Localization and Partial Characterization of Some Extracellular Matrix Molecules Which May Be Involved In Gut Morphogenesis of the Starfish, *Pisaster ochraceus*

by

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We accept this thesis as conforming to the required standard

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Date __Jan 12, 1999__
The extracellular matrix (ECM) is important in normal development by influencing cell adhesion, migration, and differentiation. If one includes the oocyte jelly coat as a part of the ECM, it is present from before fertilization. ECM is made up of a variety of molecules, many of which are uncharacterized in lower invertebrates. In this study, a polyclonal antibody (PJC-1) which recognizes several high molecular weight ECM molecules of the starfish *Pisaster ochraceus* was used in conjunction with a variety of techniques to study the ECM. Using immunofluorescence light microscopy and immunogold transmission electron microscopy, it was determined that PJC-1 recognizes molecules found in the acrosomal granule and in the external mucous ECM of sperm, and in the jelly coat, vitelline membrane, cortical granules, yolk granules, cell membrane and cytoplasm of mature oocytes. In embryo stages before, during, and after gastrulation, molecules recognized by PJC-1 are found in vesicles in ectodermal, mesenchymal, and endodermal cells. These molecules were more highly expressed in the hyaline layer, blastocoel ECM, and in particular the ECM of the gut lumen as development progressed. Immunoblots of extracts from different developmental stages showed that an increase of a 160 kDa (non-reduced) molecule correlated with the onset of gastrulation, while the upregulation of a second molecule of about 145 kDa (reduced) coincided with segmentation and differentiation of the larval gut. Embryos raised in sea water containing PJC-1 at 2.5%, 5%, and 10% exhibited perturbation of the formation of the distal gut, indicated by the development of a prolapsed anus. When viewed together, the perturbation,
immunoblot, immunogold and immunofluorescence data suggest that PJC-1 recognizes at least one molecule which may be involved in the morphogenesis of the digestive tract.
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LIST OF ABBREVIATIONS

BSA  bovine serum albumin
BL   basal lamina
BM   basement membrane
Brij-56  polyoxyethylene 10 cetyl ether
CAPS  3-[cyclohexylamino]-1-propanesulfonic acid
c\(\text{H}_2\text{O}\)  sea water
DABCO 1,4-diazabicyclo[2.2.2]octane
d\(\text{H}_2\text{O}\)  distilled water
ddH\(_2\text{O}\)  Milli-Q filtered distilled water
DIC  diffraction interference phase contrast
ECL  enhanced chemiluminescence (ECL is a trademark of Amersham Pharmacia Biotech Inc.)
ECM  extracellular matrix
EDTA  ethylenediaminetetraacetic acid
FITC  fluorescein isothiocyanate
\(g\)  normal earth gravity
g.i.  gastrointestinal
GV   germinal vesicle
HL   hyaline layer
HRP  horseradish peroxidase
IgG  Immunoglobulin class G
<table>
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<tr>
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<tr>
<td>kDa</td>
<td>kilodaltons</td>
</tr>
<tr>
<td>kV</td>
<td>kilovolts</td>
</tr>
<tr>
<td>M</td>
<td>molar (moles per litre)</td>
</tr>
<tr>
<td>1-MA</td>
<td>1-methyladenine</td>
</tr>
<tr>
<td>M_r</td>
<td>relative mobility</td>
</tr>
<tr>
<td>NGS</td>
<td>normal goat serum</td>
</tr>
<tr>
<td>NRS</td>
<td>normal rabbit serum</td>
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<td>PAGE</td>
<td>polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PA/S</td>
<td>periodic acid/Schiff reagent</td>
</tr>
<tr>
<td>pl</td>
<td>isoelectric point (in pH units)</td>
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<td>PJC-1 polyclonal antibody from rabbit 1, bleed #6</td>
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<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PBS/blotto</td>
<td>PBS containing skim milk powder</td>
</tr>
<tr>
<td>PMSF</td>
<td>phenylmethylsulfonyl fluoride</td>
</tr>
<tr>
<td>PVDF</td>
<td>polyvinylidene difluoride</td>
</tr>
<tr>
<td>rpm</td>
<td>revolutions per minute</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecylsulfate</td>
</tr>
<tr>
<td>Tris</td>
<td>tris(hydroxymethyl)aminomethane</td>
</tr>
<tr>
<td>TBST</td>
<td>Tris-buffered saline with Tween</td>
</tr>
<tr>
<td>TEM</td>
<td>transmission electron microscope (microscopy)</td>
</tr>
<tr>
<td>TEMED</td>
<td>N,N,N',N'-tetramethylethylenediamine</td>
</tr>
<tr>
<td>UV</td>
<td>ultraviolet</td>
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I would like to thank first my supervisor, Dr. Bruce Crawford for giving me a chance and for the guidance, support, and encouragement he has shown me over the past three and a half years. I would also like to thank the members of my supervisory committee, Dr. Linda Matsuuchi and Dr. Calvin Roskelley for their continued support and guidance. The members of the Crawford lab, both past and present, have done much work without which this thesis would not have been possible: Lincoln Crichlow for developing the antibody I used in this study; Dr. Corinne Reimer for the figure on early development; Craig Martin for his technical help; Turen Pang for allowing me to use some of his material; and Bita Maghsoodi get thanks for putting up with me as I wrote this. You have all made the lab a fun place to work. Although all the graduate students in the department have been good friends, I most especially wish to thank Candace Hofmann, Leslie Kerr, David Mulholland, and Aruna Somasiri for help of a technical nature and for supplying some of the chemicals used. I wish to thank the many people whom I have met and have made my time in Vancouver enjoyable. However I owe the biggest thanks to my family and most especially my fiancée; without their support I would not have been able to do this. Marcelle, I'm coming home!

I wish to dedicate this thesis to the memory of my father,

Stavros Leventis

(1945-1995)
I. INTRODUCTION

1. Overview

Over the course of their early development, organisms undergo cell division, cell migration, differentiation, and reorganization. These processes, termed morphogenesis, lead to a more complex multicellular three dimensional organization complete with organs and organ systems. The forces driving morphogenesis occur at a cellular level in part due to of changes in cell-cell and cell-substrate, or extracellular matrix (ECM), interactions. The ECM, which is secreted by the cells, provides an environment into which both inhibitory and permissive molecular signals are secreted. It also has the ability to directly influence cells by way of cellular receptors which bind to ECM molecules. Different such receptors trigger different signaling cascades within the cells which ultimately alter them. Cells also use the ECM as an environment through or along which they can migrate by way of adhesion to the ECM substrate.

The ECM is not static throughout development, but is in a state of constant change as cells secrete new molecules into it, modify existing ECM molecules (e.g. Vafa et al., 1996), or alter the physical characteristics of it by their passage through it (Hay, 1990; Hay, 1995). Frequently, the appearance of new molecules in the ECM correlates with a change in the organization of nearby cells. This may indicate that these new ECM components have a direct role in these changes in cellular
organization. The study of the ECM has often been performed by examining where and when its components are produced and secreted, and what morphogenetic changes are occurring nearby both temporally and spatially.

Because the embryos of many organisms are opaque, the direct observation of morphological changes is difficult. Most early work in embryology focused on the study of sections of fixed material to determine when specific morphological events occurred. Tissue culture techniques, which are continuing to become more refined, have been used for many decades to study cell-cell and cell-ECM interactions. Cells are grown in or on gels which attempt to mimic part or all of the ECM. Although this is a powerful method of study, cells in culture do not behave identically to cells in vivo in part because not all the components of the ECM, or the signaling molecules secreted into it, are contained in the artificial gels. This may be by design or because some of the omitted molecules are unknown. However, the use of organisms from the invertebrate classes, which are frequently transparent and/or translucent for at least a part of their early development, allows for the direct observation of morphological change in a living system. Many of the known vertebrate ECM components have homologues in invertebrate animals. Here, the experimental animal is the embryo of the starfish *Pisaster ochraceus* (Echinodermata: Asteroidea) which develops from a fertilized oocyte to a feeding larva in about 6 d and is translucent throughout this time. This allows for the direct observation of morphogenetic events in intact animals. In addition, the fact that development is synchronous allows large numbers of embryos to be raised for biochemistry. Using a polyclonal antibody developed in this
laboratory against an isolated, periodic acid/Schiff reagent positive, high molecular weight (~250-350 kDa) ECM molecule, changes in some elements of the extracellular matrix were studied over the course of the first 7 d of development during which most of the gastrointestinal tract is formed.

2. Gametes of *Pisaster ochraceus*

Like most starfish, *Pisaster ochraceus* has separate sexes. In nature, the gonads mature annually based upon photoperiod and the gametes are released into sea between May and August (Strathmann, 1987). Because they reproduce by free-spawning, large numbers of gametes must be released, hence they are small.

(a) Sperm

All asteroid sperm, including those of *Pisaster ochraceus*, have a spherical head with the acrosome at the anterior end of the nucleus in a depression (see Chia and Bickell, 1983). The acrosome, a membrane bound vesicle, is spherical in shape with a diameter of about 0.7 μm (personal observation). By transmission electron microscopy, it is seen to consist of a dense outer cortex and a less dense inner region. Between the acrosome and the nucleus is a small amount of amorphous material of known to be comprised mostly of monomeric G-actin which is postulated to play a role in fertilization (Chia and Bickell, 1983) termed the periacrosomal material. Posterior to the nucleus is the middle segment containing a single large
mitochondrion, which forms a band around the sperm, and the centrioles (see Chia and Bickell, 1983). The diameter of the head, including the middle segment, is 2.0 μm (Chia et al., 1975), while the tail is about 8-10 μm in length (personal observation).

(b) Oocytes

*Pisaster ochraceus* oocytes are 150-160 μm in diameter and are pale orange in colour (Strathmann, 1987). They are covered in several layers of extracellular matrix material which are secreted while the oocyte develops. Immediately outside the cell membrane lies the thin vitelline membrane which is about 0.5 μm in thickness (Crawford and Abed, 1986). In sea urchin, it is known to consist of 8 major glycoproteins (Correa and Carroll, 1997b). Short microvilli project toward and are embedded into this layer. The jelly coat, a mucous-like coating of ECM material which swells when the oocyte is released into sea water, varies in thickness, but is seen to be about 2.5 μm thick by light microscopy. It is composed of three regions. The innermost portion consists of a fine network of fibers and granular material, and is about 1 μm across. The thin middle region is only about 0.2-0.3 μm thick, and contains granular material and a loose network of fibers thicker than those of the inner layer. The outer layer is the thickest (1.2-1.3 μm) and consists of dense, thick fibers and granular material (Crawford and Abed, 1986). Immediately inside the plasma membrane lies a well defined cortex containing many vesicles 0.8-1.1 μm in diameter (Crawford and Abed, 1986). These cortical granules contain proteins which are exocytosed immediately upon fertilization to form the fertilization membrane and
the early hyaline layer (Holland, 1980; Hylander and Summers, 1982). Other granules of similar size but with different staining characteristics are also found here. Just deeper to the cortical granules are found large, relatively lightly staining vesicles with no known function in starfish. Deeper and throughout the cytoplasm of the oocyte are many yolk granules of various sizes, and other granules similar in size and at least partial antibody characteristics to cortical granules (Reimer, 1994; Reimer and Crawford, 1995). Immature oocytes have a large nucleus with a prominent nucleolus. Together, these structures form the so-called germinal vesicle (GV).

(c) Gametes at fertilization

The process of fertilization in Pisaster ochraceus is quite a complex one; what follows is a brief description. 1-methyladenine (1-MA) from either an exogenous source (i.e. when culturing embryos) or when released from the radial nerve in the starfish, causes maturation and ejection of the oocytes (Kanatani, 1973). Approximately 90 min following the application of 1-MA, the GV breaks down and the oocyte is considered to be mature. It is also at about this time that the ovary contracts to eject the oocytes (Kanatani, 1973). When a sperm encounters an oocyte, several molecules found in jelly coat together trigger the acrosome reaction in sea urchin and at least some starfish. The jelly coat was first noted to play a role in triggering the acrosome reaction in sea urchins (Dan, 1956) and molecules thought to be involved in this were recently isolated from sea urchin egg jelly (Keller and Vacquier, 1994). Three groups of molecules which cause the acrosome reaction in the starfish
Asterina pectinifera have been identified. They are: acrosome reaction-inducing substance (ARIS), a sulfated glycoprotein (Hoshi et al., 1990); a group of sulfated steroid saponins (Co-ARIS; Hoshi et al., 1990); and a group of sperm-activating peptides (asterosaps; Nishigaki et al., 1996). Soon after encountering these molecules, the acrosome granule exocytoses, releasing substances which allow the sperm to more easily penetrate the jelly coat and vitelline membrane, and exposing an oocyte binding molecule (Hoshi, et al., 1990). The supply of G-actin present within the periacrosomal material rapidly polymerizes in the direction of the surface of the oocyte, forming the fertilization rod and pushing some of the sperm cell membrane with it (Chia and Bickell, 1983). Molecules on the surface of this cell membrane interact with sperm binding proteins found on the surface of the oocyte, which are about 305 kDa in the sea urchin (Correa and Carroll, 1997a). The membranes fuse, and the sperm head enters the oocyte. Almost immediately, there is a membrane depolarization in the oocyte and a rapid exocytosis of the cortical granules into the perivitelline space. The fertilization membrane is formed by the interaction of the vitelline membrane and the contents of the cortical granules. In sea urchin, cortical granules are known to include hyalin, a 330 kDa glycoprotein which is the major constituent of the hyaline layer, proteases, a β-glucanase, and a peroxidase which serves to harden the fertilization membrane (McClay et al., 1990). Other vesicles containing different molecules sequentially release their contents, including the cell adhesion molecule cadherin, into the perivitelline space between the fertilization membrane and the cell surface for greater than 30 min following fertilization (Matese et al., 1997). After it forms, the fertilization membrane rises off the
plasma membrane and the microvilli are no longer embedded. The jelly coat becomes reduced to about 1.5 μm thickness (Crawford and Abed, 1986). Within the oocyte, the cortex becomes less well defined with the large clear vesicles assuming the position vacated by the cortical granules. The cortical granule-like vesicles within the substance of the cytoplasm are not exocytosed (Reimer, 1994; Reimer and Crawford, 1995).

