling.

(A) The unhatched blastula shows the presence of an electron dense PC3H2-positive granule, which resembles the cortical granules of the unfertilized egg. Gold particles are not associated with the fertilization membrane (fm). However some gold particles are found in the region just below it over elements of the developing hyaline layer (arrows). Bar = 0.6 μm.

(B) A section through an early gastrula stage embryo showing positive staining with the PC3H2 antibody of the basal lamina (bl) of endodermal cells, as well as over elements of the matrix in the blastocoel (*). Note that the electron dense granule (cg) that is positioned near the base of the cell also has gold label associated with it. Bar = 0.6 μm.

(C) This figure shows the apical region of 2 ectoderm cells of the same embryo as (B). Gold label is found over a cortical granule (cg) as well as over regions of 2 Golgi complexes (gc). As particles are found just outside the cg membrane, material is either being secreted or is being taken up by the granule for storage. In addition, one region of the hyaline layer (hl) is also decorated with PC3H2 immunogold (arrows). Bar = 0.6 μm.
granules observed in the oocytes were also present in early development, including the blastula stage (2 day old) and gastrula stage (4 day old) embryos (Fig. 26 A-D). The granules were stained with intensities that were comparable to those of the egg, suggesting that the amount of protein in the granules did not change substantially over the this time period (Fig. 26A-D). These putative yolk granules were seen in all three germ layers, including the ectoderm, endoderm and mesenchyme cells of the gastrula (Fig. 26D). Labelling was confined to the intracellular granules, with no label apparent in extracellular spaces or other areas of the cells. Stained granules were still present in all three germ layers of the early larva (Fig. 26E), although the intensity of the stain was reduced in comparison with that in the earlier stages of development (Fig. 26A-D). It was only at the late bipinnaria larval stage (12 days post fertilization) when the complete depletion of this material was observed (Fig. 26F). Labelling with PY4F8 was never seen outside of these granules, either within the cells or in extracellular locations.

(b) Immunogold

TEM observations of the early larva revealed granules that were intermediate in staining density with a morphology that resembled the yolk granules found in the oocyte. These granules also labelled with PY4F8-immunogold (Fig. 27), confirming the results with immunofluorescence. In addition to these granules, several other membrane-bound structures, including Golgi associated vesicles and electron translucent vacuoles were observed. No evidence of gold label was apparent outside of the granules either in the cytoplasm or in the extracellular matrices, suggesting that the material was not being released into the cell cytoplasm or extracellular spaces.
Fig. 26. Immunofluorescence localization of yolk proteins in the early embryo and larva.

In this series, eggs and embryos of various stages of development have been fixed by freeze-substitution into ethanol, and thin sections (1.5 μm) of JB4-embedded material have been stained with the PY4F8 antibody. Bar = 50 μm.

(A) The immature oocyte showing bright staining throughout the cytoplasm in round granules, but which is excluded from the nucleus (n).

(B) A 2-cell stage embryo, showing cells with abundant stained granules throughout the cytoplasm.

(C) A blastula stage embryo, showing intense labelling of granules in the blastomeres. The unstained regions in the cell apices represent nuclei. b = blastocoel.

(D) A mid-sagittal section through a gastrula, in which differentiation of the archenteron into the different regions of the digestive tract has begun. Labelled granules are still seen throughout the organism in all 3 germ layers, including the ectoderm (ec), the endoderm (en) and mesenchyme cells (mc).

(E) An early larval stage of development, showing a well differentiated digestive tract, with a mouth (m), esophagus (e), stomach (s) and intestine (i). Note that although many granules are still present throughout the organism, the staining intensity of these granules has decreased dramatically over this period of development.

(F) A late bipinnaria larva, showing some elements of the digestive tract, including the esophagus (e), stomach (s), intestine (i) as well as a coelomic pouch (cp). At this later stage in development, no labelled granules are present in the organism, indicating that the material has been completely depleted, and that no new synthesis of this material is occurring. This figure has been over-exposed during photography compared to those preceding it in order to increase the contrast.
Fig. 27. Immunogold localization of yolk proteins in the gastrula.

A TEM showing ectodermal cells of an early bipinnaria larva, prepared by freeze-substitution into Alcian blue-saturated ethanol, and labelled with PY4F8-gold. Gold particles are located over a single putative yolk granule (y), which has an intermediate staining density. Label is not located over smaller granules of similar staining density (g'), nor is staining observed in any other areas of the cell. Other structures in the cell include mitochondria (m), a nucleus (n), a cillum rootlet (c), lipid vacuoles (v) and on the apical cell surface, the hyaline layer (hl). Bar = 0.8 μm.
6. Immunoblot analysis of the PY4F8 and PC3H2 antigens

Detergent extracted material from immature oocytes was subject to Western blot analysis to identify the proteins constituent of the 2 granules types. Material probed with the PC3H2 antibody (cortical granule-specific) identified a single band of 125 kDa (Fig. 28A). When the same extract was probed with the PY4F8 antibody (yolk granule-specific), 3 bands were stained: a major band of 164 kDa, a minor band of 100 kDa and a doublet at 70 kDa. (Fig. 28A). To investigate the subunit nature of the yolk antigens, non-reduced samples (prepared without β-mercaptoethanol) were transferred to PVDF and probed with the PY4F8 antibody for comparison with reduced extracts (Fig. 28B). Under non-reducing condition, the same 3 major bands were present as those found in the reduced lane, indicating that these 3 yolk proteins are not present as a disulfide-linked multimer in the immature oocyte. In addition, a new band of 45 kDa was present under these conditions. As this band was not present under reducing conditions, it is possible that the β-mercaptoethanol, while not breaking inter-chain disulfide bonds, was altering immunogenicity by destroying intra-chain disulfide bonds of the 45 kDa protein. Furthermore, there was a significant difference in the antigenicity of the yolk proteins prepared under reducing and non-reducing conditions. Blots from non-reducing gels were several fold more antigenic, i.e., 0.2 µg total oocyte extract under non-reducing conditions had a comparable band signal as 1 µg protein in the reducing gels when probed with PY4F8 antibody. This further suggests that the β-mercaptoethanol altered the antigenicity of the yolk proteins, and indicates that PY4F8 may be directed towards a protein rather than a carbohydrate epitope.
Fig. 28. Western blot analysis of yolk and cortical granule antigens in the oocyte.

The figure shows detergent-extracted material from immature oocytes which has been subject to SDS-PAGE on 3-10% acrylamide gels under reducing (A) and non-reducing (B) conditions, and then transferred to PVDF for immunoblotting.

(A) Lane 1 was stained for total protein with Coomassie blue. Lane 2 represents 5 µg total protein which was immunostained with PC3H2, revealing a single band at 125 kDa. Lane 3 represents 1 µg of total protein which was probed with PY4F8 and shows 4 major bands at 164 kDa, 100 kDa and a doublet at 74 and 64 kDa.

(B) In this figure, material was prepared for electrophoresis without β-mercaptoethanol: Lane 1 was stained for total protein with Coomassie blue; lane 2 represents 0.2 µg of oocyte extract probed with PY4F8, showing a similar band profile as that observed under reducing conditions (A, lane 3). One additional band is stained at 48 kDa under non-reducing conditions.
7. Immunoblot analysis of PY4F8-yolk antigens during development

Detergent extracts from 4 different developmental stages, immature oocytes, blastulae, gastrulae and bipinnaria larvae were separated by SDS-PAGE gels under reducing conditions, and the proteins were transferred to PVDF and probed with PY4F8. Coomassie blue staining of the extractable proteins revealed a general decrease in proteins larger than 120 kDa during embryonic and larval development (Fig. 29A). PY4F8 immunoreactive bands were present in all the embryonic stages although no bands were observed in the larval stage extracts (Fig. 29B). In the unfertilized oocyte (O), 3 bands were present at 164 kDa, 100 kDa and a doublet at 70 kDa; at the blastulae stage (B), the 100 kDa band was no longer present, however the other 2 bands were identical to those of the oocyte. At the gastrula stage (G), only a broad band between 64-74 kDa remained, although a few minor bands were evident at 116 and 100 kDa. At the advanced larval stage (L), there was no evidence of immunoreactive bands. These results are consistent with the immunohistochemical findings, and suggest that the PY4F8 antigens are progressively depleted and/or broken down over embryonic and larval development.

8. Ontogeny of the major yolk proteins

To determine the possible source of the yolk antigens, both the adult intestine and coelomic fluids were examined for PY4F8-reactive proteins. The 3 major yolk proteins of the oocyte were present in both the intestine and coelomic fluids, suggesting that synthesis of the yolk proteins may be occurring in these locations (Fig. 30). In addition, under non-reducing conditions, a region of immunoreactivity at 400 kDa, not seen in the oocyte, was present in both intestinal extract and coelomic fluid (Fig. 30, arrows). Under reducing conditions, however, this 400 kDa band was not present, suggesting that this high molecular mass protein may be a disulfide-linked complex.
Fig. 29. Western blot analysis of yolk proteins in early development.

Western blot analysis with PY4F8, in which detergent extracted proteins from either unfertilized oocytes (O), blastulae (BI), gastrulae (G) and bipinnaria larva (L) were separated on SDS-PAGE (3-10% gradient gels) under reducing conditions, and transferred to PVDF membranes.

(A) Coomassie blue-stained membrane, in which 10 µg protein was loaded/lane showed that the higher molecular mass proteins are gradually lost during development.

(B) Western blot in which 1 µg total protein/lane was probed with the PY4F8 antibody. In the oocyte (O), 4 major bands are present at 164 kDa, 100 kDa and a doublet at 64 and 74 kDa. At the blastula stage (BI), the decrease of the 100 kDa band was apparent, and at the gastrula stage (G), only 1 major broad band remained between 64-74 kDa. However a few faint bands between 100 and 160 kDa were still present. Extract from the larval stage (L) showed no immunoreactive bands.
Mr  O  B  I  G  L

420
205
116
97
66
45

Coomassie

OBI  G  L

Western
Fig. 30. Western blot analysis of yolk proteins in the adult intestine and coelomic fluid.

The figure shows PY4F8 immunoreactivity to components of the adult intestine (A) and coelomic fluid (B). Intestinal extract and coelomic fluid was separated by electrophoresis on 3-10% gradient gels under both reducing and non-reducing conditions, and then transferred to PVDF and either stained with Coomassie blue (lanes 1 and 3) or probed with PY4F8 (lanes 2 and 4).

(A) Intestinal extract prepared without β-mercaptoethanol and stained with PY4F8 showed several immunoreactive bands at 400, 164, 120, 100 and 74 kDa (lane 2). Under reducing conditions, only 2 major bands at 164 and 74 kDa were apparent (lane 4), suggesting that the intestine contains a large (400 kDa) multimeric disulfide linked yolk protein.

(B) A band profile similar to that observed in the non-reduced intestinal extract (A, lane 2) was observed when coelomic fluid is probed with PY4F8 under non-reducing conditions (lane 2). Again, a region of immunoreactivity was present around 400 kDa (arrow), although it was a broader band than in the intestine; in addition, the 4 bands with lower molecular masses were also present. Under reducing conditions (lane 4), immunoreactivity was restricted to a single band migrating as a doublet at 164 kDa.
9. Lectin characterization of the yolk proteins

Of the different FITC-conjugated lectins used in this study, only WGA and Con A stained granules resembling the PY4F8-specific yolk granules (Fig. 31). The staining pattern was essentially the same for both of these lectins, characterized by strong labelling of the ubiquitous yolk granules of the oocytes. Positive labelling with Con A suggested that the granules contained glycoproteins of the high mannose or complex type, while positive labelling with WGA suggested that glycoproteins with terminal neuraminic acid or N-acetyl glucosamine residues were also contained within these granules.

To determine if the PY4F8 yolk antigens were in fact the yolk constituents being recognized by Con A and WGA, the antigens were isolated by immunoprecipitation from homogenized oocytes, and were then transferred to PVDF where they were probed with Con A and WGA. Coomassie blue staining of the immunoprecipitated PY4F8 antigens from oocyte extracts revealed 2 major bands at 164 kDa and 116 kDa, and 3 minor bands at 400 kDa, 100 kDa and 74 kDa (Fig. 32A, lane 1). In addition, immunoprecipitating IgG fragments were present at 55 kDa in both the PY4F8 immunoprecipitation and control lanes (Fig. 32A, lane 2). When the same samples were probed with PY4F8 antibody, similar bands were detected; however, no staining of the 116 kDa band was observed, indicating that this major protein may have co-precipitated together with the yolk antigens (Fig. 32A, lane 3). Alternatively, this protein may have represented a subunit lacking the PY4F8 epitope but derived from one of the larger yolk antigens. When the immunoprecipitations were probed with Con A, all of the PY4F8 yolk antigens were stained, including bands at 400, 164, 100, 74 and 64 (Fig. 32A, lane 5) as well as the 116 kDa band. With WGA, the 2 larger yolk antigens (400 and 164 kDa) stained positive, but no labelling of the 100, 74 or 64 kDa proteins was observed (Fig. 32A, lane 7). Control lectin blots revealed no binding under the same conditions. These results suggested that: (1) the yolk antigens were indeed glycoproteins; (2) all of the antigens contained N-linked high mannose or complex type (Con A-reactive) oligosaccharide chains; and (3) the larger yolk antigens also contained WGA-reactive sites, indicating the presence of neuraminic acid.
Fig. 31. Con A and WGA binding in the unfertilized oocyte.