3. Development of the gastrointestinal tract

Following fertilization, the zygote undergoes a number of cleavages to form a hollow embryo, called a blastula, which hatches out of the fertilization membrane at about 45-48 h. The morphogenesis of the gastrointestinal tract in *Pisaster ochraceus* (Crawford and Abed 1983; Abed and Crawford, 1986; summarized in figure 1) begins at about 45 h of development with the commencement of the invagination of ectodermal cells at the vegetal end of the (usually) hatched blastula. As the cells push into the blastocoel, they create a hollow blind-ended tube called the archenteron, or primitive gut, lined with endodermal cells. The hyaline layer, the external ECM surrounding the embryo, is pulled into the archenteron with the cells. As it grows, the tip of the archenteron becomes expanded. At about 72 h post-fertilization, some of the endodermal cells at the tip of the archenteron undergo an epithelial-mesenchymal transformation, lose their adhesion to the hyaline layer, and migrate into the blastocoel as mesenchymal cells. These cells contribute to the
Figure 1: Development of the gut in *Pisaster ochraceus*.
(from Reimer, 1994)

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

(B) At 48 h, gastrulation begins in the hatched blastula, with an invagination of cells at the vegetal region that form the archenteron (a) or primitive gut.

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

(D) The embryo and the archenteron continue to elongate, and a blister of basement membrane (bm) forms at the archenteron tip (Crawford and Abed, 1983); although the bm 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 end from which a second population of mesenchyme cells emerge.

(E) Side view at a slightly later stage. While the bm cannot be seen, its position is marked by the flattened surfaces of cells located within it (arrows), and extends over to the presumptive stomodeal ectoderm (se).

(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 (arrows).

(continued...)
(G) At 5 d post-fertilization, the gut is segmented into a mouth (m), esophageal (e), stomach (s), and intestinal (i) regions. The intestine has started to take on a bent shape and its opening is beginning to move from a posterior position to a more ventral one. 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 d feeding bipinnaria larva has a well-developed segmented digestive tract, including a mouth (m), esophagus (e), stomach (s), and intestine (i). The intestine is now J-shaped, longer, and its aperture is now placed ventrally. Bar = 50 μm.
secretion and reorganization of the ECM (Reimer, 1994) and ultimately form a variety of structures, including neurons and muscle cells. As the archenteron elongates, a blister of basement membrane (BM) is formed at its tip and fuses with the presumptive ectoderm of the stomodeum (Crawford and Abed, 1983). This newly formed BM appears to act as a guide for the migration of endodermal and ectodermal cells of the stomodeum. The mouth is formed by 4.5-5 d post-fertilization and the animal is considered to have entered the bipinnaria larval stage. At the same time as the blister of BM is forming, two pouch-like coeloms begin to form as outpocketings of the lateral archenteron tip. A second group of endodermal cells in this region transform into mesenchymal cells, migrate into the blastocoel, and settle on the esophagus to form the esophageal musculature (Crawford, 1990). After the formation of the mouth, there is some further development of the digestive tract. The archenteron becomes segmented into a short esophagus, a round stomach, and a J-shaped intestine. Between 5 d and 7 d post-fertilization, the anal opening moves from the posterior end to a more ventral position. During the process of mouth formation, the coelomic pouches detach from the gut, assume a more dorsal position, and remain suspended in the blastocoel.

4. Extracellular matrix in echinoderm development

Extracellular matrices of animals consist of a network of molecules including collagens, large glycoproteins, such as fibronectin, laminin, and tenascin, and
proteoglycans, such as heparan sulfate and chondroitin sulfate (reviewed by Hay, 1991). Together, these molecules form a complex three dimensional web of interconnecting fibers. The ECM molecules have binding sites for like and unlike ECM molecules, and they also have sites for the binding of cell surface molecules, such as the integrins and cell surface glycoproteins (reviewed in Adams and Watt, 1993). This allows for the cell to get information from the ECM and for the ECM to influence development (Adams and Watt, 1993; Juliano, 1996).

Integrins bind a variety of ECM molecules, including fibronectin, laminin, collagens, entactin, tenascin, and vitronectin (reviewed in Ruoslahti, 1991; Adams and Watt, 1993) by recognizing short peptide sequences. They are heterodimers composed of an $\alpha$ and a $\beta$ subunit, both of which are cell surface molecules with a trans membrane and a cytoplasmic domain (Ruoslahti, 1991). Integrin-ligand binding initiates signal transduction which allows information originating in the ECM to cause changes within the cell (Juliano, 1996). Since integrins also play a part in the organization of the actin cytoskeleton, they help to mediate changes in cell movement and shape (Yamada, 1997).

From the outset, all embryos consist of a cellular component and an extracellular matrix component. Echinoderm embryos have ECMs both inside the animal in the basement membranes and the blastocoel, and external ECMs, those of the hyaline layer and later the gut ECM. These ECMs have the same basic make up as do those of vertebrates. ECMs in sea urchin are known to contain a collagen
network (Crise-Benson and Benson, 1972; Spiegel and Spiegel, 1979; Wessel and McClay, 1987; Benson et al., 1990; Suzuki et al., 1997), fibronectin and laminin (Katow et al., 1982; Wessel et al., 1984; McCarthy and Burger, 1987; Hawkins et al., 1995), as well as tenasin-like molecules (Anstrom et al., 1990). Proteoglycans and glycosaminoglycans are also present (Karp and Solursh, 1974; Solursh and Katow, 1982; Lane and Solursh, 1991) and a novel large acidic glycan different from proteoglycans and apparently unique to echinoderms has also been described (Papakonstantinou et al., 1994). The ECM of the starfish is not as well characterized as is that of sea urchin, but the blastocoel ECM contains large glycoproteins (Crawford and Crawford, 1992) and proteoglycan-like molecules (Reimer and Crawford, 1997). Like cells in most animals, echinoderm cells are in contact with a basement membrane. Epithelial cells are also in contact with a similar structure which is part of the hyaline layer called the apical lamina (see Hardin, 1996). Analogous structures in the starfish hyaline layer (Campbell and Crawford, 1991) suggest that the apical lamina is also present in starfish.

Like other animals (reviewed in Adams and Watt, 1993), echinoderm ECMs are known to play a role in development. Gastrulation is one of the most studied early events in development because perturbation of this causes defects which are easily seen and usually not very subtle. Evidence exists for the importance of both the blastocoel and the hyaline layer for normal gastrulation. It has been shown that both ectodermal and endodermal cells in sea urchin maintain cellular contact with hyalin throughout embryogenesis (McClay and Fink, 1982; Wessel et al., 1998). In
sea urchin embryos, removal of the hyaline layer in the blastula prevents gastrulation (reviewed in Hardin, 1996). Sea urchin embryos grown in sea water containing antibodies to hyalin, a major component of the hyaline layer, do not gastrulate (Adelson and Humphreys, 1988). Nectin, one of the substance released by the oocyte immediately after fertilization (Matese et al., 1997), is found in the hyaline layer and is required for spiculogenesis (Zito et al., 1998). Preliminary work done in this lab suggests that one anti-hyaline layer antibody in starfish may cause exogastrulation (B. J. Crawford, personal communication).

The other ECMs are also known to play a role in morphogenesis. In sea urchin, antibodies to a large sulfated proteoglycan found in the basement membrane and blastocoel ECM blocked normal cell movements and rearrangements during segmentation of the gut (Ingersoll and Ettensohn, 1994). Antibodies directed against a large proteoglycan-like molecule present in the blastocoel ECM of starfish prevent the archenteron from finding the presumptive stomodeum (Reimer and Crawford, 1997).

5. Statement of the problem

Like any model in research, insights gained in the study in starfish of ECM-cell interactions during gut morphogenesis are not directly relevant to those processes in other species. However, homologies in composition, structure, and function of the
ECMs of all animals mean that the data provided here should provide insights into similar interactions in other animals. Before the mechanisms of cell-ECM interactions can be elucidated, some information must be known about the molecules present in starfish ECM. Relatively few such molecules have been characterized in starfish. A polyclonal antibody, PJC-1, developed against an isolated 250-350 kDa band from a gel run with homogenate from mature oocytes, was known to recognize the jelly coat by immunofluorescence. The present study used immunofluorescence microscopy and immunogold transmission electron microscopy of sperm, oocytes, and some embryo stages to localize the molecules recognized by PJC-1 to the ECM compartments, and to examine their patterns of secretion during the first 7 d of development. Electrophoresis and immunoblotting were used to achieve basic characterization of the antigens which react with PJC-1, and a simple perturbation experiment was performed to see if the molecules played an active role in the normal morphogenesis of the embryo. The results were then compared and analyzed in an attempt to determine the role of the individual molecules recognized by PJC-1 in early starfish morphogenesis.
II. MATERIALS AND METHODS

1. Animals and embryo preparation

Ripe adult starfish were collected in late spring and early summer from the intertidal zone at Stanley Park, Vancouver and at Moses Point near Sydney, BC. They were maintained at 12°C in salt water aquaria in the Department of Anatomy at UBC on a 12 h light-dark cycle in order to prevent spawning. They were fed on live mussels that were collected either at Kitsilano Beach, Vancouver or purchased commercially (Safeway). Sea water (cH$_2$O) for use in both embryo culturing and in maintaining the adult starfish was collected at the Canadian Department of Fisheries and Oceans station in West Vancouver. When used for embryo culturing, cH$_2$O was filtered with a Whatman #1 filter and aerated for more than 30 s prior to use. Embryo cultures were prepared as previously described (Crawford and Abed, 1983) as follows: Excised ovaries were placed in 0.1 mg/ml 1-methyl adenine (1-MA; Sigma, Oakville, ON) for 90 min. This caused maturation (completion of meiosis) and ejection of the oocytes (Stevens, 1970; Kanatani, 1973). Maturation was determined by examining the oocytes for the breakdown of the germinal vesicle (nucleus and nucleolus). This was observed using a Leitz inverted phase contrast microscope. The oocytes were then rinsed in fresh cH$_2$O for about 30 min. Following this, enough eggs to cover one third to one half of the bottom were placed into 1 L plastic tri-pour beakers containing about 400 ml of freshly filtered and aerated cH$_2$O. A sperm suspension was made by diluting about five drops of "dry" sperm (i.e. as secreted
from an excised testis) in about 50 ml of CH$_2$O. Following confirmation of sperm motility by use of an inverted phase contrast microscope, five to seven drops of this suspension were added to each beaker containing the oocytes to fertilize them. The water was then agitated several times using a meat baster to ensure rapid and complete mixing of oocytes and sperm. Fertilization was confirmed after 15 min by examination of the eggs for the presence of a fertilization membrane using the inverted microscope. Additional drops of the sperm suspension were added if the amount of fertilization was adjudged to be low (<90%). Embryos of the correct age/stage were harvested with a pasteur pipet and placed into 50 ml plastic centrifuge tubes (Fisher) on ice to settle. After about 15 min, the settled embryos were transferred to 15 ml glass centrifuge tubes and concentrated by gentle centrifugation (125 g for 2 min on a clinical centrifuge). Those required for homogenization were stored at -70°C until needed, while those for use in histochemical analysis were cryopreserved and freeze-substituted (see below).

2. Cryopreservation and tissue embedding

In order to preserve both maximum immunoreactivity and tissue structure, typical aldehyde-based tissue fixation was not used. Instead, sperm, oocytes and embryos were fixed by freeze substitution as described by Campell et al. (1991) with some modifications as outlined below. For further details, see appendix 1.
(a) *Oocytes and embryos*

Embryos or oocytes were collected on ice in 15 ml glass centrifuge tubes and packed by centrifugation at 125 g for 1 min using a clinical bench top centrifuge. All the embryonic stages, except the oocytes, were placed into a cryoprotective solution consisting of 15% 2,3-butanediol (Sigma) in cH$_2$O for 15 min followed by another brief centrifugation to repack them. Oocytes were not cryoprotected as this solution causes the discharge of the cortical granules and raising of a partial fertilization membrane (B. J. Crawford, personal communication). A 20 μl Pipetman was used to place about 1 μl of packed embryos or oocytes (enough to provide a layer one embryo or oocyte thick) onto 50-mesh copper transmission electron microscopy (TEM) grids (Marivac, Halifax, NB). Excess water was removed using a triangle of #1 filter paper. The grids were then plunged into a small cup containing liquid propane (Home Hardware) which was cooled by a liquid nitrogen bath in a fume cabinet. After about 20 s in the propane, they were cooled further by a 5 s immersion in liquid nitrogen. Following this, the specimens were either stored in plastic cryovials (Fisher) in a liquid nitrogen refrigerator for freeze substitution at a later date or were freeze substituted immediately.

Grids containing oocytes or embryos to be freeze substituted were placed into 10 ml glass vials containing 100% ethanol which had been cooled to below -100°C with liquid nitrogen. Tissue to be examined by immunogold TEM was placed into vials containing Alcian Blue 8GX (Marivac)-saturated 100% ethanol in order to further preserve the structure of the extracellular matrix (ECM; Crawford and Abed, 1986).
The glass vials were then placed into a freezer set to -85°C for five to seven days to allow the ethanol to replace the water in the embryos. After this time, the ethanol or Alcian Blue-saturated ethanol was changed with fresh 100% ethanol and the vials were slowly warmed to room temperature by transferring them first to -20°C in a freezer, then to 4°C in a refrigerator, and finally to the bench top. Two different resins were used according to manufacturer's instructions to embed the oocytes and embryos at this time. Those to be used for immunofluorescence studies were embedded in JB-4 (Polysciences, Warrington, PA). The polymerization was catalyzed using a chemical hardener. Embryos and oocytes to be studied by TEM were embedded in LR White medium hard resin (Marivac), and heat cured overnight at 50°C in an incubator. In order to exclude air from the resins while they were polymerizing, resin (containing specimens) was placed into the space between two aluminum weigh dishes (Fisher).

(b) Sperm

Testicular secretions were collected dry using a pasteur pipet and were not subjected to either centrifugation or cryoprotection. The dry sperm was dabbed onto the TEM grids in a thin layer using a cleaned wooden toothpick and were then flash frozen and freeze substituted in 100% ethanol in the same manner as were the oocytes and embryos. Because the many resin change steps required in JB-4 embedding led to an unacceptably high amount of tissue loss, sperm were embedded in LR White for both immunofluorescence and immunogold studies. This was performed according to the manufacturer's instructions, with one modification. In
order to minimize sperm loss during embedding, the LR White resin was mixed with and allowed to infiltrate the sperm in 1.5 ml Eppendorf microcentrifuge tubes. This permitted the use of centrifugation (5 min at 2,000 g) to pack the sperm and minimize their loss during processing.

3. Antibodies used

The polyclonal antibody used in these studies was prepared by Dr. Bruce J. Crawford and Mr. Lincoln Crichlow in this lab in 1994 (see appendix 2). It was produced in rabbit from a single band electroeluted from a gel of oocyte homogenate. The antibody was developed in an attempt to examine starfish myosin, as the protocol used was designed to isolate myosin. The electroeluted band was of a molecular weight greater than known myosins (~250-350 kDa). When the antibody was originally used to probe sections of oocytes for immunofluorescence, it was discovered that it stained the jelly coat and not the areas where myosin was expected to be found. Because of this staining pattern, it was named *Pisaster* Jelly Coat-1 (PJC-1). Two rabbits were used to produce PJC-1. However, only serum collected from the sixth (and final) bleed of the first rabbit (PJC-1 1-6) was used in these experiments. Henceforth, PJC-1 1-6 will be referred to as PJC-1.
4. Immunohistochemistry

(a) Immunofluorescence

Thin (2 μm) sections of JB-4-embedded freeze-substituted embryos (28 h mid blastula, 41 h unhatched late blastula/early gastrula, 72 h mid gastrula, and 7 d early bipinnaria) and mature oocytes, and LR White-embedded sperm were cut using a dry glass knife on a Porter-Blum MT-1 ultra-microtome. One section was placed on a glass slide, covered by a drop of distilled water (dH₂O), and was gently warmed on a hot plate to evaporate the water and to ensure good adherence of the section to the slide. Each section was then pre-incubated in a moist chamber for 2 x 30 min in 2% PBS/blotto (phosphate buffered saline [PBS], pH 7.4, containing 2% Lucerne skim milk powder [blotto; Safeway] and 0.1% NaN₃ [Sigma]; see appendix 4) and 10% normal goat serum (NGS; Sigma) so as to ensure that sites susceptible to non-specific binding were blocked. This greatly decreased the amount of background fluorescence. The sections were then covered with about 40 μl of primary antibody (PJC-1) or normal rabbit serum (NRS; Sigma) diluted 1:1000 in 2% PBS/blotto and incubated for 90 min at 37°C. This concentration was chosen as it provides the best signal to background staining ratio as determined by performing a series of titrations from 1:10 to 1:10,000. Following 2 x 15 min rinses in 2% PBS/blotto, the sections were incubated for 60 min in a 1:256 dilution of FITC-conjugated goat anti-rabbit IgG (Sigma) at 37°C. They were then rinsed 4 x 5 min with PBS, pH 7.4 with 0.1% NaN₃, and were mounted with a #1 22 x 22 mm coverslip (Fisher) with a drop of Gelvatol/DABCO, a water soluble mounting medium containing 30% glycerol.
(Fisher), 16.5% gelvatol (Marivac), 0.8% 1,4-diazabicyclo[2.2.2]octane (DABCO; Sigma) in PBS, pH 7.2 (Taylor and Heimer, 1974; Johnson et al., 1982). The addition of DABCO decreases the rate of photobleaching and promotes some recovery from fluorochrome bleaching. Sections were examined and photographed on a Zeiss Photomicroscope II with a UV light source and appropriate filters. Kodak TMAX 3200 film was used for photography, with the camera set to provide an automatic exposure and the ASA set to 6500 to decrease exposure times and increase contrast (see appendix 4 for film processing details). Because of the thickness of the sections, it has been determined using scanning laser confocal microscopy that most of the immunofluorescence staining in sectioned tissue of Pisaster ochraceus is at or near the surface of the section, not through the entire thickness of the section (B. J. Crawford, personal communication).

(b) Immunogold transmission electron microscopy

The full protocol can be found in appendix 5. Ultrathin sections (50-60) nm as determined by silver-gold colour of LR White-embedded tissue (sperm, mature oocytes and 7 d bipinnariae) were cut using a Diatome diamond knife on a Porter-Blum MT-1 ultra-microtome and were collected on copper 100 mesh TEM grids (Marivac) coated in Formvar (TAAB Laboratories, Reading, England; Hayat, 1989) to provide extra support (see appendix 6 for coating details). All PBS/blotto used in the following protocol was filtered prior to use with a Millipore Millex 0.45 μm filter attached to a Becton-Dickinson 5 ml syringe (Fisher). Optimum antibody dilutions were determined by performing a series of titrations similar to that described in
section 4a above. Each grid was floated section side down on a drop of blocking solution consisting of 10% NGS in 0.2% PBS/blotto (PBS, pH 7.4, with 0.2% milk powder) for 2 x 15 min, rinsed in PBS/blotto for 2 x 5 min each, and was then floated for 90 min on a drop of 1:500 dilution of PJC-1 in 0.2% PBS/blotto. Following primary antibody incubation, the grids were rinsed four times (for 5, 5, 10, and then 15 min) in PBS/blotto, and were then incubated in secondary antibody solution (gold-conjugated goat anti-rabbit IgG [unconjugated antibody from Sigma]; see appendices 7-9) for 75 min. The grids were rinsed as above in 0.2% PBS/blotto followed by a rinse in a stream of dH₂O. They were then stained for 10 min in saturated aqueous uranyl acetate (Marivac) solution (Hayat, 1989), and then for 10 min in lead citrate (Reynolds, 1963). The grids were then viewed using a Phillips 300/301 TEM at 60 kV and electron micrographs were taken with a 2 s exposure on Kodak 5302 35 mm film (see appendix 4 for film processing details). Control grids were stained as above, with normal rabbit serum replacing PJC-1. Gold particle density comparisons were performed by placing a grid of known dimensions (1 μm²) over different regions of an electron micrograph and counting the number of gold particles within it. Means and standard deviations were calculated for similar regions. Comparisons between different regions were made using student’s t-test for unpaired samples, except for those cases where different sample sizes were used, where the Welch t' test was employed instead (Glass and Hopkins, 1996). In all cases, the 0.05 confidence level was used to indicate significance. Those t values lying between 0.1 and 0.05 were taken to indicate trend toward a difference.
5. Tissue homogenization

Oocytes, sperm or embryos at various stages (45 h hatched early gastrula, 72 h mid gastrula, 96 h late gastrula, and 7 d early bipinnaria) were collected and placed in a 10 ml Dounce tissue homogenizer on ice. An equal volume of homogenization buffer (20 mM Tris, 0.5 M NaCl, pH 7.4) containing a cocktail of protease inhibitors (1 mg/ml pepstatin A [Sigma], 1μM ethylenediaminetetraacetic acid [EDTA; BDH, Toronto ON], 1 mM iodoacetamide [Sigma], and 1 mM phenylmethylsulfonyl fluoride [PMSF; Sigma]; see table 1 for inhibitor targets and effective concentrations) and 1% Brij-56 (polyoxyethylene 10 cetyl ether; Sigma), a non-ionic detergent which does not contain any phenol rings and therefore does not interfere with UV spectrophotometric measurement of protein concentration, if used. The tissue/buffer mixture was then homogenized with 10 plunges of the Dounce homogenizer, sonicated with a Fisher probe sonic dismembrator set to 45% for 30 s, and allowed to extract on ice for 30 min. The homogenate was then centrifuged in a Beckman Optima TLX Ultracentrifuge with a Beckman TLA-100 rotor at 50,000 g for 60 min at 4°C and the supernatant was stored until needed at -70°C. Protein concentrations were determined using the Biorad DC Microplate Protein Assay using 2%, 5%, and 10% dilutions of the supernatant (see appendix 10). This assay is based on the Lowry colorimetric assay (Lowry et al., 1951).
Table 1: Protease inhibitors

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Protease Target</th>
<th>Effective Concentration</th>
<th>Stock</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>EDTA</td>
<td>Metalloproteinases (divalent cation-dependent proteases)</td>
<td>1-5 mM</td>
<td>0.5 M in water, pH 8.0</td>
<td>Add fresh at each step</td>
</tr>
<tr>
<td>PMSF</td>
<td>Serine proteinases (some thiol- and carboxyproteinases)</td>
<td>1 mM</td>
<td>5 mg/ml in acetone</td>
<td>Add fresh at each step (hydrolyzes rapidly)</td>
</tr>
<tr>
<td>Iodacetamide</td>
<td>Triosephosphate dehydrogenase (covalent thiol proteases)</td>
<td>10 mM</td>
<td>10 mg/ml in PBS</td>
<td>Add fresh at each step</td>
</tr>
</tbody>
</table>
6. SDS PAGE and immunoblotting

(a) Gels and blotting membranes

Polyacrylamide gel electrophoresis (PAGE; see appendix 10) was performed using gradient gels (4-20%) with a 3% stacking gel to allow for maximum resolution of high molecular weight bands without electrophoresing low molecular weight proteins off the gel. For immunoblotting, polyvinylidene difluoride (PVDF) membranes were used rather than nitrocellulose, as they allowed for the longer transfer times necessary for high molecular weight molecules while retaining the smaller molecules.

(b) SDS-PAGE

Protein electrophoresis was performed after the method of Laemmli (1970). Sperm, oocyte, 45 h, 72 h, 96 h, and 7d embryo homogenate samples were placed into an equal volume of either reducing or non-reducing sodium dodecyl sulfate (SDS; Biorad) based 2x sample buffer (see appendix 11). Equivalent amounts of protein were loaded into wells of an SDS-PAGE 4-20% gradient gel, along with 5 μl of biotinylated molecular weight standard (Sigma) and 5 μl of prestained multicoloured molecular weight standard (Kaleidoscope Protein Standard; Biorad). The gels were run using the Biorad Mini-Protean II system at 95 V until the dye front reached the running gel (~5 min), and then at 195 V until either the dye front was just about at the bottom of the gel (~36 min) or until the orange (29.0 kDa) band was at the bottom of the gel (~45 min). Gels were removed, marked by cutting off one
corner, and rinsed for 30 s in dH$_2$O. Those not used for immunoblotting were stained for 15 min with shaking in Coomassie Blue solution (0.25% Coomassie Brilliant Blue R 250 (Sigma), 50% methanol [Fisher], and 10% acetic acid [Fisher]; Harlow and Lane, 1988). They were then destained by shaking overnight in a solution containing 10% methanol and 10% acetic acid. A large piece of paper towel was floated on top of the destaining solution and the gel in the destaining dish. This facilitated gel destaining by binding stain released from the gel (Harlow and Lane, 1988). Gels were rinsed in several changes of dH$_2$O, placed on a moistened piece of heavy filter paper (Biorad) and were dried using a Savant Slab Gel Drier SGD 2000 at 50°C for 30 min.