The figure shows 1.5 µm sections through immature oocytes which have been prepared by freeze-substitution and stained with the FITC-labelled lectins Con A (A) and WGA (B). Both lectins appear to stain yolk granules found throughout the egg. Staining is excluded from the nuclei (n) and from the jelly coat which surrounds the oocyte. Bar = 50 µm.
or N-acetyl glucosamine terminal residues, characteristic of N-linked complex oligosaccharides and O-linked oligosaccharides (Beeley, 1985).

10. Epitope characterization of the PY4F8 yolk antigens

To further investigate the extent of glycosylation, as well as to determine the nature of the PY4F8 epitope, yolk proteins were treated with Endo F, an enzyme which removes all N-linked oligosaccharide groups from proteins. After digestion, all of the yolk antigens appeared to have a greater electrophoretic mobility than the controls (Fig. 32B, lane 2), indicating that they were sensitive to Endo F treatment and contained a considerable amount of N-linked carbohydrate. Despite this, immunoreactivity after endo F treatment was retained, showing that the epitope was not dependent on the these oligosaccharide moieties, but rather was directed to the protein core and/or to O-linked oligosaccharides.
Fig. 32. Characterization of PY4F8-immunoprecipitate with lectins and endoglycosidase F digestion.

(A) This figure demonstrates the binding patterns of Con A and WGA on immunoprecipitated yolk (PY4F8) antigens from immature oocytes. Immunoprecipitations and their controls were run on 3-
12 % gradient gels, transferred to PVDF, and stained as follows: Lanes 1 and 2 show Coomassie
blue staining of PY4F8 immunoprecipitated antigens (IP) and the control immunoprecipitation (IP-
C). In addition to the immunoprecipitated IgG fragments (50 kDa; arrow), which were present in
both lanes 1 and 2, several other bands were apparent in lane 1, including strong bands at 164
and 116 kDa, and weaker bands at 400, 100, and 74 kDa. In lane 3, the immunoprecipitated
antigens were probed with PY4F8 hybridoma supernatant, which detected bands at 164, 100, 74
and 64 kDa, in addition to the fragmented IgG (arrow). Lane 4 represents the same PY4F8-
immunoprecipitation as in lane 3 which was probed with a control monoclonal antibody, and which
showed specificity only for the fragmented IgG bands. The 116 kDa Coomassie stained band
present in lane 1 (arrow) was not present in lane 3, indicating that this protein co-precipitated with
the PY4F8 antigens, but was not a PY4F8-specific yolk protein. Lanes 5 and 6 show Con A-
staining of the immunoprecipitated antigens (Con A) and the control immunoprecipitation (Con A-
C). Several Con A-positive bands were present, including all of the bands detected in lanes 1 and
3, indicating that they were all glycosylated and contain terminal mannose residues. No bands
were apparent in the control lane 6. Lanes 7 and 8 represent blots identical to those in lanes 5
and 6, but stained with WGA. In this case, only the 2 larger yolk antigens and the 116 kDa co-
precipitated band were stained, indicating that these glycoproteins also contain terminal
neuraminic acid and/or N-acetylglucosamine residues.

(B) This figure shows the effects of Endoglycosidase F on PY4F8 yolk antigens. Detergent
extracted proteins from blastulae were digested for 18 hours at 37°C, separated by SDS-PAGE
under reducing conditions, and transferred to PVDF for immunodetection with PY4F8
supernatant. Controls were incubated without the enzyme and treated as above. When probed
with PY4F8, 3 major bands were present in the control digest (lane 1), including a band at 164
kDa, a doublet at 100 kDa, and a broad band at 70 kDa, which was representative of the typical
band profile observed in early embryos. After digestion with Endo F, several immunoreactive
bands were present (lane 2), indicating that the PY4F8 epitope was not dependent on the N-
linked oligosaccharides. In addition, the Endo F-digested proteins migrated with a greater
electrophoretic mobility, and there were no major bands which corresponded to those of the
control, indicating that most if not all of the yolk proteins recognized by PY4F8 were sensitive to
Endo F digestion and contained N-linked oligosaccharides.
IV. DISCUSSION

PART A: The PM1 proteoglycan in starfish gut morphogenesis

(I) Notes on methodology

1. Monoclonal antibody production

In this study, monoclonal antibodies were generated to isolate, characterize and study a morphogenetically active blastocoel-specific component in starfish gut morphogenesis using immunochemical and in vivo perturbation techniques. Monoclonal antibodies were chosen as a research tool, because with this technique a relatively impure antigen can be used to generate a host of antibodies, and then the particular one(s) of interest can then be selected for. Previously, monoclonal antibodies have been generated to blastocoel matrix components of P. ochraceus embryos; these antibodies, however, also shared epitopes with elements of the hyaline layer (Crawford and Crawford, 1992). In using antibodies such as these for function blocking studies, it was difficult to separate effects on the hyaline layer from those on blastocoel matrix components. For this reason, it was necessary to generate new monoclonals with blastocoel-specific epitopes. Attempts at reducing the number of antibodies generated against the hyaline layer by immunizing mice with a hyaline layer-deficient embryo homogenate were not successful, as many antibodies with specificities for the hyaline layer were still generated. Hence a new approach was used to prepare the immunogen in the present study. This involved obtaining a glycoconjugate-enriched fraction from embryo homogenates using a Con A-Sepharose affinity column. While many positive clones were generated, it was not possible to use conventional techniques such as the ELISA (enzyme-linked immunosorbent assay) in screening the various hybridoma supernatants, because this procedure did not permit the level of resolution required to select for blastocoel-specific immunoreactivity. Instead, supernatant from all positive wells (wells containing successful fusions) was tested on thin (1.5μm) sections of gastrula-stage embryos using indirect immunofluorescence; in this way, antibodies specific for the blastocoel ECM could be distinguished from those which had dual specificity for
both the hyaline layer and the ECM, or other specificities. The resolution afforded by the plastic-embedded sections was very advantageous; at the light microscopic level, labelling of the blastocoel matrix could be distinguished readily from that of basement membranes, the hyaline layer, and in fact different regions of the hyaline layer. In addition, labelling of intracellular granules was easy to detect, and therefore the synthesis and storage of matrix components could be investigated. When this technique was used in combination with tissue that was fixed by freeze-substitution in ethanol, antigenicity of many components did not appear to be compromised, as monoclonals having both protein and carbohydrate specificities labelled the tissue strongly. This was, however, a very labor intensive procedure, as the hyaline layer was particularly antigenic; in some cases, the entire 96 well plate of hybridomas was positive, and while almost all produced antibodies directed against the hyaline layer, 1 clone was generated that produced the blastocoel-specific antibody, *Pisaster matrix 1* (PM1).

2. Antibody perturbation studies

Studies involving the use of antibodies to block the functions of ECM components have been performed extensively in both cell culture and *in vivo* systems. When performed in the whole organism, perturbation studies of this type usually involve micro-injecting the antibodies directly into tissue to ensure that they are delivered to a specific location. Alternately, if the targeted structure is on the outer aspect of cells, as is the hyaline layer of echinoderms, perturbation experiments can be performed by simply incubating the embryos in sea water containing antibodies. A similar approach was used in the present study, that of transferring embryos into sea water containing PM1 antibody or antibody fragments. Although it was not assumed that the antibody would gain access to the blastocoel, embryos treated in this way were severely affected in their development, while controls incubated in normal mouse IgM were not. This suggested that the PM1 antibody was having a specific effect on a developmental process, resulting in the disruption of morphogenesis. Subsequent experiments were therefore designed to show that this effect on development was the result of the PM1 antibody binding to and interfering with the PM1 antigen in the blastocoel. This involved probing the tissues with an FITC-labelled anti-IgM
antibody shortly after the embryos were transferred into sea water containing the PM1 antibody. The results showed specific labelling of material in the blastocoel and endodermal cells but not in other areas of the embryo or hyaline layer, which demonstrated that the PM1 antibody did indeed gain access to the blastocoel when embryos were simply transferred into sea water containing it. This suggests that the effect on development was a result of a specific interaction between the PM1 antigen and antibody. It is unclear whether the intact or fragmented IgM was actively taken up by the epithelial cells, or whether the antibody simply diffused into the blastocoel under these conditions. Since the epithelial cells are often loosely attached in the region of the endoderm, it is possible that during archenteron expansion, their junctional complexes are transient, permitting antibody to leak through into the blastocoel.

3. Affinity purification of the PM1 antigen

Several factors owing to the biochemical properties of the PM1 antibody (IgM subclass; 900 kDa) and the large size of the PM1 antigen (>600 kDa) made the affinity purification of this molecule somewhat challenging. Initially, the antibody was coupled to CNBr-activated Sepharose 4B. However for undetermined reasons, the coupling efficiency with this support resin was extremely low. Affi-gel 10 provided a reasonable alternative, although intact IgM was still difficult to couple efficiently. Attempts were made to first fragment the IgM with pepsin, and this improved the coupling efficiency somewhat. A second difficulty was the size of the antigen (>600 kDa). With a molecule of this size, the binding of a single epitope is not strong enough to withstand the vigorous column washings that are required in the affinity purification procedure, to ensure that non-specific proteins are eluted from the column. Therefore it is likely that a significant amount of the PM1 antigen was lost during the washing procedures. A further factor was the buffer conditions required for affinity chromatography. Even though 4 M guanidine chloride was used to extract the PM1 proteoglycan from the embryos, this buffer was not compatible with affinity chromatography, as it would have denatured the column, and instead Tris buffer was used. It is probably that a considerable amount of material was lost due to insolubility in the Tris buffer. For these
reasons, up to 30 column runs were required to obtain enough material to carry out the experiments in the present study. The benefits of obtaining a pure antigen did, however, outweigh the above negative factors.

(II) Biochemical Characterizations of the PM1 antigen

1. Evidence that the PM1 antigen is a proteoglycan.

Several experimental findings led to the suggestion that the PM1 antigen is a carbohydrate-containing molecule and that it shares structural features that are commonly associated with those of proteoglycans. These findings are discussed in the following section.

(a) Localization in the Golgi suggests the PM1 antigen contains sugar residues

The PM1 antigen is first detected in the trans Golgi network and in Golgi associated vesicles. Studies show that carbohydrates are usually modified and/or added to protein or lipid cores in this region of the Golgi complex (for review see Kornfeld and Kornfeld, 1985). These include: (1) oligosaccharides linked to asparagine residues (N-linked), that are characteristic of glycoproteins; (2) oligosaccharides linked to serine, threonine or hydroxylysine residues (O-linked) that are characteristic of mucins; (3) GAG chains typical of proteoglycans. The localization of PM1 immunogold particles to the trans Golgi region and post Golgi secretory vesicles and not to the cis Golgi or ribosomes suggests that the antibody is directed against a region of the antigen which is undergoing processing or modifications of its carbohydrate region. The nature of the sugar groups cannot, however, be determined from these observations.
(b) Carbohydrate groups not typical of glycoproteins

Several experimental findings indicated that the carbohydrate groups associated with the PM1 antigen are not those typically associated with glycoproteins. First, glycoproteins generally contain N-linked oligosaccharides that are of the high mannose or complex type. Endoglycosidase F, an enzyme which specifically cleaves such N-linked oligosaccharides (Elder and Alexander, 1982), did not affect the PM1 antigen, as both PM1 immunoreactivity as well as the apparent Mr of affinity pure PM1 were unchanged after treatment, while control glycoproteins showed a definite mobility shift after treatment. Secondly, the vicinal hydroxyl groups of such N-linked oligosaccharides can be efficiently oxidized with periodic acid to generate aldehyde groups, which can then be reduced with sodium borohydride (Bobbitt, 1956). This treatment changes the conformation of the carbohydrate groups, and often will affect antigenicity of an antibody that is directed towards a carbohydrate epitope (Woodward et al., 1985). Periodate oxidation of acidic polysaccharides (GAGs), however, proceeds very slowly because of the strong repulsion between periodate ion and the polyanions. This effect can be overcome by increasing the ionic strength of the oxidizing buffer, which decreases the degree of ionic repulsion between periodate ions and highly charged GAG groups (Scott and Harbinson, 1968). In the present study, when PM1 was oxidized under conditions of low ionic strength, little or no effect was observed, suggesting that it does not contain oligosaccharides characteristic of glycoproteins. However, when the ionic strength was increased with 0.2 M sodium perchlorate, immunoreactivity was reduced significantly. This strongly suggests that the epitope is dependent upon highly charged acidic groups such as GAGs.