(c) Immunoblots

After proteins had been subjected to PAGE, the proteins were transferred to 0.45 μm PVDF membranes for detection with either the ECL enhanced chemiluminescence system (Amersham) or with Coomassie Blue solution. Gels were first rinsed twice with dH$_2$O, and then were placed into prechilled transfer buffer (10 mM CAPS [3-[cyclohexylamino]-1-propanesulfonic acid; Sigma], 5% methanol, pH 11.0) to equilibrate for at least 20 min. The gel was then placed into the transfer cassette (see appendix 11 for details) together with a piece of PVDF membrane. The cassette was then placed into the transfer apparatus along with the frozen cooling block and the reservoir was filled with transfer buffer pre-chilled to 4°C. The sealed unit was placed into an ice-water bath to help maintain a low temperature during transfer. The transfer was performed at 100 V for 60 min. The use of the coloured
molecular weight standard helped to confirm that protein transfer had in fact taken place. The membranes were then briefly rinsed twice in dH₂O and 10 min in TBST (Tris-buffered saline with Tween; 20 mM Tris [tris(hydroxymethyl)aminomethane; Sigma], 150 mM NaCl [Sigma], 0.1% Tween-20 [BDH], 0.05% NaN₃ [Fisher], pH 7.4) before being placed into the blocking solution (4% fetal bovine serum [Gibco], 3% bovine serum albumin [BSA; Sigma], 2% NGS in TBST) overnight. Following blocking, the blots were rinsed with TBST twice briefly, and then for 15 min and 2 x 5 min prior to being placed into 25 ml of the primary antibody solution (1:10,000 PJC-1 in TBST, as determined by testing various concentrations for maximal signal with minimal background) for 90 min. The membranes were then rinsed as above and placed into the secondary antibody solution (1:7,500 biotinylated goat anti-rabbit IgG [Sigma] in TBST) for 60 min. The membranes were again rinsed as above and were placed into a tertiary antibody solution (1:15,000 streptavidin-horseradish peroxidase [HRP] conjugate [Sigma] in TBST) for 60 min. They were rinsed again as above except with TBST containing no sodium azide (NaN₃) as this interferes with the action of HRP. All of the rinses and incubations were performed at room temperature on an orbital shaker. Detection was performed with the Amersham ECL kit as per manufacturer’s instructions (see appendix 13), and the membranes were exposed onto Kodak X-OMAT-AR film (Medtec, Vancouver, BC) for an appropriate amount of time (see appendix 4 for processing details). Membranes were stripped of the antibodies in stripping buffer (100 mM 2-mercaptoethanol [Sigma], 2% SDS, 62.5 mM Tris, pH 6.7) for 30 min at 50°C with occasional agitation. They were then blocked and reprobed as above with control primary solution (1:10,000 NRS in
TBST) replacing the PJC-1 antibody solution. The blots were then detected with ECL.

7. Calculation of apparent band weights

Using a processed film of an immunoblot, the apparent molecular weight of visible bands were calculated as follows. The top of the gel was marked on a photocopy of the immunoblot. The distance from the top to the middle of each of the bands of the molecular weight markers was measured and divided by the distance traveled by the dye front. This number was plotted against \( \log_{10}(\text{molecular weight}) \) using the application Cricket Graph on an Apple Power Macintosh 7300/200 computer. An equation for the line of best fit was determined with the curve fit feature. The distance from the top of the gel to the middle of each band of interest could then be used with this equation to determine its relative mobility (\( M_r \)), which is roughly equivalent to the molecular weight (Beeley, 1985).

8. Perturbation studies

All perturbation studies (see below) were performed in plastic 24-well tissue culture plates (Fisher). The wells were soaked with dH\(_2\)O for 1 h to ensure they were free of any contaminants and then dried. Rinsed oocytes (see section 1 above) were
placed into a 50 ml plastic specimen cup containing approximately 40 ml of cH₂O, and were suspended by gently swirling the cup. The volume of this suspension required to deliver about 100 oocytes was determined and was placed into the wells of the 24-well plate. Experiments were performed with each well containing 1 ml total volume of cH₂O with oocytes and antibody.

(a) Sperm

Sperm suspension as described in section 1 was incubated with varying concentrations (0.5%, 1%, 2.5%, 5%, and 10% in cH₂O) of PJC-1 or NRS in 1.5 ml Eppendorf microcentrifuge tubes for 30 min, 1 h, or 2 h at 12°C. Approximately 4 µl of this sperm was then used to fertilize approximately 100 oocytes placed into wells of a 24-well plate prepared as per above. For control-treated sperm, NRS replaced the PJC-1.

(b) Oocytes

Approximately 100 oocytes were incubated in each well of a 24-well tissue culture plate with various concentrations of PJC-1 or NRS (0%, 1%, 2.5%, 5%, 10%, and 20% in cH₂O) for 1 h at 12°C. About 2 µl of sperm suspension prepared as above was added to each well to fertilize the oocytes. The resulting embryos were observed over the course of 7 d, after which they were fixed in 1% glutaraldehyde (Marivac) in cH₂O for 1 h, rinsed in cH₂O, and stored in cH₂O containing 0.1% NaN₃ as a preservative. The percentage of embryos showing developmental defects was estimated following observation with a dissecting microscope. Individual embryos
were placed on a glass slide and covered with a #1 coverslip with small amounts of plasticine on each corner to prevent compression of the embryo. Photographs of these glutaraldehyde-fixed whole embryos were taken using a Zeiss microscope set up for DIC using Kodak TMAX 100 film (see appendix 4 for film processing details).
III. RESULTS

1. Immunolocalization of the PJC-1 antigens

(a) Immunofluorescence light microscopy

(i) Sperm

Following staining using PJC-1 and a FITC-conjugated secondary antibody, sperm exhibit fluorescence staining on the anterior region of the head, along the tails, and at the cell surface (figure 2A). From the immunofluorescence data, it is unclear whether this surface staining is on the membrane or on the extracellular jelly which coats the sperm (Bickell et al., 1980). Only a few examples of each of these locations of fluorescence are visible in any one section because immunofluorescence staining of *Pisaster ochraceus* tissue occurs only at or near the surface of the section, not through its entire thickness. This has been determined using scanning laser confocal microscopy (B. J. Crawford, personal communication). Normal rabbit serum (NRS) control-treated sperm do not show any staining (not shown).

(ii) Mature oocyte

PJC-1 labels the jelly coat and vitelline membrane, as well as the cortical granules and some other granules within the oocyte (figure 3A). The jelly coat appears to stain quite strongly through its entire thickness and is granular in appearance. The vitelline membrane also stains, but does so more uniformly than does the jelly coat. The cortical granules stain homogeneously, as do granules of a
Figure 2: Immunofluorescence localization of PJC-1 antigens in sperm.

Sperm which have been fixed in 100% ethanol by freeze substitution, were embedded in LR White, and sectioned to 2-2.5 μm. Sections were stained with PJC-1 and FITC-conjugated secondary antibody. Bar = 2 μm.

(A) Sperm in this section are stained either in a large granule which is ostensibly the acrosome (arrow heads), on the surface of the head (thin arrows), or on the tails (thick arrows).

(B) A phase contrast image of the section in (A).
Figure 3: Immunofluorescence localization of PJC-1 antigens in the mature oocyte.

This shows an oocyte which has been fixed in 100% ethanol by freeze substitution, embedded in JB-4, and sectioned to 2-2.5 μm. Sections were stained with PJC-1 and a FITC-conjugated secondary antibody.

(A) The oocyte has staining localized to most of the granules in the cortical region (cg), as well as in the jelly coat (jc) and vitelline membrane (vi). Although the matrix has a very low level of staining, some granules dispersed throughout it are brightly stained (ig). There are several large granules completely devoid of staining near the surface (dg). Bar = 10 μm.

(B) A phase contrast view of (A). Bar = 10 μm.
similar size toward the middle of the oocyte. The large, dark (on immunofluorescence micrographs) vesicles near the surface do not stain at all. Oocytes stained with NRS (not shown) exhibit no fluorescence.

(iii) Mid blastula

Fluorescence is limited to the cells, fertilization membrane, and jelly coat. The jelly coat and fertilization membrane have a granular appearance (figure 4A) like that in the mature oocyte. The cells contain large fluorescent granules, and some cells are partially outlined in fluorescence (figure 4C). It is not possible to discern whether this staining is at the cell membrane or the region just outside the cell. The blastocoel shows a very low level of staining. The nuclei, and the region where the hyaline layer is forming remain unstained. NRS controls (not shown) exhibit no fluorescence.

(iv) Unhatched late blastula/early gastrula

Fluorescence is found in large granules within many of the cells (figure 5A, C). The nuclei are not stained. As in the mid blastula (28 h) embryos, some cells appear to have an outline of staining. The hyaline layer just adjacent to the cells is stained, as are the fertilization membrane and jelly coat in the unhatched embryo. These latter structures appear to exhibit a similar degree of staining as do their counterparts in the oocyte. Fluorescence is at a very low level in the blastocoel. Control unhatched late blastulae/early gastrulae are not stained (not shown).
Figure 4: Immunofluorescence localization of PJC-1 antigens in the mid blastula.

For figures 4-8, embryos fixed in 100% ethanol by freeze substitution were embedded in JB-4 and sectioned to 2-2.5 μm. Sections were stained with PJC-1 and a FITC-conjugated secondary antibody.

(A) This section of a 28 h mid blastula stage embryo stained with PJC-1 shows staining which is localized to granules within cells, and to the jelly coat (jc) and its underlying fertilization membrane (fm). Some cells also appear to be surrounded with staining. The blastocoel (b) contains little staining, while the region in which the hyaline layer (hl) is forming is unstained. Bar = 10 μm.

(B) A phase contrast micrograph of (A). Bar = 10 μm.

(C) A high magnification view of a different section from the same embryo in (A). Here, the intense staining of the jelly coat, fertilization membrane, and granules along with the pericellular staining (arrow heads) is easily seen. The nuclei (n) and forming hyaline layer do not stain. Bar = 5 μm.

(D) A phase contrast micrograph of (B). Bar = 5 μm.
Figure 5: Immunofluorescence localization of PJC-1 antigens in the late blastula/early gastrula.

Embryos were processed as described in the caption to figure 4.

(A) In this low magnification view of most of an unhatched 41 h embryo which has just started to gastrulate, most of the fluorescence is seen to be in the fertilization membrane (fm), jelly coat (jc), and hyaline layer (hl). However granules of fluorescence are visible within cells. There is no fluorescence present in the few small regions of blastocoel (b) visible here. Bar = 20 μm.

(B) A phase contrast view of (A). The presence of the inner endodermal cells surrounding a lumen in addition to the outer ectodermal cells indicates that gastrulation has commenced. Bar = 20 μm.

(C) At higher magnification, the detail of the staining in the jelly coat, fertilization membrane, and the forming hyaline layer is evident. The granules within cells are more readily visible, and some cells can be seen to have a thin amount of fluorescence surrounding part of them (arrow heads). Bar = 5 μm.

(D) A phase contrast image of (C). Bar = 5 μm.
(v) Mid gastrula

In the mid gastrula stage embryos, the fluorescence is found in large granules within cells of both the ectoderm and endoderm (figure 6A). The nuclei remain unstained. Staining is also found in the extracellular matrix of the blastocoel, although this region is collapsed in many embryos. Some staining is seen in the hyaline layer and gut ECM in some embryos, although this is not very evident in this embryo. NRS-treated mid gastrula stage (72 h) embryos show no staining (figure 6C).

(vi) Early bipinnaria

The ECM lining the lumen of the mouth, esophagus, stomach, coeloms, and intestines is richly stained, particularly that of the stomach and intestines (figure 7A). This intense staining leads to an underexposure of the hyaline layer and blastocoel ECM which are stained more prominently than at earlier stages but still much less prominently than is the g.i. tract lumen. Both have a granular appearance, but that of the hyaline layer is denser than the ECM of the blastocoel. The large fluorescent granules seen in the earlier stages are found here in many ectodermal, endodermal (figures 7A, 8A), and mesodermal cells (figure 8A inset). The nuclei of all cells remain unstained. Bipinnariae stained with NRS did not exhibit any fluorescence (figure 7C).
Figure 6: Immunofluorescence localization of PJC-1 antigens in the mid gastrula.

Embryos were processed as described in the caption to figure 4.

(A) A micrograph of the posterior half of a 72 h mid gastrula stage embryo stained with PJC-1. Fluorescence is found in granules in the ectoderm (thick arrow heads) and endoderm (thin arrow heads). Some cells appear to have staining either on the surface or in the adjacent extracellular matrix (thin arrows). The blastocoel, although collapsed in this embryo, does contain some staining (thick arrows), but the lumen of the archenteron (al) does not. Bar = 10 μm.

(B) A phase contrast micrograph of the section in (A) showing the ectoderm (ec), endoderm (en), and archenteron lumen. Bar = 10 μm.

(C) A control (normal rabbit serum-stained) embryo showing no fluorescence. Bar = 10 μm.
Figure 7: Immunofluorescence localization of PJC-1 antigens in the early bipinnaria -- low magnification.

Embryos were processed as described in the caption to figure 4.

(A) An immunofluorescence micrograph of the posterior 2/3 of an early bipinnaria at 7 d post-fertilization showing that the most intense staining is present in the lumen of the gastrointestinal tract: o = oral; c = coelom; s = stomach; and i = intestine. There is also staining present in the ectodermal (ec), mesenchymal (mc), and endodermal cells which line the gastrointestinal tract. The hyaline layer and blastocoel extracellular matrix though stained weakly are not visible in this micrograph. Bar = 20 μm.

(B) A phase-contrast micrograph of the section in (A). Bar = 20 μm.

(C) A higher magnification view of a section stained using normal rabbit serum. There is no fluorescence present. Bar = 10 μm.
Figure 8: Immunofluorescence localization of PJC-1 antigens in the early bipinnaria -- high magnification.

Embryos were processed as described in the caption to figure 4.

(A) Immunofluorescence of the same coelom visible in the top of all three parts of figure 7. Here it is evident that there is staining within the coelom itself, adjacent to the endodermal cells. Granules of fluorescence are visible within these cells, as well as in the ectodermal cells (ec). The hyaline layer (hl) stains moderately, and although the blastocoel is stained, very little is visible in this view (arrows). Both are somewhat granular in appearance. Bar = 5 μm.