Experiments in which embryos were treated with tunicamycin provided yet further evidence to support the argument that PM1 is not a glycoprotein. Tunicamycin is a drug which inhibits the first step in the formation of N-linked oligosaccharide synthesis, and is often used to inhibit glycoprotein synthesis (Duskin and Mahoney, 1982). Embryos that were grown in the presence of very low concentrations of tunicamycin (0.2 μg/ml), although severely affected by the drug, were still capable of synthesizing and
secreting the PM1 antigen. This indicated that the antigen was not significantly affected by this inhibitor of glycoprotein synthesis and that it is therefore probably not a glycoprotein.

Finally, the PAS reaction, which involves first the oxidation of vicinol diols to generate dialdehydes, and then the visualization of these dialdehydes with the Schiff reagent (McManus, 1946), is used to identify oligosaccharides typical of glycoproteins, as N-linked oligosaccharides are rich in vicinol diols. Staining of the affinity-purified PM1 antigen with PAS was negative, again suggesting that the PM1 antigen is not a glycoprotein. Staining was observed, however, with Alcian blue, a cationic dye which binds strongly to polyanionic groups such as GAGs (Scott, 1972). The reaction can be made more specific by adding 0.5 M magnesium chloride to the Alcian blue solution. When this is done, all commonly known GAGs are stained, but nonspecific stain uptake by negatively charged polyion species found in other proteins does not occur (Wall and Gyi, 1988). The specific staining of the PM1 antigen therefore suggests that it contains highly acidic groups typical of those found in GAGs. Furthermore, despite the fact that it does not stain with Coomassie blue, the antigen is sensitive to tryptic digestion which indicates that these highly acidic groups are indeed linked to a polypeptide core, as they are in proteoglycans.

(c) Carbohydrate groups not typical of known vertebrate proteoglycans

Proteoglycans are common elements of ECMs, and have been described in a variety of vertebrate and non-vertebrate systems (reviewed by Hardingham and Fosang, 1992; Goetinck and Winterbottom, 1991, Har-El and Tanzer, 1993). While many proteoglycans contain some N and O-linked oligosaccharides (aggrecan from a rat chondrosarcoma, KSPG from monkey cornea Nilsson et al., 1982; 1983), all contain GAG chains (Roden, 1980). There are different classes of vertebrate GAGs, which include the chondroitin sulfates, dermatan sulfate, keratan sulfate, and heparin sulfates, and they are high in glucuronic residues, as well as N-acetylgalactosamine, N-acetyl glucosamine, and galactose. These GAGs can be experimentally removed from their protein core with enzymes that are available for each GAG. This provides a convenient diagnostic tool for their identification. In addition, as mentioned above, the
negative polyanionic structure of the GAG enables them to bind strongly to cationic dyes such as Alcian blue, and this binding is often used to identify proteoglycans and hyaluronic acid in tissues.

When the PM1 antigen was treated with the vertebrate GAG-degrading enzymes chondroitinase ABC, chondroitinase AC, heparitinase and ovine testicular hyaluronidase, and then separated by SDS PAGE and stained with Alcian blue, no significant effect was observed despite the fact that under similar conditions, known standards were degraded. There did appear to be a slight reduction in staining intensity after digestion with chondroitinase ABC, indicating that there may be some dermatan sulfate groups present on the PM1 proteoglycan. However, no change in the electrophoretic mobility of the band was apparent, indicating that a very small amount if any had been digested away. Western blots of the PM1 antigen digested with each of the enzymes listed above showed that immunoreactivity and electrophoretic mobility was also essentially unchanged when compared with the controls. This is consistent with earlier observations in the same species (*P. ochraceus*) in which no proteoglycans sensitive to the above enzymes could be identified (Crawford and Crawford, 1992).

Although it is known that GAGs from echinoderms contain glucuronic acid, N-acetylgalactosamine and sulfate (Motohiro, 1960; Vieira and Mourão, 1988; Kariya et al., 1990), suggesting that they are similar to known vertebrate GAGs such as the chondroitin sulfates, the observations in this study suggest that PM1 is quite distinct from known vertebrate proteoglycans. In other studies of invertebrate proteoglycans, the analysis of several chondroitin sulfate proteoglycans isolated from the sea cucumber and sponge have indicated that they have fucose-containing branched GAGs which are insensitive to digestions with chondroitinases (Kariya, et al., 1990; Vieira and Mourao, 1988; Misevic and Burger, 1990). This does not appear to be the case for PM1, as digestion of the antigen with α-fucosidase prior to chondroitinase digestion failed to have any effect.
d) β-xyloside blocks PM1 antigen synthesis

Although the PM1 antigen contains highly negatively charged groups characteristic of GAGs, which are linked to a polypeptide core, the GAGs do not resemble those commonly found in vertebrate systems. In spite of this, the β-xyloside inhibition experiment provided strong evidence that the PM1 antigen shares structural characteristics of known vertebrate GAGs. As previously mentioned, β-xyloside competes with core protein xylosides for GAG chain attachment, thereby creating a free xyloside-GAG molecule (Okayama et al., 1973; Galligani et al., 1975) and inhibiting the assembly of proteoglycans. When embryos were grown in the presence of this chemical, the level of PM1 proteoglycan synthesis and/or secretion was reduced significantly. This indicated that although these starfish GAGs appear different from other known GAGs, their attachment to the polypeptide core involves a xylose-mediated linkage. This type of linkage is characteristic of known proteoglycans, such as those containing chondroitin sulfate, dermatan sulfate and heparan sulfate residues.

(III) Role of PM1 proteoglycan in gut morphogenesis

1. Localization and secretion of the PM1 proteoglycan in the blastocoel matrix

The first indication that the PM1 proteoglycan may play a specific role in gut morphogenesis was the observation of its secretion into the matrix of the blastocoel during the time at which digestive tract development and differentiation occurs. Immunofluorescence labelling showed the PM proteoglycan had a granular appearance and was distributed throughout the blastocoel, which suggested that it was localized in the network of matrix fibers present in the blastocoel ECM. Ultrastructural immunogold labelling studies were pursued in order to define the exact localization of this proteoglycan in the matrix network. Previous studies in *P. ochraceus* have demonstrated that matrix fixed with Alcian blue and examined by TEM appears as a loose meshwork containing fibers of intermediate electron density that are encrusted with short dense fibers. These shorter dense fibers change to granules after fixation with
ruthenium red (Crawford, 1989; Strathman, 1989), indicating that they contain highly anionic structures such as GAGs (Thyberg et al., 1973). Although these components have not been previously characterized or localized in the starfish, it has been suggested that the ECM fibers may contain collagen type I and II, since they have a similar appearance to those described and characterized in other embryonic systems, such as the developing chick cornea and tibia (Hendrix et al., 1981; Hay, 1978), and that the alcian blue/ruthenium red-stained material contains proteoglycans. In the present study after immunogold PM1 staining, gold particles were present primarily on the short filamentous aggregates throughout the matrix, but not on the thicker fibrils, suggesting that these aggregates are indeed representative of proteoglycans.

Stronger evidence for the role of the PM1 proteoglycan as a morphogenetically important molecule for gut development comes from the observations of its developmentally regulated pattern of secretion into the blastocoel matrix during development. It can not be determined from the present studies whether the synthesis of the proteoglycan is also regulated, since the means of detection was with the PM1 antibody, which is directed to a carbohydrate epitope, and therefore detects the proteoglycan when it is glycosylated to its mature form prior to secretion. While early in development there are only trace amounts of the proteoglycan in the blastocoel, the amount of detectable antigen increases markedly during mid-gastrulation, so that over a 24 hour time period, there is a rapid accumulation of material in both the endodermal cells and the blastocoel. At the same time, development of the digestive tract begins. This involves the formation of mesenchyme cells, which are active in migration and possible remodelling of the matrix, and are involved with esophageal muscle formation. In addition, the elongation of the primitive gut tube (archenteron), and its differentiation, including epithelial segmentation and bending, leading to mouth and coelom formation occurs. The time at which the PM1 proteoglycan appears in the blastocoel suggests that it may be a "gastrulation specific" matrix component and that its functions may be centered around events occurring during gut formation. It is important to note that the secretion of the PM1 proteoglycan into the blastocoel is not simply an indication of the time at which all blastocoel matrix components are secreted. Several other matrix components, including the PC3H2 antigen (discussed
later), are abundant in the blastocoel of the blastula-stage embryo. Therefore, the observation of PM1 secretion at the gastrula stage indicates that it is developmentally regulated, and the fact that it is produced in endoderm and mesenchyme cells but not by ectodermal cells may also indicate that it has functions relating to the specific activities of these cell populations.

That the PM1 proteoglycan is secreted into the blastocoel at the time during which gut morphogenesis is active suggests that it may participate in cell-ECM interactions during this time. Double label experiments with the PM1 antibody and the P212 antibody (directed against a cell-surface antigen) have suggested that the mesenchyme and epithelial cells have a close physical interaction with the PM1 component of the blastocoel matrix during this time. These observations have important ramifications, as it is know from other systems that cellular behavior, including migration and differentiation during morphogenesis, is controlled not only by its developmental lineage, but also by other cells and by the ECM in the milieu (Stoker et al., 1990). In vitro, the dramatic effects of ECM on the differentiation state of numerous cell populations have been observed, including hepatocytes, mammary cells, keratinocytes, Sertoli cells, granulosa cells, myoblasts, myocytes and endothelial cell, to name only a few. In every case, the addition of specific ECM molecules dramatically alters both the morphology and the function of these cells (reviewed by Damsky and Werb, 1992). For example, fibronectin has been shown to be a regulator of keratinocyte differentiation, in that contact with fibronectin prevents the cells from undergoing terminal differentiation, and from expressing a marker of terminal differentiation, involucrin (Green, 1977; Watt, 1984; Nicholson and Watt, 1991). Other studies have shown that mammary epithelial cells are regulated in their capacity to produce β-casein by the ECM (Streuli and Bissell, 1990). And yet other studies have shown that laminin can regulate hepatocyte differentiation as measured by albumin transcription (Caron, 1990).

Evidence for the role of ECM in promoting cell migration has been shown in several in vivo systems. Studies using function-blocking antibodies have shown that fibronectin is important in avian neural crest cell migration (Sheppard et al., 1991) and amphibian mesenchyme cell migration (Boucaut and Darribère,
1983; Boucaut et al., 1984). In addition, thrombospondin and tenascin are important in stimulating cerebellar granule cell migration in the brain (O'Shea et al., 1990; Erickson and Bourdon, 1989), to name only a few. Although the detailed mechanisms responsible for ECM influences on cell migration and differentiation are not yet known in full, the general hypothesis for the ECM-cell regulatory mechanism is as follows: Extracellular signals are transmitted across the cell membrane via transmembrane receptors, such as integrins, which recognize ECM molecules (Hynes, 1987). The changes in these receptors, triggered by the ligand binding, in turn, cause rearrangements of the cytoskeletal network. This affects the intracellular cascade of signal transduction leading to changes in gene expression (Bissel and Aggeler, 1987; Adams and Watt, 1993), and therefore in the growth and differentiation state of the cells.

These studies provide evidence that cells are regulated by glycoprotein constituents of their ECM environment, and it is possible that the PM1 proteoglycan, which appears in the matrix just at the time when gut morphogenesis occurs, may play a similar role in starfish gut development. However, proteoglycans are structurally very different from glycoproteins, and it can not be assumed that their presence in extracellular matrices qualifies them to be key players in controlling cell migration and differentiation. There is, however, some evidence from in vitro systems to indicate that proteoglycans are important for cell migration. One study has shown that migrating endothelial cells exhibit increased chondroitin sulfate and dermatan sulfate proteoglycan synthesis as compared to sessile cells in vitro (Kinsella and Wight, 1986). Another study has shown that the removal of these proteoglycans from early rat embryos inhibits emigration of cranial neural crest cells in vivo (Morris-Kay and Tuckett, 1989). However, somewhat contradictory are the findings that a mixture of the proteoglycans decorin and biglycan inhibits the attachment of fibroblasts to fibronectin. Similarly, a chondroitin sulfate proteoglycan has been shown to inhibit neural crest cell migration on several ECMs, and this inhibition is mediated by interactions with cell-surface associated hyaluronic acid (Perris and Johansson, 1990). In these situations, the proteoglycans may inhibit migration by binding to the cell surface and hindering other cell adhesion receptors or by binding matrix proteins and thereby masking cell attachment sites (Ruoslahti, 1989), presumably by binding to fibronectin and in doing so, sterically hindering the fibronectin-integrin
interactions. (Lewandowska et al., 1987). These studies show that proteoglycans are certainly legitimate candidates as ECM regulatory proteins in processes of cell migration, and therefore a case can be made for the PM1 proteoglycan as a key participant in gut morphogenesis.

2. **In vivo perturbation studies with the PM1 proteoglycan**

The localization of the PM1 proteoglycan in the blastocoel matrix at the time at which gut morphogenesis is occurring, although a "pre-requisite" if it is to be considered as a morphogenetically important molecule, is not an evidence in itself. However, function blocking studies with the PM1 antibody did provide strong evidence for its putative role as an important matrix component in development of the gut. Embryos that were subject to treatment with the PM1 antibody at the early gastrula stage are affected in several ways. Their overall size represents only 60% of that of the controls, indicating that blastocoel expansion has been limited. The most noticeable feature of these embryos is, however, the lack of differentiation of the digestive tract. Unlike the control embryos, which have developed segments in the endodermal gut tube to yield distinct regions representing the esophagus, stomach and intestine, the PM1 perturbed embryos have a very shortened non-partitioned endoderm which extends approximately 50% of the length of the embryo. They fail to form the blister of BL from the tip of the archenteron that in normal development may guide the archenteron to the presumptive mouth region of ectoderm. In spite of this, mesenchyme cell formation from the tip of the archenteron does not appear to be interrupted, and several migrating cells can be observed throughout the blastocoel. Although it is not known how the PM1 antibody interferes with development, it does so presumably by a specific interacting with the PM1 proteoglycan, and thereby blocking the normal functions of this matrix component. This could be in the form of a direct interference whereby a cell matrix interaction is physically obstructed. Alternatively, the PM1 antibody could be preventing interactions with other matrix components that are required for the general organization and/or assembly of the matrix, and which would then affect cell migration and differentiation adversely.
Although few other reports exist that demonstrate the effects of function-blocking antibodies against proteoglycans *in vivo*, some studies have shown that proteoglycans are important for morphogenesis by using competition and enzymatic digestion procedures. The injection of heparin, which interferes with heparan sulphate proteoglycan-mediated interactions, retards gastrulation, and affects neural development in *Xenopus* (Mitani, 1989), while injection of heparitinase into *Xenopus* blastulae randomizes the development of right/left asymmetry (Yost, 1992), although it is not known whether cell surface associated or extracellular heparan sulfate is being interfered with in these studies. Further work is required to clarify this area of research, but these studies do support the idea that proteoglycans can play key roles in controlling the events of cell migration during morphogenesis. It therefore seems possible that similarly, during starfish development, the mesenchyme and epithelial cells involved with gut formation could be receiving important cues required for their migration and differentiation from the elements of the matrix located in the blastocoel, such as the PM1 proteoglycan.

3. Conclusions and further work

This study has shown that a large proteoglycan, PM1, is secreted into the blastocoel matrix of early gastrulae during the time at which development of the digestive tract begins, and that it is involved with the morphogenetic events that occur during this time. Since PM1 proteoglycan-binding proteins or receptors have not been identified, its specific role in development remains unknown. Possible functions based on what is known about proteoglycans could include providing resilience for blastocoel expansion, playing a role in the organization of the blastocoel matrix, binding other ECM components and growth factors, or providing a substrate for migrating cells. The present observations indicate that the PM1 antibody is directed to a carbohydrate region on the proteoglycan, and furthermore that in binding to this region, digestive tract morphogenesis can be inhibited *in vivo*. This suggests that the sugar chains on the PM1 proteoglycan may act to facilitate cell migration, differentiation and/or matrix assembly during mouth
and digestive tract formation. Further understanding of this process clearly depends on a better knowledge of the structure of the PM1 proteoglycan, as well how it interacts with other elements of the ECM or with cell surface proteins. Although the enzymatic removal of sugar groups was unsuccessful, further attempts could employ the use of chemical deglycosylation techniques, such as hydrolysis with hydrogen fluoride or trifluoromethane sulphonic acid. In this way, a thorough analysis of the sugar constituents of the PM1 proteoglycan could be carried out. Furthermore, sequencing of the PM1 proteoglycan core protein would make possible its comparison with other known proteoglycans; in this way, common structural motifs could be identified, such as such as the arginine-glycine-asparagine cell binding domain, or regions having growth factor activity. As the PM1 antibody is directed towards a sugar epitope, this antibody could not be used to screen a cDNA expression library. However, a polyclonal antibody could be developed against the affinity-purified proteoglycan, and then used to screen for the PM1 clone. Other studies could investigate PM1 proteoglycan binding proteins and receptors with the use of PM1 proteoglycan affinity columns, or with co-immunoprecipitation techniques. Knowing what the binding sites of the PM1 proteoglycan are and how they are related to other known proteoglycans would aid in establishing the mechanisms by which it affects digestive tract morphogenesis.

PART B: Starfish yolk and cortical granule proteins

(I) Yolk proteins of P. ochraceus

Yolk proteins of oviparous species are conserved through a wide range of phylogenetically diverse species, which suggests that they perform similar functions in many organisms. These are thought to eventually undergo catabolism to provide raw materials (amino acids, carbohydrates, and lipids) for use by the developing embryo; however, little direct evidence exists for this. A major objective of this part of the study was to use the monoclonal antibody Pisaster yolk 4F8 to investigate yolk proteins of the starfish P. ochraceus. The immunogen used to generate the Pisaster yolk monoclonal was derived from a digest of
gastrula-stage embryos. The predominant yolk species in the starfish at this stage are the 64-74 kDa proteins, and it is therefore probable that the antibody was generated against these low molecular mass components. In a fashion similar to the PM1 antibody studies, the anti-yolk antibody was then used to biochemically characterize and localize the yolk proteins in tissue during early embryonic and larval development as well as in the adult starfish, to understand more clearly the role that yolk serves in the developing organism. This study represents a starting point for the characterization of starfish yolk proteins.

1. Specificity of the *Pisaster* yolk 4F8 antibody

The PY4F8 antibody stains the granules that take up the majority of the egg volume, indicating that the antigens are stored here. These granules are distributed throughout the egg with a decreased incidence in the cortical cytoplasm, where the predominant organelles are the cortical granules. When oocytes were viewed with the electron microscope after immunogold labelling with PY4F8 antibody, gold particles were associated with granules of an intermediate staining density which approach 4 μm in diameter. When embryos at various stages of development were stained with the PY4F8 antibody, positive-stained granules were present throughout the embryonic life span of the starfish. During the early larval stage, however, the staining intensity of the granules was significantly reduced, and at the same time, it did not appear as though this material was undergoing secretion, as no immunolabelling was present in any other regions of the embryo. Western blots of egg homogenates demonstrated that the PY4F8 antibody recognized several proteins having apparent molecular masses of 164, 100, 74 and 64 kDa. These proteins, although different in size, shared a common structural feature in that they were all recognized by the same monoclonal antibody. This type of pattern, that of a family of proteins in the egg which share a common epitope, is characteristic of yolk proteins in other species, including nematodes (Sharrock, 1984), goldfish (de Vlaming et al., 1980) and chicken (Wallace and Morgan, 1986). In these species, the proteins are stored in yolk platelets or granules, which range from 1-4 μm in diameter and are the
predominant organelle in the egg. Typically, such yolk platelets and proteins are progressively degraded through development (Anderson, 1974). The similar pattern of molecular composition, localization and depletion during development that is observed in *P. ochraceus* eggs suggests that the PY4F8 antibody recognizes starfish yolk proteins which are stored in yolk granules.

2. **PY4F8 Immunoreactivity during development.**

The PY4F8-stained granules are present through the early stages of development. The granules apparent at the gastrula stage were comparable in intensity to those present in the unfertilized egg, indicating that a major loss of proteins and granules is not occurring at this stage. Although the staining intensity of the yolk granules remained strong through early development, the starfish PY4F8-antigens changed in molecular composition during this time. While in the oocyte, 4 major proteins were present, with the development to the blastula, there was a loss of the 100 kDa species. Further development to the gastrula resulted in the loss of the 164 kDa species, leaving a considerable amount of lower molecular mass material which migrated as a broad band between 64-74 kDa. Although densitometry studies were not performed, the immunoblot profiles indicated that as the larger proteins were lost during early development, the amount of staining around the 64-74 kDa region steadily increased before finally decreasing at the larval stage. Direct observations of the yolk proteins in embryonic tissue with immunogold labelling showed that no labelling was present outside of the yolk granules, suggesting that the 164 and 100 kDa proteins were not being secreted into other subcellular organelles or into extracellular spaces to become part of matrices.

This pattern of change in molecular masses has been observed in the yolk proteins of the sea urchin *S. purpuratus*, where along with the disappearance of the 160 kDa yolk protein of the egg, glycoproteins with a lower molecular masses appear (115, 108, 90, 83 and 68 kDa) and are gradually depleted during development (Scott and Lennarz, 1989). Homologous sets of proteins with similar molecular mass have
been observed in the embryos of several other sea urchins such as *L. pictus* and *A. punctulata* (Kari and Rottmann, 1985; Scott and Lennarz, 1989; Lee et al., 1989), *Hemicentrotus pulcherrimus* and *Anthocidaris crassispina* (Yokota and Kato, 1988), as well as in the sand dollar *Dendraster excentricus* (Scott and Lennarz, 1989). The biochemical composition and depletion profile during development add additional evidence that the PY4F8 antigens of *P. ochraceus* are yolk proteins.

It is unclear how the change in the molecular compositions of yolk proteins through development occurs in this organism. In other organisms, such as the sea urchin *S. purpuratus*, it has been shown that yolk proteins undergo a stepwise proteolysis, such that a single limited proteolytic cleavage of a 180 kDa egg yolk protein gives rise to 2 intermediate molecular weight glycoproteins during embryogenesis (Lee et al., 1989). In *P. ochraceus*, the amount of the 164 kDa protein decreases while the 64-74 kDa proteins appear to steadily accumulate during embryogenesis, which suggests that these lower Mr proteins may be derived from the breakdown of the larger one. This assumption can, however, not be made based on the present data, and further biochemical analysis of the individual proteins is necessary to determine whether they are related and if so, what the possible cleavage patterns are.

3. Ontogeny of the *Pisaster* yolk proteins

(a) Identification of a starfish vitellogenin

Yolk proteins of other species are known to be synthesized as high molecular mass precursors (vitellogenins) in organs distant from the ovary; in particular, an echinoderm yolk protein precursor vitellogenin has been located in coelomic fluid (Harrington and Ozaki, 1986) and intestines (Shyu et al., 1986) of adult sea urchin. Attempts were made to locate and identify a precursor vitellogenin of the starfish mature yolk proteins, and to do this, coelomic fluid and intestinal tissue was examined for PY4F8-reactive proteins. These investigations revealed a 400 kDa protein was present in both intestine and coelomic fluid isolated from adult females. Rather than a single defined band, there were several bands
giving rise to a broad area of immunoreactivity around the 400 kDa region on the blot, possibly as a result of varying degrees of glycosylation on the same protein. In addition, several smaller proteins were detected, which correspond to the mature yolk proteins of the egg. The shared immunoreactivity implied that these high molecular mass proteins share structural features with the lower molecular mass yolk proteins of the egg. This suggests that the smaller molecular mass proteins of the egg are derivatives of this large protein of the intestines and coelomic fluid, and supported identification of the 400 kDa species as a starfish vitellogenin.

(b) Effect of β-mercaptoethanol on PY4F8 immunoreactivity

Coelomic and intestinal extracts which were treated with β-mercaptoethanol prior to immunoblot analysis did not exhibit the 400 kDa PY4F8 immunoreactive bands seen in untreated material. This suggested that the starfish vitellogenin contained disulfide-linked subunits which were broken up into monomers under reducing conditions. However, in the PY4F8 immunoprecipitate derived from oocyte-extracted proteins, a 400 kDa band was present even after treatment in reducing buffer. This protein was not immunoreactive to PY4F8, but stained with Coomassie blue and with the lectins Con A and WGA. This implies that treatment with β-mercaptoethanol destroys the epitope, perhaps by cleaving intra-chain disulfide bonds, rather than causing the breakdown of the 400 kDa complexes. Further to this argument is the observation that although a similar Western blot band profile was observed in oocyte extract under both reducing and non-reducing conditions, the antigenicity of the non-reduced sample was at least 5-fold greater, indicating that the treatment with β-mercaptoethanol alters the immunoreactivity of the monomeric yolk proteins in some way.

(c) Vitellogenin processing and transport

Vitellogenins of other species vary in molecular mass from 170 kDa in the nematode (Sharrock, 1984), and 380 kDa in the goldfish (DeVlaming et al., 1980), to 460 kDa in the toad (Wiley and Wallace, 1981).
After synthesis in an organ distant from the ovary such as the liver, intestine or by coelomocytes, they are secreted into a body fluid such as blood, hemolymph or coelomic fluid, and then transported to the ovary where they are taken up by receptor-mediated endocytosis. The time and location of processing of vitellogenins varies among species, and can occur prior to their secretion, during their transit to the oocyte, and/or following their selective uptake (Wang et al., 1983; Sharrock, 1984), giving rise to mature glycoproteins. The fact that very little of the 400 kDa species was observed in the oocyte indicates that the amount of vitellogenin taken up by the oocytes was probably small in comparison with the lower molecular mass derivatives, or if large quantities are taken up, they are rapidly processed to the smaller molecular mass forms. This suggests that in *P. ochraceus*, vitellogenin is processed into the mature yolk proteins primarily in the intestine or coelomic fluid, and that these mature proteins are then transported to the ovary where they are taken up by the oocytes.