(inset) A mesodermal cell (mc) located near the ectoderm in the blastocoel of the anterior part of a bipinnaria is seen to contain two fluorescent granules. Granules are also visible in the ectodermal cells. The hyaline layer (hl) and diffuse staining in the blastocoel (arrows) are also evident. Bar = 10 μm.

(B) Phase contrast micrograph of the section in (A). Bar = 5 μm.
(b) Immunogold transmission electron microscopy

(i) Sperm

When stained with PJC-1 and a gold-conjugated secondary antibody, gold labeling is found in many structures in the sperm. The acrosomal granule has a large amount of gold label on the membrane and in other parts of the cortical region, as well as in the acrosomal medulla (figures 9A, B). The extracellular matrix adjacent to the flagellum, and perhaps the cell membrane of the flagellum, is also labeled. The periacrosomal material, nucleus, cell membrane, mitochondrial region and flagellar contents are unlabelled. Control sperm stained with normal rabbit serum exhibited a very low level of labeling only in the cortical region of the acrosomal granules (figure 9C). When compared to those stained with PJC-1 this level was statistically insignificant in both the heads (p<0.01) and the tails (p<0.01; table 2).

(ii) Mature oocyte

The gold labels the jelly coat matrix (figures 10, 11A) fairly uniformly throughout, although the outermost part appears slightly less stained. The vitelline membrane is labeled with perhaps more gold particles on the outer surface. The cell membrane, including that of the microvilli, also has a row of gold particles along most of its extent perhaps more so on its inner aspect. The cortical granules are richly labeled especially in the densest regions (figure 11A) with several gold particles often clumped together. Several other vesicles, including the yolk granules, are labeled (figures 10, 11A). The large electron lucent vesicles (figure 10) are likely the same as the dark granules in the immunofluorescence photograph (figure 3A). The
Figure 9: Immunogold TEM localization of the PJC-1 antigens to the acrosomal granule and flagellum of sperm.

Sperm fixed by freeze substitution in absolute ethanol without the addition of any cryoprotectant solution were embedded in LR-White resin and section to 50-60 nm. Following probing with PJC-1 and gold conjugated secondary antibody, sections were stained with uranyl acetate and lead citrate. Osmium tetroxide was not used, so membranes are not easily visualized.

(A) This figure shows several sperm stained with PJC-1. The gold label is most concentrated in the acrosomal granule (a) on the outer surface and within the electron dense cortical area and in the electron lucent central region. The periacrosomal material (p), nucleus (n), mitochondrial region (m), and flagellar contents are not stained. There is staining along the tail membrane or perhaps adjacent extracellular material (arrows). Bar = 0.5 μm.

(B) Detail of one sperm highlighting the gold labeling the outer part of the electron dense cortical region of the acrosomal granule (ac), its electron lucent medulla (am) and the staining along the flagellar membrane on extracellular material (arrows). Other labels are as in (A) above. Bar = 0.25 μm.

(C) NRS-stained sperm. The small amount of gold present in the acrosomal cortex in some sperm is not statistically significant. Labels are as above in (A). Bar = 1 μm.
Table 2: Gold particle counts and t-test results for sperm and oocytes

Number of gold particles per \( \mu m^2 \) in each of the following locations is listed below.

<table>
<thead>
<tr>
<th>Location</th>
<th>Treatment</th>
<th>Mean</th>
<th>StDev</th>
<th>n</th>
<th>t value</th>
<th>result</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sperm - heads (total)</td>
<td>PJC-1</td>
<td>25.31</td>
<td>12.09</td>
<td>16</td>
<td>3.03</td>
<td>p&lt;0.01</td>
</tr>
<tr>
<td></td>
<td>NRS</td>
<td>1.88</td>
<td>1.67</td>
<td>16</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sperm - tails</td>
<td>PJC-1</td>
<td>10.64</td>
<td>3.20</td>
<td>14</td>
<td>3.39</td>
<td>p&lt;0.01</td>
</tr>
<tr>
<td></td>
<td>NRS</td>
<td>0.21</td>
<td>0.43</td>
<td>14</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oocyte - jelly coat</td>
<td>PJC-1</td>
<td>34.27</td>
<td>12.67</td>
<td>15</td>
<td>3.47</td>
<td>p&lt;0.002</td>
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<tr>
<td></td>
<td>NRS</td>
<td>0.47</td>
<td>0.92</td>
<td>15</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oocyte - cell membrane</td>
<td>PJC-1</td>
<td>4.77</td>
<td>2.83</td>
<td>13</td>
<td>3.01</td>
<td>p&lt;0.01</td>
</tr>
<tr>
<td></td>
<td>NRS</td>
<td>0.00</td>
<td>0.00</td>
<td>13</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oocyte - cortical granules</td>
<td>PJC-1</td>
<td>23.00</td>
<td>4.62</td>
<td>7</td>
<td>2.27</td>
<td>p&lt;0.05</td>
</tr>
<tr>
<td></td>
<td>NRS</td>
<td>1.14</td>
<td>3.02</td>
<td>7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oocyte - medium density vesicles</td>
<td>PJC-1</td>
<td>8.67</td>
<td>5.20</td>
<td>15</td>
<td>2.75</td>
<td>p&lt;0.02</td>
</tr>
</tbody>
</table>
Figure 10: Immunogold TEM localization of the PJC-1 antigens in the jelly coat, cortical granules, and other vesicles of mature oocytes.

Mature oocytes were fixed by freeze substitution in Alcian Blue-saturated ethanol without the addition of any cryoprotectant solution, embedded in LR-White resin, and sectioned to 50-60 nm. Following probing with PJC-1 antibody and gold conjugated secondary antibody, sections were stained with uranyl acetate and lead citrate. Osmium tetroxide was not used, so membranes are not easily visualized.

This figure shows the jelly coat, cortical region and subcortical regions of a mature unfertilized oocyte. There is a high number of gold particles throughout the jelly coat (J), on the outer aspect of the vitelline membrane (vi), along the cell membrane (arrows), microvilli (arrowheads), cortical granules (cg), putative yolk granules (y), and other vesicles (v). The particles visible here in the cytoplasm are associated with structures which are not apparent at this magnification. Bar = 1 μm.
Figure 11: Detail of PJC-1 localization to the cortical granules, membrane, and jelly coat of mature oocytes.

Oocytes treated as described in the caption to figure 9.

(A) Detail of the middle right region of the oocyte shown in figure 9. The gold particles are associated with the structure of the jelly coat (J), vitelline membrane (vi), the densest parts of the cortical granules (cg) and their membranes (thin arrows), and the cell membrane (thick arrows). Other particles found in the cytoplasm of the oocyte appear to be associated with small organelles or other structures (arrowheads). Bar = 0.25 μm.

(B) Control (normal rabbit serum) probed mature oocyte showing no staining of any of the structures visible in (A) above, notwithstanding the presence of an occasional gold particle (arrows). Bar = 0.25 μm.
oocyte cytoplasm also has some sporadic labeling, however much of this appears to be associated with small granules or other structures (figure 11A). The lack of good visualization of the membranes makes it difficult to see membrane-bound organelles. NRS-stained control oocytes (figure 11B) remain essentially unlabeled. The difference in the amount of labeling was statistically significant (p<0.02) in all cases (table 2).

(iii) Early bipinnaria

The hyaline layer is labeled uniformly throughout with perhaps some sporadic areas of greater labeling (figure 12A). Some large vesicles (~1 μm diameter) of low to intermediate density located both apically and basally (figure 12A) in ectodermal cells are labeled. Mesenchymal cells also exhibited labeling in similar vesicles (figure 12B). The blastocoel ECM, consisting of fibers arranged parallel to the cell bases, is labeled fairly uniformly throughout (figure 12C), although the ECM immediately adjacent to the endodermal cells appears to have more labeling. This apparent difference is only significant at p<0.1 and so is indicative merely of a trend (table 3). The basal lamina of the endodermal cells has some gold particles associated with it (figure 12C). Endodermal cells appear to exhibit more gold labeling than do the ectodermal cells. The majority of label is found in small to intermediate sized moderately electron-dense vesicles (~0.3 μm diameter) apically, and basally in large relatively electron-lucent vesicles (~1 μm diameter) similar in size and appearance to those in the ectodermal and mesodermal cells. There is significantly more staining in the apical part of these cells than in the basal portion
Figure 12: PJC-1 antigen localization to vesicles in ectodermal and mesodermal cells, and to the hyaline layer and blastocoel ECM of bipinnaria by TEM.

For this figure and the one following (figure 12), bipinnaria stage embryos were fixed by freeze substitution in Alcian Blue-saturated ethanol with the addition of a cryoprotectant solution, embedded in LR-White resin, and sectioned to 50-60 nm. Following probing with PJC-1 antibody and gold conjugated secondary antibody, sections were stained with uranyl acetate and lead citrate. Osmium tetroxide was not used, so membranes are not easily visualized.

(A) This figure shows that the hyaline layer (hl) is labeled throughout, and that the endodermal cells have gold label in the large (~1 μm diameter) vesicles (v). As in the cells of the endoderm, the nucleus and endoplasmic reticulum (er) remain unstained. The blastocoel ECM at right (b) is labeled. Bar = 0.5 μm.

(B) This mesodermal cell, which is adjacent to an endodermal cell (e) and surrounded by the blastocoel (b), exhibits PJC-1/gold staining in large vesicles (v) similar to those in the ectodermal cells in figure 12A. Bar = 0.5 μm.

(C) In the blastocoel, gold labels the extracellular matrix and the basal lamina (bl) of the endodermal cell in the lower right corner. The apparent difference in staining near the endoderm (lower right) as compared to the rest of the blastocoel is not statistically significant. Bar = 1 μm.
Table 3: Gold particle counts and t-test results for PJC-1 7 day early bipinnaria embryos

Number of gold particles per μm² in each of the following locations is listed below.

<table>
<thead>
<tr>
<th>Location</th>
<th>Mean</th>
<th>StDev</th>
<th>n</th>
<th>t value</th>
<th>Deg. of freedom</th>
<th>result</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gut ECM - adjacent to cells</td>
<td>30.70</td>
<td>7.53</td>
<td>10</td>
<td>2.12*</td>
<td>13</td>
<td>0.10&gt;p&gt;0.05</td>
</tr>
<tr>
<td>Gut ECM - middle of lumen</td>
<td>12.33</td>
<td>4.32</td>
<td>6</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Blastocoel ECM - endoderm side</td>
<td>40.20</td>
<td>11.19</td>
<td>5</td>
<td>2.15*</td>
<td>4</td>
<td>0.10&gt;p&gt;0.05</td>
</tr>
<tr>
<td>Blastocoel ECM - elsewhere</td>
<td>15.33</td>
<td>2.85</td>
<td>18</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Endoderm - apical vesicles</td>
<td>48.26</td>
<td>12.02</td>
<td>19</td>
<td>2.70*</td>
<td>27</td>
<td>p&lt;0.02</td>
</tr>
<tr>
<td>Endoderm - basal vesicles</td>
<td>20.67</td>
<td>9.47</td>
<td>15</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ectoderm - basal vesicles</td>
<td>11.86</td>
<td>4.52</td>
<td>7</td>
<td>1.28</td>
<td>12</td>
<td>p&gt;0.10</td>
</tr>
<tr>
<td>Endoderm - basal vesicles</td>
<td>30.14</td>
<td>13.35</td>
<td>7</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* performed with the Welch t’ test
was the cis-golgi or endoplasmic reticulum stained. In all cell types, the nuclei, cytoplasm and membranes were unlabeled. The gut ECM appears to be labeled most prominently near the cell surfaces (figure 13A), but the apparent differences were not statistically significant (table 3). NRS controls exhibited only an occasional gold particle (figure 13B). This level of staining was in all cases calculated to be statistically insignificant (table 4).

2. Antigen characterization

(a) SDS-PAGE

Coomassie blue-stained 4-20% gradient SDS-PAGE gels of both non-reduced (figure 14A) and reduced (figure 15A) samples exhibit multiple bands from the top of the gel to the bottom, although the lane containing sperm homogenate has fewer bands present than do the other lanes. This demonstrates the presence of a large number of proteins, protein/polysaccharide molecules in the extracts (Harlow and Lane, 1988). The lane containing 7 d homogenate appears to be slightly underloaded.

(b) Immunoblotting and molecular weight calculation

(i) Non-reduced samples

All samples reveal several bands. About five are much larger than myosin (205 kDa) and appear near the top of the gel (figure 14B). One of these is very
Figure 13: Immunogold TEM localization of PJC-1 antigens to vesicles in endodermal cells of 7 d bipinnaria embryos.

The sections used in this figure were prepared as detailed in figure 11.

(A) Here the endodermal cells stained with PJC-1/gold are shown surrounding a cross section of the gut lumen (L). Gold label is found associated with the ECM within the gut lumen. In the apical region of the cells, the gold is within small (~0.3 μm diameter) vesicles (av), and in the golgi complex (gc). Basally, gold is found in large (~1 μm diameter) vesicles (bv) which appear similar to those found in the ectodermal and mesenchymal cells (figures 12A, B). The nucleus and endoplasmic reticulum (er) are not stained. Bar = 1 μm.