4. Characterization of the starfish yolk proteins

Vitellogenins and mature yolk proteins are typically glycoproteins rich in lipids and phosphate groups. The results of this study suggest that the extent of glycosylation of *P. ochraceus* yolk proteins is very substantial. Following treatment with Endo F, an enzyme which enzymatically cleaves asparagine (N)-linked oligosaccharides chains (typical of glycoproteins) from the core protein, major bands were present at 100 kDa, a doublet at 55 kDa, a doublet at 45 kDa and a single band at 32 kDa, whereas in the control, bands were present at 164, 100 (doublet) and 64/74 kDa. Although direct correlations between glycosylated and unglycosylated bands can not be made, the fact that the largest protein in the enzyme-treated lane migrates at 100 kDa suggests that the 164 kDa major yolk protein has undergone a mobility shift of at least 60 kDa, which represents a substantial loss in apparent molecular mass. This is even greater than that observed in the sea urchin by Armant *et al.* (1986), who observed a shift of 35 kDa after the enzymatic deglycosylation of the major yolk protein in *A. punctulata*. 
Further evidence that the yolk antigens are glycoproteins comes from lectin binding studies. Lectins are sugar-binding proteins of non-immune origin that bind to or precipitate glycoconjugates (Goldstein et al., 1973). The ability of lectins to bind specifically and reversibly to carbohydrates has been exploited in a wide variety of techniques used to study glycoproteins and oligosaccharides. There are 2 general classes of N-glycosidically linked oligosaccharide chains, one containing a polymannose chain and a second composed of a complex chain that includes mannose residues but which terminates in sialic acid units. Those oligosaccharide chains linked by serine/threonine residues (O-linked) generally do not contain mannose residues (Lennarz, 1983). Con A binds to α-mannose and a glucose residues (So and Goldstein, 1969), and as N-linked oligosaccharides of both the polymannose and complex types are rich in mannose residues, Con A is often used to identify glycoproteins with these sugar chains (Beeley, 1985). Con A binding of the Pisaster yolk proteins revealed that both the mature yolk proteins as well as the vitellogenin are N-linked polymannose containing glycoproteins, as are yolk proteins from other species, including nematodes (Winter, 1992) the leech (Baert et al., 1991), and sea urchins (Scott and Lennarz, 1989).

Another lectin, WGA, has a different sugar-binding specificity, in that it preferentially binds to sialic acid and N-acetyl glucosamine residues (Allen et al., 1973; Greenaway and LeVine, 1973). As mentioned above, terminal sialic acid residues are characteristic of complex type N-linked oligosaccharides and of O-linked oligosaccharides, but not of polymannose-type chains. When the Pisaster yolk proteins were stained with WGA, only the 400 and 164 kDa forms stain positively, suggesting that the lower molecular mass proteins are of the polymannose type (i.e. do not contain terminal sialic acid residues). This indicates that although the family of Pisaster yolk proteins share structural similarities, in that they all contain the PY4F8 epitope, there appear to be variations within their oligosaccharide chains.
5. Utilization of yolk proteins

Although yolk granules in certain other embryonic systems are known to provide a store of nutrients and energy (Williams, 1967), the exact mode of utilization of the major yolk proteins by the growing embryo has not been clearly established. There have been several reports that material stored in yolk granules is secreted to the ECM. For example, the protein toposome identified in sea urchin eggs (Noll et al., 1985) is stored in yolk granules, and is released to become part of the hyaline layer during embryogenesis (Gratwohl et al., 1991). Another study (Outenreath et al., 1988) identified a lectin in yolk granules of *Xenopus* that was secreted into the ECM of early embryos. In the present study, proteins recognized by the PY4F8 antigen were found exclusively in the yolk granules of oocytes, and during embryogenesis, localization of the yolk proteins remained in these granules. No evidence of the yolk protein was evident in other subcellular compartments or in extracellular matrices, suggesting that the yolk proteins recognized by PY4F8 are not secreted into these areas, or if they are, the secreted antigens are altered such that the epitope is no longer present. This does not rule out the possibility that other proteins are stored in the yolk granules that may be secreted in a similar way to those described above. While *Pisaster* eggs do contain at least one protein that is secreted into extracellular matrices during development, much like the above-mentioned proteins (the PC3H2 antigen; see below), this protein is not stored in yolk granules but is a component of the cortical granules.

While the traditional notion of yolk is that it is serves as a nutrient store during early development, there seems to be little change in the amount of yolk present in the early stages of *Pisaster*. The proteins and the yolk granules appear to undergo depletion only once the organism has reached the feeding larval stage. This suggests that the principle function of yolk may be as a nutrient store for the larva, rather than for the early embryo. A similar observation has been made of sea urchin yolk proteins, where it has been further observed that larvae given a supply of food deplete their yolk much more efficiently than those which are starved (Scott et al., 1990). This could indicate that when no food is available for the feeding larvae, the yolk material may be used sparingly, to ensure survival until which time the larvae obtains a
suitable external food source. However, if the larvae have a readily available supply of food, the yolk material may in such circumstances, be used towards rapid growth and development, as there would be no requirement for an internal nutrient store. It would be of interest to study the yolk of non-feeding larvae, such as Crossaster papposus or Hipasteria spinosa, and compare the structures and depletion profiles of their yolk proteins with those of feeding larvae. These species give rise to large eggs (about 1 mm in diameter) which are very yolky compared with eggs of feeding larvae. The fact that these eggs are about 5 times as large, and contain much more yolk than those which undergo a feeding larval stage (Strathmann, 1987), would seem to indicate that the yolk in this case is required for nourishment during the period of larval development, since they are unable to feed. Further characterization of starfish vitellogenin and its derivatives, as well as examining different conditions under which yolk proteins of the starfish are depleted and the rates at which this occurs may provide further information on how these proteins are utilized for nutrition.

6. Conclusions

Although yolk proteins from many species have been studied extensively, and as a result, their ontogeny, transport and uptake mechanisms including receptor characterization is known, a key question still remains in the field of yolk biology, and that centers around the biochemistry of yolk protein utilization. The depletion profile of the yolk in *P. ochraceus* is suggestive that, like several other species, yolk is probably not important during early stages of development, the time during which it is generally assumed to be utilized. The study has also shown that a family of yolk proteins recognized by the PY4F8 antibody are not secreted into the ECM or other subcellular organelles, suggesting that they are degraded for nutritive stores. Since the depletion is not obvious until the feeding larval stage is reached, the yolk may represent a safety store of food in the case that the larva does not encounter external food sources at this stage in development. Further studies are necessary to understand the biology of yolk utilization in starfish and in other species. In this respect, the isolation of yolk granule enzymes, which may be
responsible for the limited proteolysis of the yolk proteins, may provide clues to their depletion and how it is regulated.

(II) A cortical granule antigen of *P. ochraceus*

This final part of the study involved the identification and immunohistochemical analysis of a cortical granule protein in the unfertilized egg and early embryo. This was of interest for 3 major reasons. First, immunohistochemistry was used to investigate the storage, synthesis and secretion patterns of another blastocoel ECM component, and to compare this with that of the PM1 proteoglycan. Secondly, it was of interest to compare the storage and secretion of another type of granule in the egg with that of the ubiquitous yolk granules. And finally, the investigation of a component of starfish cortical granules was undertaken to study possible functions based on its localization during egg activation and early development, since cortical granule proteins have not been identified in the starfish until now.

1. The PC3H2-antigen is a cortical granule protein.

A general characteristic of cortical granules is their diverse morphology, including shapes resembling spindles, tubules, dumb-bells or pears (reviewed by Anderson, 1972). There are 2 common features among cortical granules of many different species. The first is that they are positioned in the cortical cytoplasm of mature eggs, and the second is that they have a very electron-dense component to them that is unmatched by any other organelle in the oocyte (Anderson, 1968; Bal, 1970; Schuel, 1978). In the present study, the PC3H2 antibody-labelled granules were located primarily in the peripheral cytoplasm of *Pisaster* eggs, and had a distribution similar to those of cortical granules. When eggs were viewed with the electron microscope, gold-labelled antibody identified granules of about 1.5 μm in diameter having a varied morphology consisting of an electron dense outer component, and an electron translucent
component towards the core with patches of intermediate stain density interspersed throughout the granules. These are morphologically similar to cortical granules that have previously been described in starfish eggs, including *Patiria miniata* (Holland, 1980), *Pisaster ochraceus* (Crawford and Abed, 1986), and *Marthasterias glacialis* (Sousa and Azevedo 1989), and somewhat similar to cortical granules in other species such as hamsters (Cherr et al., 1988) and sheep (Cran et al., 1988). These observations therefore indicate that the PC3H2 antibody is directed towards a component of starfish cortical granules.

2. The PC3H2 antigen is released during the cortical reaction

Since the observations of Harvey (1911) that the cortical granules of sea urchins are no longer visible subsequent to egg activation, investigators have agreed that the cortical granules of many organisms are released during fertilization and play a role in blocking polyspermy. However, only a few components of cortical granules from a handful of species have been characterized, and many of their functions during the cortical reaction remain unknown. Furthermore, several organisms have cortical granules which do not participate in the cortical reaction at all, such as the annelid *Chaetopterus*, the amphineuran mollusc *Moalia* and the mussel *Mytilus* (reviewed by Anderson, 1972). Some of the components of cortical granules are involved with the formation of the fertilization membrane. For example, the cortical granule lectin of *Xenopus* eggs is a metalloglycoprotein that combines with jelly coat components to form the fertilization membrane (Nishihara et al., 1986). Other cortical granule constituents identified include fucosyl and sialyl-rich glycoconjugates (Lee et al., 1988), and ovoperoxidase (Gulyas and Schmell, 1980) in the mouse; sulfated acid mucopolysaccharides in sea urchins and starfish (Schuel et al., 1974; Sousa and Azevedo, 1989); and proteases (Alliegro and Schuel, 1988), β-1,3-glucanase (Wessel et al., 1987), and and hyalin (Hylander and Summers, 1982), in sea urchins. In the present study, the PC3H2 antibody, which recognized a 125 kDa protein located within the cortical granules, was used to determined if *Pisaster* cortical granules undergo exocytosis during the cortical reaction, and if so, what the fate of this component was during early development. Shortly after fertilization, the PC3H2 antigen was released
from the granules located in the peripheral cytoplasm, and labelling was detected in the perivitelline space, the space intervening between the egg plasma membrane and the fertilization membrane. The antigen was not detected in the fertilization membrane, and by the blastula stage, no label in the perivitelline space was evident, indicating that it may have a transient role during fertilization.

3. The PC3H2 antigen is present in the early embryo

It was first reported by Anderson (1972) that when cortical granules are present, depending on the organism, they may or may not participate in the cortical reaction, and it was suggested that there were 2 types of cortical granules, one of fertilization, and one not yet classified. In P. ochraceus, while during egg maturation, many of the cortical granules not already at the periphery move towards it, there are several PC3H2-stained granules which remain in the subcortical and central cytoplasm, and appear to be randomly scattered, interspersed with the yolk granules. Investigations at the TEM level have shown that they have a similar morphology to those in the cortical region, indicating that they are probably members of the same granule population. This is confirmed by the fact that they are also stained with PC3H2 immunogold label. In addition, observations from several different batches of oocytes have revealed a consistent pattern, indicating it is not random. These granules that are left behind do not undergo exocytosis at fertilization, but are still present later in development in the blastula and gastrula stages, and when viewed with the electron microscope, have a similar morphology to the peripherally located granules. Furthermore, PC3H2- immunogold label was found over the same electron-dense regions, suggesting that they are the same type of granules.

However, while these cortical granules that have been "left behind" do not secrete material during egg activation, they do appear to secrete the PC3H2 antigen into the blastocoel, the basement membranes and one region of the hyaline layer during embryogenesis. In addition, immunogold electron microscopy shows gold label associated with Golgi complexes. The label is concentrated in the trans Golgi near the
lateral edges, and in most cases, the Golgi are in close proximity to at least one cortical granule. Gold particles were observed just on the outer sides of the cortical granule membranes as well, which suggests that these granules may be serving as storage sites for newly synthesized PC3H2 antigen. The present observations indicated that the starfish cortical granules release the PC3H2 antigen at 2 different times, the first occurring at egg activation, and the second during embryogenesis, where it becomes a major component of all 3 extracellular matrices in the embryo, the hyaline layer, the blastocoel ECM, and the basement membranes. A similar observation has been made in the sea urchin with the protein hyalin. This protein, although located in cortical granules which are exocytosed at fertilization, is also present in randomly distributed cortical vesicles, which do not move to the periphery during egg maturation, nor are they exocytosed during the cortical reaction (Hylander and Summers, 1982). These granules are thought to function in the early embryo as a hyalin reservoir, in the case that renewal of the hyaline layer is required.

4. Conclusions

The present observations indicate that one of the components of egg cortical granules is a 120 kDa protein that is released upon egg activation into the perivitelline space, but which is not incorporated into the fertilization envelope, as are several other components of cortical granules in other species. This indicates that while the PC3H2 antigen may be involved in blocking polyspermy, it does not contribute to a permanent structural barrier. While all cortical granules located in the peripheral egg cytoplasm appear to undergo exocytosis, some cortical granules resist movement to the cell periphery and release their contents later in development. One of these components, the PC3H2 antigen, is secreted to become a part of the ECM of the blastocoel, basement membranes, as well as one region of the hyaline layer. It is unknown whether this protein has a similar function at fertilization and during embryonic development. However, during the cortical reaction, its localization in the perivitelline space is transient, whereas during development, it clearly remains as a structural component of the ECM.
This study has investigated several components of starfish embryonic ECM and egg storage granules, with the intentions of studying how ECM affects cell behavior during morphogenesis. A major part of the work presented investigated the role of one particular matrix component in starfish gut morphogenesis. By generating monoclonal antibodies to starfish gastrula extract, an antibody specific for matrix in the blastocoel was isolated, the PM1 antibody. Using this antibody as a research tool, a large extracellular component of the blastocoel ECM (estimated Mr > 600 kDa) was identified. This component was determined to be a proteoglycan, based on the fact that it was stained with Alcian blue under conditions of high ionic strength. In addition, the antigen was sensitive to trypsin, indicating that it contained a polypeptide component. And finally, its assembly was disrupted by the inhibitor of proteoglycan synthesis, β-D-xyloside. Further investigations at the histochemical level revealed that the PM1 proteoglycan is synthesized and/or undergoes maturation first during early gastrulation. Secretion into the blastocoel does not occur until mid-gastrulation, suggesting that it may have a specific role in the morphogenesis of the digestive tract which begins here at that time. Function blocking studies using the PM1 antibody in embryo cultures resulted in severely malformed embryos when compared to the controls, with a marked effect on digestive tract morphogenesis. This suggests that the PM1 proteoglycan plays a specific role in the development, perhaps by providing resilience for blastocoel expansion, playing a role in the organization of the blastocoel matrix, binding other ECM components and growth factors, or providing a substrate for migrating cells.

Another component of the blastocoel ECM, the PM3H2 antigen, has a distribution in early development that is very different from that of the PM1 proteoglycan. This antigen is present in unfertilized oocytes, where it stored in the cortical granules. Although a significant amount of the antigen is exocytosed during the cortical reaction which occurs shortly after fertilization, it also appears to be secreted into various ECMs during the course of embryonic development. Whereas the PM1 antigen is secreted into the ECM
of the gastrula-stage embryo, the PC3H2 antigen first appears in the blastocoel at the blastula-stage. This secretion does not appear to be dependent upon new transcription, as the maternally-derived antigen stored in cortical granules is released into the ECM. This is in contrast to the PM1 antigen, which is not synthesized or matured until the gastrula stage. Although the PC3H2 antigen localized to several different ECMs, it was found in the same network of blastocoel matrix as the PM1 proteoglycan. The 2 different storage and secretion profiles of these ECM components suggest that they may function differently during morphogenesis. Further characterization of the PC3H2 antigen will bring us to a closer understanding of its function during fertilization and development.

Although there are reports of protein stored in yolk granules that contribute to ECMs during early development, this does not appear to be the case for the family of yolk glycoproteins recognized by the PY4F8 antibody. The results indicate that these Pisaster yolk proteins have a similar localization and depletion profile as those "classical" yolk proteins documented in many other species. The PY4F8 recognizes several major yolk proteins of the egg that are derived from a 400 kDa vitellogenin precursor, and like yolk proteins in other species, the vitellogenin appears to be synthesized and/or stored in the intestines and coelomic fluid. As the smaller yolk proteins are also detected in the intestine and coelomic fluid, there is the possibility that the vitellogenins are broken down at these remote sites and transported to the ovary in this form. Biochemical studies have shown that the starfish yolk proteins are considerably rich in glycoproteins, as endoglycosidase F digestion causes a significant shift in electrophoretic mobilities of the proteins. In addition, Con A and WGA binding to the proteins suggests that they contain N and maybe O-linked oligosaccharide chains. Although the PY4F8 antibody recognizes the classical yolk proteins, which are thought to provide a store of nutrients for the developing antibody by undergoing catabolism, they are not depleted to a significant degree until larval development begins, which indicates that these proteins may be represent a safety store of food for the feeding larva. This study by no means rules out the possibility that other proteins are stored in the yolk granules, which may contribute to ECMs in a similar way as has been reported in several other organisms.
REFERENCES


APPENDICES

Appendix 1: Freeze substitution of starfish oocytes and embryos
(After Campbell et al., 1991)

1. Harvest oocytes and embryos on ice and transfer to 15 ml conical glass centrifuge tubes.

2. Resuspend embryos but not oocytes in 10 ml of cryoprotectant solution, consisting of 15% 2,3-butanediol prepared in sea water, and allow to equilibrate for 30 minutes.

3. Prepare freezing apparatus in a fume hood, by filling a Dewar flask with liquid nitrogen, and placing an insert cup into the Dewar flask. Fill the cup with cooking grade propane (Mastercraft), and adjust an eddy current motor with an attached stirring rod so that the rod goes at least half way into the insert cup. LIQUID PROPANE IS VERY EXPLOSIVE-USE EXTREME CAUTION, AND ENSURE NO SPARKS COME NEAR THE CRYOGENIC APPARATUS.

4. After the embryos have equilibrated, remove as much solution as possible, and place 1 μl of the thick suspension on a 50 mesh EM grid. Use filter paper to draw off as much liquid as possible, and to ensure an even monolayer is present. Using a pair of cross-closing forceps, quickly plunge the grid with embryos deep into the propane for 10-15 seconds and then into the surrounding liquid nitrogen for 10 seconds. The grid can now be transferred into the pre-cooled substituting media (see below), or alternatively, the grids can be placed into freezer vials containing liquid nitrogen and stored in a liquid nitrogen freezer for long term storage. Eggs can be frozen in the same manner, but they should not be incubated in cryoprotectant prior to freezing.

5. To substitute the eggs or embryos the ethanol must first be pre-cooled to -90° C. To do this, place approximately 150 g of dry ice into a wide mouth thermos, and slowly add acetone until the thermos is half full. Then pour in liquid nitrogen and break up the acetone as it freezes until the bath reaches between -90 and -95° C.

6. Place 10 ml absolute ethanol or alcian blue (Marivac)-saturated ethanol into glass vials and pre-cool in the dry ice-acetone bath for at least 10 minutes.

7. Quickly transfer the grids with embryos into the pre-cooled substituting medium, place the vials quickly into the thermos, and store in a -70° freezer. Let the embryos substitute for 5 days, and maintain the temperature below -80° C with daily additions of liquid nitrogen.
8. After the substitution is complete, gradually bring the vials up to room temperature, and wash with absolute ethanol twice. The embryos are now ready to be embedded into JB4 or LR White resins.

9. Infiltrate the embryos with several changes of uncatalyzed resin for at least 24 hours. For JB4, polymerize the tissue in 9 mm aluminum dishes at room temperature, ensuring that at least 5 ml of solution is used per dish, and that a second dish has been placed over top in order to exclude air from the surface. Alternatively, polymerize the LR White in beam capsules at 55°C.
Appendix 2: Con A-Sepharose affinity chromatography

Column Volume: 3 ml
Washing buffer: 20 mM Tris-HCl, 0.5M NaCl, 0.1% Brij 56, pH 7.4
Eluent: 0.5 M methyl α-D-mannopyranoside in washing buffer
Temperature: 20° C
Elution Rate: 5 ml /hour
Protease inhibitors included in all buffers: 1 mM PMSF, 1 μg/ml pepstatin A, 10 mM EDTA

1. Place 3 ml of Con A-Sepharose in column, made from the empty 5 mm syringe barrel.

2. Wash column with 10 column volumes (30 ml) TBS (20 mM Tris, 0.15 M NaCl, pH 7.4) to remove the thimerosal, which is used as a preservative.

3. Wash with 1 column volume of 1 M NaCl in 20 mM M Tris-HCl and 0.1% Brij 56, pH 7.4, to reduce the free release of Con A into solution.

4. Wash with 1 column volume 0.1 M methyl-α-D-mannopyranoside in 20 mM Tris-HCl, 0.5 M NaCl, 0.1% Brij 56.

5. Apply 1.0 ml detergent solubilized sample (embryo extract) to the lectin column, and recycle through column 2 times. Wash with 10 column volumes (30 ml) of washing buffer, or until a flat line is achieved with the UV recorder (a$_{280}$), indicating that all unbound protein has been eluted from the column.

6. Apply 3 ml 0.2 M methyl-D-α-mannopyranoside eluting buffer to the column, and stop the column flow for 60 minutes after all 3 ml has entered the resin. This allows dissociation to take place prior to elution, and ensures a good recovery.

7. Resume column flow with washing buffer, and collect the peak in 1 ml fractions.

8. Concentrate the protein containing fractions in dialysis tubing placed over beads of PEG (MW 20,000) and store at -70°C.
Regeneration of column:

1. Wash with 10 column volumes of 0.1 M Tris-Cl, 0.5 M NaCl, pH 8.5.

2. Wash with 10 column volumes 0.1 M sodium acetate buffer, 0.5 M NaCl, pH 4.5 (with 1 mM calcium chloride, 1 mM magnesium chloride and 1 mM manganese chloride to preserve the binding activity of Con A).

3. Re-equilibrate with TBS, or store in above buffer, pH 6.0 with 0.01% thimerosal.
Appendix 3: Protein quantification

UV Detection at $\alpha$ 280:
This is a quick method of determining the concentration of a known pure protein solution, for which the extinction coefficient is known. This method has the advantage that none of the sample is destroyed during the reading. The absorbance maximum at 280 nm is due primarily to the presence of tyrosine and tryptophan residues.

1% Protein $E_{280}$ (i.e. the absorbance of a 10 mg/ml solution at 280 nm)

<table>
<thead>
<tr>
<th>Protein</th>
<th>$E_{280}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgG</td>
<td>13.6</td>
</tr>
<tr>
<td>IgM</td>
<td>11.8</td>
</tr>
</tbody>
</table>

1. Read the absorbance versus a buffer control at 280 nm.
2. Calculate the approximate concentration using the following equation:

$$\text{Concentration of sample} = \frac{\text{absorbance at 280 nm}}{\text{extinction coefficient at 280 nm}} \times 10 \text{ mg/ml}$$

For protein solutions that are contaminated with nucleic acids, an approximate concentration can be determined using the following equation:

$$\text{Protein concentration (mg/ml)} = (1.55 \times A_{280}) - (0.76 \times A_{260}).$$

Biorad DC protein assay:

This assay is based on the reaction of proteins with an alkaline copper tartrate solution and Folin reagent. i.e. the reduction of Folin reagent by copper-treated proteins. Color development occurs primarily by tryptophan and tyrosine residues.

The protocol was followed according to manufacturers directions:

1. Serial dilutions of the standard (BSA) was prepared, ranging from 1.5 mg/ml to 0.1 mg/ml.
2. One hundred $\mu$l of the standard solution (or sample solution) were placed in 10 ml test tubes.
3. Five hundred µl of reagent A (an alkaline copper tartrate solution) were added to each test tube, and the mixture was vortexed briefly.

4. Four ml reagent B (a dilute Folin regent) were added to each test tube, and the mixture was vortexed briefly.

5. Solutions were allowed to stand 15 minutes while the color reaction developed, and absorbance was read at 750 nm within the hour. The standard absorbances were plotted, and sample absorbances were determined based on comparison with the standard curve.
Appendix 4: Immunization of mice with Con A-specific embryo fraction

1. Prepare immunogen in Freund's incomplete adjuvant, using equal volumes of protein solution and adjuvant. Immunize 4 week-old BALB/c mice by subcutaneous injections of 100 μl immunogen/mouse. Each mouse should receive no more than 200 μg protein per administration.

2. Boost the mice at 1 month intervals with intraperitoneal injections of 100 μl pure antigen (no Freund's adjuvant).

3. Five days after each boost, take a test bleed from the tail as follows: use a sharp sterile razor blade to remove a small part of the tail end (2 mm), and collect 4 drops of blood by massaging the tail proximal to distal. Suspend the blood in 500 μl 2% blotto, and spin in a clinical centrifuge to remove the red blood cells. Use serum at dilutions of 1:10 and 1:100 to test for antibody titer on JB4-embedded embryo sections using indirect immunofluorescence microscopy.