(A inset) This higher magnification view of an endodermal cell in the upper left of (A) shows a golgi complex surrounded by vesicles, with the nucleus at the right edge, and the lumen of the gut at the left. There are numerous gold particles within the dense granules, the medial and trans lamellae of the golgi complex, and in the trans golgi network to the right of the golgi complex. Bar = 0.5 μm.

(B) In this control (NRS-probed) section similar to (A) but of a transverse section of the gut lumen, almost no gold particles are present. The occasional lone particle of gold found within the apical or basal vesicles (arrows) is statistically insignificant when compared to the PJC-1-probed sections. Bar = 1 μm.
Table 4: Gold particle counts and t-test results for PJC-1 and NRS stained 7 day bipinnariae

Number of gold particles per μm² in each of the following locations is listed below.

<table>
<thead>
<tr>
<th>Location</th>
<th>Treatment</th>
<th>Mean</th>
<th>StDev</th>
<th>n</th>
<th>t value</th>
<th>result</th>
</tr>
</thead>
<tbody>
<tr>
<td>Endoderm - apical vesicles</td>
<td>PJC-1</td>
<td>48.26</td>
<td>12.02</td>
<td>19</td>
<td>4.12</td>
<td>p&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>NRS</td>
<td>0.05</td>
<td>0.23</td>
<td>19</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Endoderm - basal vesicles</td>
<td>PJC-1</td>
<td>20.67</td>
<td>9.47</td>
<td>15</td>
<td>3.32</td>
<td>p&lt;0.01</td>
</tr>
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<td></td>
<td>NRS</td>
<td>0.60</td>
<td>1.30</td>
<td>15</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ectoderm - vesicles</td>
<td>PJC-1</td>
<td>14.13</td>
<td>6.69</td>
<td>15</td>
<td>3.32</td>
<td>p&lt;0.01</td>
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<tr>
<td></td>
<td>NRS</td>
<td>0.33</td>
<td>0.62</td>
<td>15</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Blastocoel ECM</td>
<td>PJC-1</td>
<td>16.75</td>
<td>5.15</td>
<td>20</td>
<td>4.01</td>
<td>p&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>NRS</td>
<td>0.20</td>
<td>0.40</td>
<td>20</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hyaline layer</td>
<td>PJC-1</td>
<td>25.08</td>
<td>5.81</td>
<td>13</td>
<td>3.316</td>
<td>p&lt;0.01</td>
</tr>
<tr>
<td></td>
<td>NRS</td>
<td>0.46</td>
<td>0.97</td>
<td>13</td>
<td></td>
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</tr>
</tbody>
</table>
Figure 14: SDS-PAGE and immunoblot: non-reduced.

Gels and immunoblots of non-reduced sperm, oocytes, early (45 h), mid (72 h), and late (96 h) gastrulae, and bipinnariae (7 d) performed using SDS-PAGE. Each well was loaded with approximately 10 µg total protein.

(A) Coomassie Blue-stained 4-20% gradient gel showing numerous bands in all homogenates.

(B) Immunoblot using PJC-1 of a 4-20% gel containing the above samples. Two intense bands are visible, one well above the 205 kDa band of myosin, and the other at 43 kDa. This latter band is not due to PJC-1 (see C below). Other bands appearing at 73, 83, 95, 116, 123, and 162 kDa also show up when the membrane is probed without PJC-1.

(C) Immunoblot of homogenate from oocytes, 45 h and 72 h embryos probed only with secondary antibody and streptavidin-HRP showing the prominent 43 kDa band along with the 73, 83, 96, 116, 123, and 162 kDa bands. This indicates that the appearance of these band is due to an interaction not related to PJC-1.

(D) The same blot in (B) stripped and reprobed using normal rabbit serum. Only the bands evident in C are visible here. The 43 kDa band does not appear. It has been observed that this band usually does not reappear once the membranes have been stripped and reprobed.
Figure 15: SDS-PAGE and immunoblot: reduced.

Gels and immunoblots of reduced sperm, oocytes, early (45 h), mid (72 h), and late (96 h) gastrulae, and bipinnariae (7 d) performed using SDS-PAGE.

(A) Coomassie Blue-stained 4-20% gradient gel.

(B) Immunoblot using PJC-1 of a 4-20% gel containing the above samples. Notice the presence of multiple bands present above the 205 kDa band of myosin. Other bands are visible at 72, 83, 96, 116, 124, and 160 kDa. With exception of the 160 kDa band and two bands midway between the top of the gel and the myosin band (most readily visible in the oocyte lane, but present in the sperm, 45 h, and 72 h lanes), these bands are not due to PJC-1.

(C) The same blot in (B) stripped and reprobed without the use of a primary antibody. Several bands seen in B are also visible here, indicating that they are due to non-specific interactions between either the secondary antibody or the streptavidin-HRP conjugate.

(D) The same blot in (B) stripped and reprobed using normal rabbit serum.
prominent in all lanes, except that of sperm, and it may obscure other nearby bands. This is likely the original immunogen. The size of these bands has not been determined due to the lack of a very high molecular weight standard. Other bands appear at a \( M_r \) of 43, 73, 83, 96, 116, 123, and 162 kDa. The most prominent of these is the 43 kDa band. Unfortunately, most of the bands appear due to interactions with either the secondary antibody or the streptavidin-HRP (figure 14C), and are not related to PJC-1. Only the prominent heavy band near the top, the 160 kDa band and perhaps the 116 kDa band are due to recognition by PJC-1. The 160 kDa band is the only one of the "real" bands which shows some obvious change in density. It is most visible in the 72 h and 96 h homogenates. Probing the blots with NRS reveals only the same bands visible when probed without a primary (figure 14D), however they appear much fainter and require a much longer exposure time to be seen. As is seen here, the 43 kDa band usually does not appear if the membrane is stripped and then reprobed.

(ii) Reduced samples

About ten bands are visible each lane in the immunoblots of reduced samples with the exception of that of sperm, which has only four bands visible (figure 15B). As with the non-reduced samples, most of these bands (\( M_r \) of 73, 83, 96, 116, and 123 kDa) appear to be due to interactions with either the secondary antibody or streptavidin-HRP (figure 15C). The only exceptions to this are two bands found midway between the top of the gel and the myosin (205 kDa) band, and one at about 145 kDa. The two bands of undetermined size are seen most prominently in the
oocyte lane, however they are also visible, albeit faintly, in the sperm, 45 h, and 72 h lanes. The upper of these two bands appears to be in the same position as the band immediately above the heavy prominent band in the non-reduced blot. The 145 kDa band appears to increase in intensity in the lane containing 7 d homogenate. Again, the NRS-probed blots (figure 15D) exhibit the same bands as do the blots probed with only the secondary and tertiary antibodies. As with the non-reduced blots, these bands are much fainter and require a longer exposure for visualization.

3. Perturbation studies

(a) Sperm

When dilute sperm solution is incubated with various dilutions of PJC-1 (0%, 0.5%, 1%, 2.5%, 5%, and 10%), there is no immediate effect of PJC-1 on the sperm except for a general increase in activity. This effect is also observed to the same degree in the control sperm (data not shown). After the 30 min, 1 h, or 2 h incubation, PJC-1 treated sperm still swim normally, and no sperm are observed to be agglutinated. The general level of activity of the sperm is decreased from when the PJC-1 was first added, but again this effect is mirrored in the NRS-treated sperm (data not shown). Oocytes fertilized with either the PJC-1 or NRS incubated sperm appear to exhibit normal development at 1 h and 18 h post fertilization when compared to untreated controls (data not shown).
(b) Oocytes

Oocytes which were incubated in various dilutions (0%, 1%, 2.5%, 5%, 10%, and 20%) of PJC-1 for 1 h, and then rinsed and fertilized with untreated sperm develop fertilization membranes and grow normally in about the same percentage of oocytes in untreated, PJC-1 treated, and control-treated groups (data not shown). Oocytes treated the same way, but fertilized without washing exhibit a normal fertilization membrane 1 h after the addition of sperm. However, it is apparent by 2 d that development is affected. Many of those in 20% PJC-1 die by 2 d, but so do the NGS-treated controls (data not shown). There is also some mortality in both 5% and 10% groups, but many continue with development. By 7 d, untreated and 1% embryos are completely normal (figures 16A, B). The type and degree of embryo defects increases with PJC-1 concentration. The archenteron becomes less well differentiated and segmented as antibody concentration increases (figures 16C, 17C). At 10% antibody concentration the embryos become severely stunted. At 20%, the embryos are either dead or are ungastrulated (not shown). These effects are mirrored by the control embryos (figure 17B) with one exception. Some PJC-1 treated embryos in the 2.5%, 5%, and 10% groups exhibit anal prolapse (figures 16D, 17A,C). The rate of occurrence of this defect is about 30% in the 2.5% and 10% groups and 60% in the 5% group, but was not observed in any of the control embryos in these groups.
Figure 16: Perturbation effects of PJC-1: 0%, 1%, 2.5%.

DIC micrographs of 8 d bipinnaria larvae after developing in sea water containing PJC-1 in various concentrations from fertilization. m = mouth; e = esophagus; s = stomach; i = intestine; a = prolapsed anus; mc = mesenchyme cell (marked only on figure 16A). Bar = 40 μm.

(A) Lateral view of an untreated (0%) bipinnaria showing normal development of the gastrointestinal tract.

(B) Bipinnaria treated with 1% PJC-1 since fertilization. Gastrointestinal development is normal. One coelom (c) is visible in this oblique view.

(C) Larva treated with 2.5% PJC-1. The stomach and intestine are less well developed and segmented than in the untreated animal. The mouth and esophagus are fairly normal, although the latter structure appears a bit shorter.

(D) Larva treated with 2.5% PJC-1. Larva from the same group as (C) above, but this one displays a prolapsed anus (a). The stomach and intestines also show a lack of segmentation and development. The proximal structures of the gut appear relatively normal.
Figure 17: Perturbation effects of PJC-1: 5%, 5% control, 10%.

DIC micrographs of 8 d bipinnaria larvae after developing in sea water containing PJC-1 in various concentrations from fertilization. m = mouth; e = esophagus; s = stomach; i = intestine; a = prolapsed anus; ar = archenteron. Bar = 40 μm.

(A) Bipinnaria grown in 5% PJC-1. As in the 2.5% larva in figure 16D, this larva has a prolapsed anus in addition to other effects on the gastrointestinal tract.

(B) Bipinnaria grown in 5% NRS. Although there are effects on the g.i. tract, there is segmentation at the proximal end with the appearance of an esophagus. The distal g.i. tract is unsegmented.

(C, D) Larvae grown in 10% PJC-1. G.I. development is severely perturbed here. There is no segmentation of the elongated archenteron, and although some do exhibit anal prolapse (C), many do not (D).
IV. DISCUSSION

1. SDS-PAGE and immunoblots

In the non-reduced samples PJC-1 specific bands are visible with M, of approximately 116 and 160 kDa. The sizes of the prominent band well above the myosin band (205 kDa) and the band immediately above it visible in the sperm lane have not been determined because of the lack of a suitable high molecular weight standard. The prominent band is likely the original immunogen for PJC-1 as it appears at about the same position. The 160 kDa band is seen to become darker in the 72 h and 96 h homogenate lanes, when gastrulation is occurring, but appears fainter again in the 7 d lane. It is apparent from the coomassie-stained gels that bands in the 7 d lane are less intense than are those in the other lanes, indicating that this lane is perhaps underloaded. This could explain the relative faintness of the 160 kDa band in the 7 d lane in the non-reduced blot. In addition, it may be that this molecule is related to gastrulation and early morphogenesis of the digestive tract completed before 7 d post-fertilization, and is either modified or degraded after events for which it is required are complete.

The prominent high molecular weight band in the non-reduced samples is not visible in the reduced samples either at the same position, or as bands of similarly intense staining lower on the blot. This indicates that PJC-1 recognizes an epitope on this molecule which is not present after reduction with 2-mercaptoethanol. This
molecule may be made up of subunits, or its tertiary structure is significantly altered. Its prominence seems uniform throughout, but the strength of its signal obscures any subtle changes in its expression. As well, this strong signal likely obscures other nearby bands which may be detected by PJC-1. For example, in the lane with the sperm homogenate where this band is much more discrete, there is another band immediately above it. It is not possible to determine whether this latter band is present in the oocyte or any of the embryo stages due masking by the prominent band.

The 145 kDa band in the blot with the reduced samples is visible in the oocyte, disappears from the 45 h and 72 h lanes, but has reappeared by 96 h. It then increases in intensity from the 96 h to the 7 d lane. (That this molecule becomes denser in the 7 d lane indicates that there must be quite an increase in its expression, seeing as this lane also appears to be underloaded.) At 96 h, the coeloms are just forming, and the archenteron tip is connected to the presumptive stomodeal ectoderm by a blister of basement membrane (Crawford and Abed, 1983; Abed and Crawford, 1986). Between about 4.5 d (110 h) and 7 d, the mouth is formed and the digestive tract becomes segmented. As the gut differentiates the amount of ECM which lines the gut increases. It is possible that the 145 kDa band forms part of this ECM. In view of the fact that the antibody affects gut morphogenesis, it is also possible that the appearance of this 145 kDa band is related to these gut morphogenetic events.
The two bands midway between the top of the blot of reduced samples and the myosin band are most visible in the oocyte, become less dense in the 45 h and 72 h lanes, and all but invisible in the 96 h and 7 d lanes. They are also visible in the sperm. This suggests that these are molecules specific to the gametes, perhaps related to fertilization, which are no longer required once the zygote is formed and are degraded. The upper of these bands is at approximately the same height as the band visible just above the prominent band in the sperm lane of the non-reduced blot. If this is the case, it would mean that this band is in fact being masked in the oocyte lane on the non-reduced blot. The 116 and 160 kDa bands from the non-reduced blot, and the reduced 145 kDa band appear in the oocyte, but not the sperm. Thus they likely represent molecules not required in the sperm, such as those for the formation of the fertilization membrane and the early hyaline layer.