4. Continue to monitor antigen response with test bleeds, always taken 5 days after the boost. When the immune response is satisfactory, harvest the spleen for hybridoma production 3-5 days following the boost.
Appendix 5: Hybridoma production for monoclonal antibodies:
(After Kannangara et al., 1989)

Growth of Myeloma cell line

1. 5-7 days before the fusion, remove 1 vial of myeloma cells from the liquid nitrogen freezer. Thaw at 45°C, and wash with 10 ml Dulbecco's Modified Eagle Medium (DMEM) with no fetal calf serum (FCS).

2. Centrifuge at 800 x g for 10 minutes.

3. Seed cells into 20% Fetal calf serum (FCS) in DMEM in sterile petri or T.C. dish.

4. Subculture into 10% FCS in DMEM when cells cover 50-70% of the surface area of dish.

Fusion Protocol

1. Harvest myeloma cells: 5-6 petri dishes (50-70% covered) are required per spleen. Place all of the cells in one 50 ml centrifuge tube and centrifuge 800 x g for 10 minutes. Decant off liquid.

2. Wash cells with 10 ml DMEM twice with gentle resuspension and centrifugation as above.

3. Prepare fusogen:
   a) Add 5 g polyethylene glycol (PEG) 4000 MW and 4 ml DMEM to a beaker.
   b) Heat gently with stirring at 45°C until dissolved.
   c) Cool to room temperature.
   d) Add 1 ml dimethyl sulfoxide (DMSO).
   e) Filter through a 0.45 μm Millipore filter into a sterile tube.
   f) Place in a 37°C water bath until required.

4. Harvest spleen from mouse as follows: sacrifice the mouse in a chamber filled with CO₂, spray the mouse with 70% ethanol and placed on a paper towel. Using sterile techniques, make a small incision in the abdominal skin and pull the skin in an anterior and posterior direction away from the
abdomen, ensuring that the skin is pulled well over the head of the mouse to maintain a sterile environment in the abdominal cavity. Dissect out the spleen from the mesentary and placed in a sterile petri dish containing 10 ml DMEM. Cover spleen with a 1 inch square of sterile gauze, and squeeze through the gauze with the sterile end of a syringe plunger. Centrifuge as in step 1.

5. Mix spleen and myeloma cells together in a round bottom 12 ml tube and centrifuge as above.

6. Remove all liquid from the pellet of mixed spleen and myeloma cells.

7. **Precise timing and volumes are required for the following steps:**
   a) Add 1 ml of fusogen to cells over a time period of 1 minute while stirring the pellet constantly with the pipette tip.
   b) Add 1 ml DMEM over a time period of 1 minute to the cells with stirring, to slowly dilute out the PEG solution.
   c) Add 2 additional ml DMEM over the next minute with stirring.
   d) Add 6 additional ml DMEM over the next 3-5 minutes with stirring.

8. Spin down cells and resuspend in 5 ml (HAT) media (media supplemented with hypoxanthine, aminopterin and thymidine), and with 20% FCS included.

9. To isolate a feeder cell population, sacrifice 2, 4-6 week BALB/c mice and remove their thymuses. Follow the same sterile techniques as for spleen removal, but in this case, after the removal of skin, use a pair of sharp scissors to open the rib cage and access the area deep to the manubrium where the thymus lies. Be careful not to rupture the heart or major vessels. Mince the thymuses through gauze using the same technique that was used on the spleen, wash in DMEM, and resuspend in 10 ml HAT media.

10. Mix thymocytes with the fused cell mixture, and dilute to 50-100 ml with HAT. Plate out at 100 µl per well into sterile 96 well plates, and incubate at 37° C with 5% CO₂.

11. After 3-4 days, add 100 µl HT media (HAT without aminopterin).

12. After 7 days, remove 180 µl media and add 180 µl fresh HT media.

13. After 10 days, test clones for antibody production by removing 30 µl of hybridoma supernatant and performing immunofluorescence microscopy on thin sections of JB4-embedded embryos.
Appendix 6: Recloning hybridomas

Once the selected hybridomas have grown to occupy a large part of the well, recloning must be carried out in order to obtain wells containing only one clone; this should be done immediately, as prolonged growth can result in selection against the particular clone of interest.

1. Sacrifice 2 BALB/c mice and remove thymuses as described in appendix 4. Suspend the cells in 50 ml DMEM supplemented with 20% FCS.

2. Add 100 μl of thymocyte suspension to each well of a 96 well plate; add 200 μl to the first well in each row (across), and 0 μl to the last well in each row.

3. Add 15 μl of cells from the original well of clones to the first well in each row (A-H). Use 4 rows /clone to ensure a positive single clone.

4. Make serial dilutions of each row by removing 100 μl from well #1 and adding to #2 and so on until well #12 now has 100 μl. When diluting, mix each well vigorously by pipetting up and down several times before removing the 100 μl.

5. Incubate plates at 37° C, and visually inspect each well daily for single colony formation. After 8-10 days, retest the supernatant from wells containing single clones.
Appendix 7: Expansion and freezing of positive clones

1. Once positive clones have been selected and have been recloned to ensure that all hybridomas are derived from a single clone, the cells can be expanded for further use. This is done by gradually increasing the surface area available to the growing cells, starting with 24 well tissue culture wells, and then sterile 60 mm, 90 mm, and finally 150 mm petri or tissue culture dishes. In every expansion, cells should be left undisturbed until the bottom of the well or plate is crowded. The cells should have smooth puffy plasma membranes, which indicates that they are still in log phase growth, as opposed to wrinkled, collapsed and irregular ones, which indicates they are dead or dying.

2. Harvest 2, 90 mm plates of cells in log phase growth; centrifuge at 800 x g for 10 minutes and resuspend the cells in 5 ml of freezing media (1 ml FCS, 0.5 ml DMSO and 3.5 ml DMEM).

3. Aliquot exactly 1 ml into 1.5 ml sterile freezer vials, and place the vials into a foam-insulated cardboard box, and then quickly into a -70° C freezer overnight to allow the cells to freeze slowly.

4. Remove vials from freezer and store them in liquid nitrogen indefinitely.

5. To resurrect the hybridomas, remove 1 vial of cells from the liquid nitrogen freezer, and gently thaw them in a 37° C water bath. Wash with 10 ml DMEM, and resuspend in 10 ml DMEM supplemented with 20% FCS. Plate all the cells in one 90 mm dish, and leave undisturbed until expansion is required.
Appendix 8: Isotyping monoclonals with the Serotec Isotyping kit.

This diagnostic test is based on red cell agglutination. Sheep red blood cells are coupled to antibodies directed to the 6 different mouse monoclonal isotypes. The blood cells are then incubated with hybridoma supernatant in microtiter plates, and positive agglutination is easily visible on the bottom of the plates, indicating the isotype of the supernatant.

**Protocol:**

1. Make 1/10 and 1/50 dilutions of hybridoma supernatants in PBS; ensure that highly cloned supernatants are used.

2. Pipette 30 μl of diluted supernatant into each of eight wells across a "U" bottomed microtiter plate.

3. Add 30 μl of each isotyping reagent (antibody-coupled sheep RBCs) to the wells containing the supernatant.

4. Cover the plate and place on a shaker for 6 seconds.

5. Leave plate on a cool flat surface with no vibration for 1 hour.

6. Read results as follows: partial or full carpet of cells over bottom of well indicates that agglutination has occurred, and is therefore a positive result; a small red circle of cells at the bottom of the well indicate that no agglutination has occurred, and therefore is a negative result.
## Appendix 9: Photography

### Microscopy

<table>
<thead>
<tr>
<th>Light and DIC microscopy:</th>
<th>Exposure time (sec.)</th>
<th>Film</th>
<th>Developer/conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>-color</td>
<td>Automatic</td>
<td>Fugicolor 100</td>
<td>Automatic, -1 density</td>
</tr>
<tr>
<td>-B/W</td>
<td>Automatic</td>
<td>Kodak T-MAX 100</td>
<td>Kodak D-76, 7 min. @ 22°C</td>
</tr>
</tbody>
</table>

| Fluorescence light microscopy: | | | |
| -color                      | 3-7 seconds          | Fugicolor 1600 | Automatic, +3 density |
| -B/W                        | 5-10 seconds         | Kodak TMAX p3200 | Kodak TMAX, 10 min. @ 22°C |

| Gels and X-rays (Reprovit with light box) | | | |
| -color                      | F8, 1/15, 1/30, 1/60 | Fugicolor 100 with 80A filter | Automatic, -2 density |
| -B/W                        | F11, 1/8, 1/15, 1/30 | Kodak high contrast copy | Kodak D-19, 6 min. @ 22°C |

| Blots (Reprovit with side illumination) | | | |
| -color                      | F8, 1/2, 1 | Fugicolor 100 with 80A filter | Automatic, -2 density |
| -B/W                        | F11, 1/2, 1/4, 1/8 | Kodak high contrast copy | Kodak D-19, 6 min. @ 22°C |

| TEM (B/W)                   | 2 seconds | Kodak fine grain (5302) | Kodak D-19, 5 min. @ 22°C |

| Reprints (Reprovit, side illumination): | | | |
| -B/W                      | F8, 1/15, 1/30 | Kodak T-MAX 100 | Kodak D-76, 7 min. @ 22°C |
| -color                    | F8, 1/2, 1, small prints | Fugicolor 100 with 80A filter | Automatic |
|                           | F8, 1, 2, large prints | Kodak fine grain (5302) | Automatic |
|                           | F8, 1/4, 1/2, immunofluorescence | | Automatic, +2 density |

### Paper:
- To print B/W prints from B/W negatives: Ilford multigrade III RC, 90s, Kodak Dektol 1:2
- To print B/W prints from color negatives: Kodak panalure select RC (FH), Kodak Dektol 1:2
Appendix 10: Preparation of colloidal gold
(After Slot and Geuze, 1985)

To make 100 ml gold sol, prepare the following solutions:

Solution I: chloroauric acid
1% HAuCl₄ 1 ml
distilled water 79 ml

Solution II: citric acid (reducing soln)
1% tri-sodium citrate (aqueous) 4 ml
1% tannic acid (aqueous) 0-0.5 ml
distilled water up to 20 ml

<table>
<thead>
<tr>
<th>1% Tannic Acid</th>
<th>1% sodium citrate</th>
<th>Particle size (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5 ml</td>
<td>4 ml</td>
<td>6 nm</td>
</tr>
<tr>
<td>0.125 ml</td>
<td>4 ml</td>
<td>10-15 nm</td>
</tr>
<tr>
<td>/</td>
<td>4 ml</td>
<td>20-25 nm</td>
</tr>
<tr>
<td>/</td>
<td>2 ml</td>
<td>30-35 nm</td>
</tr>
<tr>
<td>/</td>
<td>1 ml</td>
<td>60-70 nm</td>
</tr>
</tbody>
</table>

Procedure:

1. Heat solutions I and II separately in a 60°C water bath.
2. Add reducing solution II to solution I quickly with stirring; ensure temperature remains constant at 60°C to avoid the formation of a heterodisperse sol.
3. Keep the solution stirring until color change to red or purple is complete; the reaction time will increase proportionally to particle diameter size.
4. Store at 4°C

To size gold particles:

1. Place 1μl gold sol on a coated 100 mesh grid, and air dry.
2. Photograph gold particles with a magnification of at least x 16,000.
3. Measure the diameter size on the negative, and use the following formula:
   \[ \text{Negative measurement of diameter (mm)} \times 10^6 = \text{diameter (nm)} \]
Appendix 11: Microtitration assay for determination of the correct protein concentration for gold sol stabilization.

Protocol:

1. Add 100 µl distilled water to each of 10 1.5 ml Eppendorf tubes.

2. Prepare a 1 mg/ml solution of protein to be assayed in distilled H₂O. Dialyze if necessary against 2 mM sodium borate, pH adjusted just basic to the pI of the protein (9.0 for IgG).

3. To the first tube, add 100 µl of the protein solution. Serially dilute by removing 100 µl from first tube, adding it to the 2nd tube and pipetting up and down 3 times; then continue subsequent dilutions to the 9th tube, and leave 10th tube protein free.

4. Adjust the pH of 5.0 ml gold sol to just basic of the pI of the protein being assayed using 0.2M K₂CO₃.

5. Add 500 µl gold sol to each well and pipette stir. Stand 15 minutes.

6. Assess the resistance of mixture to salt-induced flocculation by adding 100 µl of 10% NaCl. Stand 5 minutes.

7. The last well to maintain a red color represents the end point for protein stabilized gold.

8. Calculate protein stabilizing concentration per ml gold sol and double for experimental protein stabilizing concentration.
Appendix 12: Gold conjugation to rabbit anti-mouse IgG/M

1. Dilute 100 μl of stock protein solution (2 mg/ml) up to 500 μl in 2 mM sodium borate-HCl, pH 9.0, and dialyze against borate buffer overnight.