That it labels several different bands suggests that PJC-1, which was produced by electroeluting a band from a gel prepared with extract from oocytes, either recognizes the same epitope on several molecules, or it recognizes more than one epitope. The first suggestion would require some homology between different molecules which are found in different locations, namely the various ECM compartments. However, this does not help to explain the immunogold localization to the inner surface of the plasma membrane in the oocyte. In light of this, the second suggestion, or a combination of both, seems more likely.
2. Perturbation studies

The perturbation studies demonstrate that although PJC-1 has no effect on fertilization, it seems to have an effect on development of the g.i. tract. Embryos cultured in 2.5%, 5%, and 10% PJC-1 in sea water exhibit one defect in addition to those shared with the NRS-treated controls. Specifically, these embryos have a prolapsed anus. Presumably this occurs as PJC-1 block sites on molecules which somehow provide cues for certain morphogenetic events leading to the normal formation of the distal g.i. tract. This blocking likely occurs on the gut ECM as the antibody can enter the archenteron as it develops. However an alternative possibility is that it blocks sites in the blastocoel by gaining entry to the interior of the embryo by some sort of uptake mechanism. The ability of antibodies and other large molecules such as lectins placed into the environment of the embryo to enter the blastocoel and then affect development has been seen in Pisaster ochraceus (Reimer, 1994; Reimer and Crawford, 1997), and in sea urchin (Tully et al., 1998). The mechanism of entry is unknown. Whether, following perturbation, PJC-1 is bound only in the hyaline layer and gut ECM or also within the blastocoel was not investigated. This could be determined by processing embryos grown in PJC-1 for immunogold TEM (i.e. by freeze-substitution and embedding in LR-White), and then probing sections with only a gold-conjugated anti-rabbit secondary antibody. With this technique, which has been used successfully in the past (Reimer and Crawford, 1997), colloidal gold would label any PJC-1 which was present.
3. Immunolocalization of the PJC-1 antigens

(a) Gametes

(i) Sperm

The immunolocalization data show that material recognized by PJC-1 is found on both the outer surface of the cortex and within the medullary region of the acrosomal granule, and to the surface of the tails. Much of the immunogold labeling on the surface of the flagellum and the flagellar root appears to be associated with the outer surface of the membrane. This suggests that PJC-1 may be directed against material coating the membrane surface rather than the membrane itself. Lack of visualization of the membranes makes this determination difficult. Although the surface of the head is stained with immunofluorescence, it did not label with colloidal gold. Mucoid extracellular material is known to coat echinoderm sperm (Atwood, et al., 1974; Bickell et al., 1980). This material is present when the live sperm are secreted from the testis but is mostly removed when sperm are processed for TEM. It is possible that the antibody was directed at this material, and if so, it would explain this discrepancy.

When the acrosome reaction is triggered the contents of the acrosomal granule are exocytosed into the jelly coat of the oocyte. The acrosomal granule was seen to be labeled in both immunofluorescence and immunogold experiments. Because it is reasonable to assume that the molecules recognized by PJC-1 in the acrosomal granule are released following its exocytosis, a possible role for
molecules recognized by PJC-1 in fertilization is suggested. However, no effect of PJC-1 on fertilization was observed in the perturbation experiments (see section 2). The simple explanation is that these molecules do not in fact play a role in sperm binding or fertilization. Another possibility is that the sites on these molecules which are important for these roles are not blocked by the antibody. This could be due to PJC-1 not being directed against such sites, or that the local concentration of antibody is not sufficient to block sites on the molecules involved in sperm binding or fertilization in the time between when they are released and when they act.

(ii) Mature oocyte

The immunofluorescence data and the immunogold TEM data for the oocyte are complimentary with each showing the same structures being labeled. The presence of gold particles in the electron dense region of all cortical granules is in agreement with what was seen previously with a monoclonal antibody (Reimer, 1994; Reimer and Crawford, 1995). One interesting group of observations is the presence of gold particles along much of the inner surface of the oocyte cell membrane, on the outer surface of the cortical granules, and along the surface of the acrosomal granule in sperm. Although it seems unlikely, it is possible that such apparently membrane-associated molecules recognized by PJC-1 are somehow involved in the cell trafficking events which ultimately cause trafficking of their associated vesicles to the cell membrane. Such molecules include motors (GTPases of the Rab family) which move the vesicles along the cytoskeleton (Novick and Brennwald, 1993), address proteins which target the vesicle to the correct membrane
(v-SNAREs), and docking and fusion proteins at the target membrane (t-SNAREs; Söllner et al., 1993; Weber et al., 1998). The molecules which form the vesicle trafficking machinery are conserved in eukaryotes from yeast to mammals (Bennett and Scheller, 1993). Most such proteins which have been studied are small (25-40 kDa), although some docking proteins found in the golgi complex are 115-130 kDa (Nakamura et al., 1997; Orci et al., 1998), and those of the recently described exocyst complex are in the 75-105 kDa range (Kee et al., 1997). Although the molecules recognized by PJC-1 generally appear to be too large to be involved in vesicle trafficking, the molecule with the M₉ of 116 kDa is of a size consistent with some known vesicle trafficking proteins.

There are vesicles devoid of staining in the immunofluorescence experiment and vesicles which are electron lucent in the immunogold TEM work. Given that they are both similar in terms of size and location, they are likely the same vesicles. Although the contents of these vesicles are not known in starfish, it is possible that they represent one of the four vesicles which are exocytosed following fertilization in addition to the cortical granules in sea urchin (Matese et al., 1997).

(b) Embryo stages

The embryo stages stained immunofluorescently with PJC-1 contain fluorescent label in granules within cells, around cells, and in the various extracellular matrix compartments. These are the ECM of the blastocoel, the ECM
lining the lumen of the digestive tract (gut ECM), and the hyaline layer. Until hatching when the fertilization membrane and its overlying jelly coat are lost, these structures also exhibit fluorescent staining. This is not surprising as the fertilization membrane and jelly coat also stain in the oocyte.

At all stages, the ectoderm, mesenchyme, and endodermal cells are seen to contain immunofluorescent granules. In immunogold labeled bipinnaria stage embryos, all labeling within the cells is seen to be localized to either vesicles or to the golgi complex. Since the increase in fluorescence seen in the hyaline layer, blastocoel ECM, and gut ECM during development ostensibly comes from molecules secreted from the cells, it seems likely that these vesicles are secretory in nature. It is known that proteins which are exocytoxed by cells are produced in the endoplasmic reticulum, modified in the golgi complex, and either secreted by a constitutively active exocytotic pathway or by way of secretory vesicles, in which case some signal triggers the exocytosis of the vesicle (Alberts et al., 1994). Since the vesicles seen in the cells are seen to contain PJC-1 epitope-containing molecules, it is reasonable to assume that such molecules are produced and modified by the secretory pathway mentioned above.

The appearance of gold particles in the medial- and trans-golgi suggests that PJC-1 recognizes epitopes which are either not apparent in earlier compartments due to incomplete folding or assemble of the molecules, or that they are at least in part carbohydrate in nature. Proteins are modified in the golgi complex by the
addition and modification of carbohydrate moieties (Alberts et al., 1994). Previous work performed when the antibody was developed demonstrated that the band used to create PJC-1 stained positive with PA/S suggesting that it contained a glycoprotein (B. J. Crawford and L. Crichlow, unpublished observation).

The golgi also plays a role in ensuring proteins are folded correctly, and some assembly of multimeric molecules occurs in the golgi. These may be other explanations for the lack of recognition of molecules by PJC-1 until they are present in the golgi complex.

Both surfaces of the polarized epithelia in the starfish embryo are associated with ECMs which show an increase in their levels of PJC-1 epitope expression throughout development. This suggests that molecules recognized by PJC-1 are being secreted from the vesicles present in the cells. The position of these suggests that some vesicles are exocytyosed apically by the ectoderm cells and endodermal cells into the hyaline layer and the gut ECM respectively. Both cell types appear to be releasing material from their basal surfaces into the ECM of the blastocoel, suggesting that both cell types contribute PJC-1 reactive molecules into the ECM of the blastocoel.

In the early bipinnaria, the PJC-1 positive vesicles in the endodermal cells are of two sizes. The smaller vesicles (~0.3 μm diameter) are localized toward the apical surface and the larger ones (~1 μm diameter) are found in the basal region. Whether
the larger vesicles are formed by the fusion of the smaller ones, or have separate histories is not obvious from the TEM data. Clearly the large vesicles are much too large to bud off the golgi complex directly, suggesting that they are produced by the fusion of smaller vesicles. Interestingly, those PJC-1 positive vesicles found in both the ectoderm and mesenchyme cells strongly resemble the larger basal vesicles of the endoderm. Since the vesicles of the mesenchymal cells, and the basal regions of both the ectodermal and endodermal cells are probably exocytosed into the ECM of the blastocoel, it is possible that the products contained in these vesicles are the same or have similar functions with respect to modification of the blastocoel ECM.

Pericellular staining is observed most prominently in the mid blastula, somewhat less so in the late blastula/early gastrula, and is not evident in later stages. This may occur because the pericellular staining is overwhelmed by the overall increase in fluorescence as development progresses. The molecules being stained may also diffuse away from the cells into the blastocoel to provide the staining seen there. It is likely that both of these explanations play a role in the apparent loss of pericellular fluorescence. Immunogold TEM of mid and late blastulae would possibly provide more information as to just what is being stained. The pericellular staining is an indication of the exocytosis of PJC-1 epitope-containing material at the lateral and basal surfaces.

Some fluorescence positive granules are not released at fertilization but appear to be secreted into the blastocoel once it is formed. Although some of the
material being secreted may come from these maternally produced granules, the amount that is found between the cells of the mid-blastula stage suggest that new synthesis is occurring. It seems possible that embryonic expression of these molecules commences by the mid-blastula stage. Again, if molecules recognized by PJC-1 were found to be in the golgi complex of these embryos by immunogold TEM, this would provide better evidence that embryonic expression of such molecules has commenced.

The hyaline layer, which starts to form soon after fertilization (Holland, 1980) is not fully organized until the embryo hatches in the late blastula/early gastrula stage (Crawford and Abed, 1986). In sea urchin, deployment of molecules to the hyaline layer is also seen to begin with fertilization (Spiegel et al., 1989) and is seen to continue through early morphogenesis (reviewed in Alliegro et al., 1992). PJC-1 only begins to stain the hyaline layer at or just after hatching. This level of fluorescence staining increases as gastrulation progresses. This shows that there is secretion into the hyaline layer beyond the hatching stage, as suggested by Crawford and Abed (1986). The presence of material labeled with PJC-1/gold in apically situated vesicles in the ectoderm of 7 d early bipinnaria suggests that secretion of molecules into the hyaline layer is still occurring at this stage. It is likely that such secretion continues through larval development.

It is at the onset of gastrulation that fluorescence first appears in the blastocoel ECM. Immunogold labeling shows that PJC-1 is seen to be associated with the fibers
in the blastocoel suggesting that it is staining the ECM itself rather than being passively localized there. As the g.i. tract develops, the blastocoel ECM becomes more strongly stained. However, of all the regions which stain, it is the ECM which lines the gut that exhibits the largest change in strength of fluorescent signal during development. There is little or no fluorescence observed within the lumen of the archenteron until the mid gastrula stage. However in the early bipinnaria, where segmentation of the archenteron is essentially complete, the gut ECM along the lumen of the entire g.i. tract is labeled quite strongly. Labeling is especially strong in the stomach and intestine. The high degree of expression in the gut ECM as compared with the other locations indicates that the endodermal cells produce a greater amount of some or all of these PJC-1 epitope-containing molecules than do the other cell types between the mid gastrula and early bipinnaria stages. In the perturbation studies, PJC-1 is seen to affect gut development. Unfortunately, at present it is impossible to determine just which of these ECM molecules is responsible for the perturbation of gut development observed in these studies.

Previous work has shown that disruption of blastocoel ECM molecules causes perturbation of normal gastrulation in sea urchin (Govindarajan et al., 1995; Berg et al., 1996) and in starfish (Reimer and Crawford, 1997). Incubation of pre-gastrula stage embryos in sea water containing PJC-1 (see section 2) appears to affect development of the g.i. tract. The evidence, although circumstantial, suggests that the blastocoel ECM may be necessary for proper development and segmentation of the larval g.i. tract.
4. Conclusions and further work

This study has shown that the polyclonal antibody PJC-1 recognizes several high molecular weight molecules. These molecules are found in a variety of vesicles and extracellular matrix structures in sperm, mature oocytes, and various stages of embryos of the starfish. Although their presence in the acrosome of sperm and in the jelly coat, vitelline membrane, and cell membrane of oocyte, and that two high molecular weight molecules are found most in the gametes suggests a possible role for some of these molecules in fertilization, attempts to interrupt the fertilization process have so far failed to confirm this.