2. Adjust the pH of 10 ml gold sol to 9.0 with 0.2 M K₂CO₃.

3. Add 0.5 ml dialyzed protein all at once to stirring gold sol, and stir for 30 minutes.

4. Add 0.5 ml 10% BSA to stirring gold solution and continue to stir a further 5 minutes.

5. Place in 1.5 ml Eppendorf tubes and centrifuge 15,000 x g for 45 minutes.

6. Resuspend in 10 mM Tris with 50 mM NaCl, 0.1% BSA (pH 8.2), and gradually increase the salt concentration to 150 mM NaCl by dialysis over 24 hours.

7. Add sodium azide to a final concentration of 0.02%, and store at 4°C for 1 year.
Appendix 13: Gradient gels
(Use distilled water and electrophoresis grade reagents throughout)

Reagents:

Lower Tris (1.5 M) Dissolve 36.34 g Tris base and 0.8 g SDS in 150 ml H₂O. Titrate pH to 8.8 with 6N HCl. Add H₂O to final volume of 200 ml, and Millipore filter (0.45µ).

Upper Tris (0.5M) Dissolve 12.11 g Tris base and 0.8 g SDS in 150 ml H₂O. Titrate pH to 6.8 with 6N HCl. Add H₂O to final volume of 200 ml, and Millipore filter (0.45µ).

30% Acrylamide (2.5% cross-linker) Dissolve 7.5 g acrylamide and 0.2 g bis-acrylamide in H₂O to a final volume of 25 ml, and Millipore filter (0.45µ).

Electrophoresis Buffer: Dissolve 3.03 g Tris base, 14.41 g glycine and 1 g SDS in 1L H₂O.

10% Ammonium. Persulfate Dissolve 100 mg in 1 ml H₂O. Store the solution at 4°C and discard after one day.

To Pour 7 Mini Slab Gradient Gels:

<table>
<thead>
<tr>
<th>Stock Solutions</th>
<th>3%</th>
<th>12%</th>
</tr>
</thead>
<tbody>
<tr>
<td>30% acrylamide</td>
<td>2.0 ml</td>
<td>8.0 ml</td>
</tr>
<tr>
<td>Lower Tris (1.5 M)</td>
<td>5.0 ml</td>
<td>5.0 ml</td>
</tr>
<tr>
<td>H₂O</td>
<td>13.0 ml</td>
<td>7.0 ml</td>
</tr>
</tbody>
</table>

Degas for 5 minutes.

TEMED 10 µl 10 µl
10% ammonium persulfate 75 µl 75 µl

Protocol:
1. Prepare acrylamide solutions and degas for 10 minutes.

2. To set up gradient gel apparatus, place the gradient maker on magnetic stirrer, place a small stir bar in the column with outflow tubing attached, and ensure that connecting port is closed off.

3. Add TEMED and APS to acrylamide solutions, swirl gently, and pour into gradient maker, ensuring that the 3% acrylamide solution is poured into the side with outflow tubing.

4. Attach outflow tubing to multi-gel apparatus, open connecting port on gradient maker, turn on the magnetic stirrer, and lastly open stopcock on outflow tubing. Allow the acrylamide to pour in slowly, so that the all the solution has entered within 2-3 minutes.
5. Disconnect outflow tubing, and rinse out gradient apparatus immediately. Then place 200 µl water-saturated isobutanol on each gel to ensure a flat even surface during polymerization.

6. Rinse the isobutanol off the gels after 1 hour, and continue to polymerise for a further hour; store gels in plastic bags at 4°C.

7. Prior to PAGE run, allow gels to equilibrate to room temperature and pour a stacking gel over top.

**Stacking gels: (makes 2)**

<table>
<thead>
<tr>
<th>Stock Solutions</th>
<th>2.5%</th>
</tr>
</thead>
<tbody>
<tr>
<td>30% acrylamide</td>
<td>0.5 ml</td>
</tr>
<tr>
<td>Upper tris (0.5 M)</td>
<td>1.5 ml</td>
</tr>
<tr>
<td>H₂O</td>
<td>4.0 ml</td>
</tr>
<tr>
<td>TEMED</td>
<td>6µl</td>
</tr>
<tr>
<td>10% ammonium persulfate</td>
<td>45µl</td>
</tr>
</tbody>
</table>

Stacking gels are poured according to manufacturers instructions (BioRad) by first placing a comb (either for multilane or preparative runs) at a 45° angle on top of the running gel, and then slowly pouring the acrylamide in to avoid bubble formation.
Appendix 14: PM1-Imunoaffinity column preparation and chromatography

**Coupling Protocol:**

Affi-Gel 10 (BioRad) is a N-hydroxysuccinimide ester of a derivatized cross-linked agarose gel bead support, which allows spontaneous coupling of ligands in a quick 1-step procedure. The coupling protocol of PM1 to Affi-Gel 10 was based on the manufacturers specifications, however because PM1 is an IgM class monoclonal, some alterations in time, pH and buffer strength were necessary. All procedures were performed at 4°C.

1. Purified PM1 antibody from 2 ml ascites fluid was dialyzed against the coupling buffer (0.25 M sodium bicarbonate, pH 8.7) overnight, and 100 μl were removed and saved for later use to determine the efficiency of protein coupling.

2. Affi-Gel 10 was removed from the freezer, and 3 ml of the slurry suspension in isopropyl alcohol were washed with 20 ml ice-cold distilled water using a Buchner filter with Whatman #54 hardened filter paper. One ml of the washed and now activated gel was quickly transferred to a 10 ml snap cap round bottom vial and stored on ice.

3. The cold buffered IgM protein solution was diluted to 3 ml in coupling buffer (see above) and then added to the gel; the vial was rotated end over end for 1 hour at 4°C, and then for a further 4 hours at room temperature. At this time, 100 μl of supernatant were removed and checked for coupling efficiency, using UV absorption at 280 nm. For this, the supernatant was diluted in 0.01 N HCl to lower the pH to prevent coupling by-products (hydroxysuccinimide) from interfering with the absorption reading.

4. After coupling efficiency was established, 0.1 ml 1 M ethanolamine HCl (pH 8.0) were added to the slurry to block any remaining active ester groups, and rotation was continued for 1 hour.
Chromatography protocol:

1. The coupled gel was transferred to a 3 ml column made from an empty syringe barrel which was connected to a Pharmacia ultraviolet detector (α 280). The column was washed with coupling buffer until a flat line on the UV recorder was obtained.

2. Prior to sample application, the column was pre-equilibrated with 20 column volumes of TBS, pH 7.4. Guanidine HCl embryo extract (0.5 ml), which had been dialyzed against TBS overnight and freshly supplemented with 1 mM PMSF, was then applied to the column at a flow rate of 5 ml/hour. The extract was recycled through the column 2 times, and the column was then washed with TBS until a flat line was achieved, indicating that all unbound protein was washed from the column.

3. The PM1 antigen was then eluted off the column using 2 ml of 0.1 M triethylamine, pH 11.0. The peak was collected in 1.0 ml fractions into tubes containing 100 µl 1 M Tris, pH 6.0. Fractions were immediately pooled and concentrated 10 fold via ultrafiltration using Centricon-50 filters (Amicon), which have a molecular cutoff of 50 kD. The affinity-purified PM1 antigen was stored in small aliquots at -70° C.

4. The PM1 affinity-column was washed extensively with TBS and re-used successfully for 5 additional chromatography runs, with only a minor decrease in peak size. For storage of the column, 0.2% sodium azide was added to the TBS to prevent bacterial growth.
Appendix 15: Immunoprecipitations

Reagents:

Embryo extraction buffer: 10 mM Tris, 1% triton X-100, 0.5% sodium deoxycholate, 0.1% SDS (sodium dodecyl sulphate), pH 7.3.

Immunoprecipitation buffer: 20 mM Tris, 150 mM NaCl, 0.1% triton X-100, pH 7.8.

Protease inhibitors: 25 mM EDTA, 1 mM iodoacetamide, 1 mM PMSF, 1 μg/ml pepstatin A, and 1 mM EGTA

Perform all procedures at 4°C in 1.5 ml Eppendorf tubes.

1. Pre-wash 50 μl settled Protein A-Sepharose 4B fast flow (Sigma) with embryo extraction buffer by rotation end over end for a total of 3, 10 minute washes. After each wash, settle the Protein A-beads with a brief (10 seconds) gentle spin in a clinical centrifuge.

2. Incubate the washed Protein A-Sepharose with the linker antibody (goat anti-mouse IgG) for 1 hour (add 20 μl of a 1 in 40 dilution of whole serum).

3. Wash with immunoprecipitation buffer as above three times over 30 minutes.

4. Incubate with 10-100 μl hybridoma supernatant for 1 hour with constant rotation.

5. Wash as in step 3, and save 25 μl of the bead suspension to be used later as a control immunoprecipitation (i.e. without embryo extract).

6. Incubate with 200 μl embryo extract 1-2 hours with rotation. Be sure to add a fresh dose of PMSF (10 μl of a 1 mg/ml stock of PMSF in acetone) at this time.

7. Wash with immunoprecipitation buffer thoroughly (3-5 times over 30 minutes).

8. Resuspend beads in 50 μl reducing sample SDS-PAGE buffer, and heat to 85°C for 15 minutes. Centrifuge 1 minute at 125 x g, and carefully remove supernatant from bead pellet. Use immediately for SDS-PAGE, or store at -20°C.
Appendix 16: Buffers

Phosphate Buffered Saline (PBS)
(Crawford, 1972)

Use 50 ml Stock V and 50 ml Stock VI and bring up to 1 L with distilled H₂O; adjust pH to 7.4

**Stock V**

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
<th>Stock VI</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>160.0g (0.14M)</td>
<td>MgSO₄·7H₂O 15.4g (0.003M)</td>
</tr>
<tr>
<td>KCl</td>
<td>8.0g (0.005M)</td>
<td>CaCl₂·2H₂O 1.6g (0.0005M) or</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>3.0g (0.001M)</td>
<td>CaCl₂·6H₂O 2.4g (0.0005M)</td>
</tr>
<tr>
<td>Na₂HPO₄·7H₂O</td>
<td>5.8g (0.001M)</td>
<td>or</td>
</tr>
<tr>
<td>or anhydrous</td>
<td>3.1g (0.001M)</td>
<td></td>
</tr>
</tbody>
</table>

Dissolve in distilled H₂O and make up to 1 L.

**2% Blotto:**

Dissolve 2 g of Carnation non-fat milk powder in 1 liter PBS with 0.1% sodium azide; adjust pH to 7.4.

Tris Buffered Saline (TBS)
(Sigma)

TBS ranges commonly from 20-50 mM with 0.15 M NaCl. The pH varies with the temperature, therefore to make a 1 L solution of 50 mM Tris, follow the table below:

<table>
<thead>
<tr>
<th>pH@5°C</th>
<th>pH@25°C</th>
<th>pH@37°C</th>
<th>Trizma HCl</th>
<th>Trizma Base</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.76</td>
<td>7.20</td>
<td>6.91</td>
<td>7.02</td>
<td>0.67</td>
</tr>
<tr>
<td>7.97</td>
<td>7.40</td>
<td>7.12</td>
<td>6.61</td>
<td>0.97</td>
</tr>
<tr>
<td>8.18</td>
<td>7.60</td>
<td>7.30</td>
<td>6.06</td>
<td>1.39</td>
</tr>
<tr>
<td>8.37</td>
<td>7.80</td>
<td>7.52</td>
<td>5.32</td>
<td>1.97</td>
</tr>
<tr>
<td>8.58</td>
<td>8.00</td>
<td>7.71</td>
<td>4.44</td>
<td>2.65</td>
</tr>
</tbody>
</table>
Citrate-Phosphate Buffer, pH 5.6:

For 100 ml of buffer, mix 42 ml 0.1 M citric acid stock with 58 ml 0.2 M Na₂HPO₄ stock.

Sodium Phosphate Buffer:

(Gomori, after Sorensen, 1955)

<table>
<thead>
<tr>
<th>pH @ 25° C</th>
<th>x ml 0.2 M Na₂HPO₄</th>
<th>y ml 0.2 M NaH₂PO₄</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.8</td>
<td>4.0</td>
<td></td>
</tr>
<tr>
<td>6.0</td>
<td>6.15</td>
<td></td>
</tr>
<tr>
<td>6.6</td>
<td>18.75</td>
<td></td>
</tr>
<tr>
<td>7.2</td>
<td>36.0</td>
<td>14.0</td>
</tr>
<tr>
<td>7.6</td>
<td>43.5</td>
<td>6.5</td>
</tr>
<tr>
<td>8.0</td>
<td>47.35</td>
<td>2.65</td>
</tr>
</tbody>
</table>