The molecules with PJC-1 epitopes on them are first secreted into the blastocoel and hyaline layer at the beginning of gastrulation. Later, large quantities of them are secreted into the lumen of the developing g.i. tract. A 160 kDa molecule (in non-reducing conditions) shows an increase in expression over the course of early gastrulation while a 145 kDa molecule (in reducing conditions) is most prevalent in the bipinnaria when segmentation is just complete and the intestine is migrating ventrally. Embryos grown in sea water containing PJC-1 caused anal prolapse. These observations suggest that these molecules might play a role in gut morphogenesis. Just what the exact role is of these molecules in the development of the g.i. tract is not clear. Presumably some have a role in cell-ECM interaction which allows for normal development of the gut either directly as a substrate for cell
adhesion or indirectly by changing the environment of the ECM for other structural or signaling molecules.

That PJC-1 only recognizes molecules after some processing in the golgi complex and not in the endoplasmic reticulum suggests that the epitopes are recognized only after some carbohydrate modification, or correct folding or assembly has occurred. Part of the difficulty of using PJC-1 is that it does not recognize only one molecule, but several, thus it is impossible to determine the functions of any one molecule. Future work should endeavour to isolate the molecules recognized by PJC-1 and develop monoclonal antibodies. Using such antibodies would allow each molecule to be studied individually. Additionally, immunofluorescence data of the stages between mid gastrula and early bipinnaria, and immunogold data of more embryonic stages would probably provide more information on exactly when these molecules begin to be synthesized. Ultimately any monoclonal antibodies which are developed that recognize protein epitopes could be used to probe a cDNA library to attempt to find out if there are already any known homologues of such molecules.


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

Note: Sperm were collected “dry,” that is as secreted from the testis, and steps 1-3 were omitted.

1. Harvest oocytes and embryos on ice in 50 ml plastic centrifuge tubes. Transfer to 15 ml conical glass centrifuge tubes when settled.

2. Centrifuge in clinical centrifuge 2 min at 125 g and remove water.

3. Resuspend embryos, but not oocytes, in 10 ml of cryoprotectant solution (15% 2,3-butenediol in CH₂O) and allow to equilibrate for 15 min on ice.

4. Prepare freezing apparatus in a fume hood. Fill the Dewar flask with liquid nitrogen and place the metal insert cup, suspended from a retort stand, into the Dewar flask. Fill the cup with cooking grade liquid propane from an inverted propane tank with an attached spout. Adjust a brushless eddy current motor with an attached stirring rod so that the rod goes at least halfway into the propane. Use extreme caution!! Liquid propane is highly explosive and readily dissolves oxygen from the air. Keep all sparks away from the apparatus.

5. Remove as much liquid as possible from the embryos/oocytes and place 1 μl of the thick suspension onto a 50 mesh TEM grid (Marivac) held by a pair of cross-close forceps. Use a triangle of #1 filter paper to remove as much of the liquid as possible. If the embryos/oocytes are in more than one layer, gently touch another TEM grid, held by cross-close forceps, to the grid. This will transfer some embryos/oocytes to the new grid. Collect dry sperm and smear
a small amount onto grids using a clean toothpick. Quickly plunge one grid at a time deep into the stirring liquid propane for 15-20 s and then rapidly transfer it into the surrounding liquid nitrogen for 5-10 s. The grid can now be transferred to the pre-cooled substitution medium (see below) or to a plastic cryovial in liquid nitrogen for storage in a liquid nitrogen refrigerator.

6. To substitute sperm, oocytes, or embryos, pre-chill 100% ethanol, or Alcian Blue 8X saturated ethanol, in 10 ml scintillation vials to below -80°C either in a -85°C freezer or with a liquid nitrogen bath. Note that marking the vials with masking tape or markers is often not permanent. It is best to use a diamond pen to mark the vial directly.

7. Quickly transfer the grids with sperm, oocytes, or embryos (4-6 grids per vial is usually sufficient) to the pre-chilled substitution medium and place the vials into the -85°C freezer for 5-7 d.

8. After the substitution period is complete, replace the substitution solution with fresh -85°C 100% ethanol. Then slowly bring the vials to room temperature by transferring them for 2 h first to a -20°C freezer, then to a 4°C refrigerator, and finally to the bench top. The embryos/oocytes are now ready to be embedded in either JB-4 or LR White resin.
Appendix 2: Production of PJC-1 antibody
(Performed by B. J. Crawford and L. Crichlow)

Using a protocol devised to isolate myosin (Kane, 1986), protein isolated from starfish oocytes was placed into non-reducing sample buffer containing 20 mM iodoacetamide and run on a preparative 3-12% gradient SDS-PAGE gel. The gel was stained for 10 min with 0.05% Coomassie Blue R 250 solution and then destained. The band of interest was excised from the gel, and homogenized by being passed repeatedly between two syringes fitted with 21 G needles with the addition of a small quantity of PBS, pH 7.4. The gel homogenate was then mixed with Incomplete Freund's Adjuvant and 400 µl was injected subcutaneously into six sites on each of two New Zealand White rabbits. Serum was collected six times from each rabbit over the course of several weeks prior to sacrifice. The polyclonal antibody was tested on muscle myosin and oocytes using an immunofluorescence protocol. Myosin staining was not observed, and instead the jelly coat of the oocyte was noted to be brightly stained. Because of this, the antibody was named *Pisaster* Jelly Coat-1.
Appendix 3: Buffers

**Phosphate Buffered Saline (PBS)**

*(After Crawford, 1972)*

20x stock solution:

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>160.00 g</td>
<td>140 mM</td>
</tr>
<tr>
<td>KCl</td>
<td>8.0 g</td>
<td>5 mM</td>
</tr>
<tr>
<td>KH$_2$PO$_4$</td>
<td>3.0 g</td>
<td>1 mM</td>
</tr>
<tr>
<td>Na$_2$HPO$_4$-7H$_2$O</td>
<td>5.8 g</td>
<td>1 mM</td>
</tr>
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</table>

Dissolve in distilled H$_2$O and make up to 1 L. Store at 4°C.

Use 5 ml stock solution and bring up to 100 ml. Add NaN$_3$ if required and adjust pH to 7.4.

**PBS/blotto**

Add from 0.2 g (immunogold TEM) or 2 g skim milk powder (immunofluorescence light microscopy) to 5 ml PBS 20x stock and 1 ml of a 10% NaN$_3$ solution, and bring up to 100 ml with dH$_2$O. Adjust pH to 7.4 (final solution is PBS, pH 7.4, 0.2% or 2% milk powder, 0.1% NaN$_3$). Store at 4°C and check pH before each use. If it has changed by more than 0.1 pH units, discard.

**Tris Buffered Saline (TBS)**

10x stock solution

<table>
<thead>
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<tr>
<td>0.2 M Tris</td>
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<tr>
<td>Tris Base</td>
<td>26.44 g</td>
</tr>
<tr>
<td>Tris HCl</td>
<td>87.66 g</td>
</tr>
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</table>
Dissolve in dH$_2$O and bring volume to 1 L. Store at 4°C.

Dilute 100 ml 10x TBS stock solution in 900 ml dH$_2$O and add sodium azide, if required. The pH should be at 7.4.

The following table (Sigma) details how to make a 50 mM tris solution with an exact pH at specific temperatures. The quantity of reagents used can be adjusted for any required concentration. Trizma is Sigma's brand name for tris (tris[hydroxymethyl]aminomethane).

<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>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>(g/L)</td>
<td>(g/L)</td>
</tr>
<tr>
<td>7.76</td>
<td>7.20</td>
<td>6.91</td>
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<td>7.71</td>
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</tr>
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</table>
Appendix 4: Photography

<table>
<thead>
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<th>Use</th>
<th>Exposure Time (s)</th>
<th>Film/Paper</th>
<th>Developer/Conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phase and DIC light microscopy</td>
<td>Automatic</td>
<td>Kodak TMAX 100</td>
<td>Kodak D-76, 7 min @ 22°C</td>
</tr>
<tr>
<td>Fluorescence light microscopy</td>
<td>Automatic</td>
<td>Kodak TMAX P3200 (ASA 6300)</td>
<td>Kodak TMAX, 10 min @ 22°C</td>
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<tr>
<td>Transmission electron microscopy</td>
<td>2 s</td>
<td>Kodak 5302 fine grain</td>
<td>Kodak D-19, 5 min @ 22°C</td>
</tr>
<tr>
<td>Reprints (Reprovit, side illumination)</td>
<td>1/15, 1/30 (f8)</td>
<td>Kodak TMAX 100</td>
<td>Kodak D-76, 7 min @ 22°C</td>
</tr>
<tr>
<td>Printing from negatives</td>
<td>Variable</td>
<td>Kodak Polycontrast III RC glossy</td>
<td>Kodak Dektol 1:2, 1.5 min</td>
</tr>
<tr>
<td>ECL Detection of Immunoblots</td>
<td>Variable</td>
<td>Kodak X-OMAT-AR</td>
<td>Kodak D-19, 4 min @ 22°C</td>
</tr>
</tbody>
</table>

Fixer: Kodak Fixer, 10 min for film, 5 min for paper

Rinse: Running tap water, 20 min for film, 5 min for paper
Appendix 5: Staining protocol for immunogold electron microscopy

- All solutions, except water, should contain 0.05-0.1% sodium azide.
- All solutions should be filtered prior to use with a Millex 0.45 μm filter on a syringe.
- All time steps below are performed by floating the grid section side down on a drop (~40 μl) of solution.

1. Pre-incubate each grid for 2 x 15 min with 10% normal goat serum in PBS/blotto (phosphate buffered saline containing 0.2% milk powder, and 0.1% NaN₃, pH 7.4).

2. Rinse 2 x 5 min in PBS/blotto.

3. Incubate grid 75 min on a drop of PJC-1 1-6 1:1000 in PBS/blotto (use 1:250 for sperm). Use normal rabbit serum as a control.

4. Rinse grid on drops of PBS/blotto for 2 x 5 min, 10 min, 15 min.

5. Incubate grid 75 min on a drop of undiluted goat anti-mouse IgG-gold.

6. Repeat step 4, then rinse grid for 2 x 1 min with dH₂O, then with a stream of dH₂O.

7. Place grid section side up on a triangle of #1 filter paper to dry for 1-2 min using another triangle of filter paper to absorb any water between the jaws of the forceps.

8. Stain grid 10 min with saturated aqueous uranyl acetate.

9. Rinse for 1 min in dH₂O and then in a stream of dH₂O.
10. Repeat step 7.

11. Stain grid 10 min in Reynolds' lead citrate.

12. Repeat steps 8 and 7.
Appendix 6: Coating TEM grids with Formvar
(After Hayat, 1989)

- Use acid-cleaned glassware washed in distilled water.

1. Place grids to be used, shiny side down, on a small pad of paper.

2. Make 10 ml of a 3% solution of polyvinyl formol (Formvar) in chloroform in a screw-top flask by adding the Formvar powder to the surface of the chloroform and letting it stand overnight.

3. Introduce 3 ml of the 3% solution to the bottom of a 100 ml graduated cylinder.

4. Tilt the cylinder and slowly add chloroform to the 25 ml mark without forming bubbles. Cover the cylinder with aluminum foil and mix gently. Let it stand overnight.

5. Wipe a pre-cleaned glass microscope slide with a lint-free, non-oily paper (e.g. Kimwipes). Handle one end only and make certain that this end never touches the Formvar solution.

6. Fill a cleaned glass 1000 ml container with dH₂O.

7. Attach a 30 cm piece of string to a small paper clip.

8. Attach the paper clip to the handled end of the cleaned glass slide.

9. Lift the cover from the cylinder and lower the slide so that the bottom 2/3 of it is immersed in the Formvar solution.
10. Raise the slide and let it stay in the vapours above the Formvar solution. Replace the cover for 30 s.

11. Remove the cover, lift the slide from the cylinder and allow any remaining liquid to dry. Detach the clip from the slide.

12. Scrape the three edges of one side of the slide with a sharp-edged tool (e.g. a razor blade) making certain that each side is face down while being scraped so that dust will not settle onto the film. Score the film just below the upper extent of the film.

13. Breathe gently onto the scored surface of the film.

14. Immediately, while the film is still moist from the breath, insert the slide into the dH₂O at a 45° angle so that the Formvar film floats free of the slide. Remove the slide.

15. Place the grids shiny side down onto the surface of the floating film over areas which are light silver in appearance (indicating 40-50 nm thickness) when viewed with reflected fluorescent light against a black background.

16. If the film appears colourless, the Formvar solution is too dilute; add stock solution. If the film is coloured, the Formvar solution is too concentrated; dilute it with more chloroform. A good film may have some clear areas and some yellow areas, but only silver areas should be used.

17. Insert a 50 ml glass beaker into the water with the open end up and slightly tilted so that the edge of the film makes contact with the edge of the beaker and the entire film is pressed down into the water by the beaker. Rock the beaker back and lift it so that the edge which entered the water first is out first. This is done so that the grids are pressed between the Formvar film and the end of the beaker.
18. Lay the beaker on its side on a clean absorbant surface in a 50°C incubator.

19. When all the water has evaporated (30 min), stand the beaker grid side up and cover it with a 100 ml beaker to keep off any dust.

20. Remove grids one at a time as needed.
Appendix 7: Preparation of colloidal gold
(After Frens, 1973; and Slot and Geuze, 1985)

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

Solution II: citric acid (reducing solution)
1% tri-sodium citrate (aqueous) 4 ml
1% tannic acid (aqueous) see below
distilled water up to 20 ml

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

To make 100 ml gold sol:

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 a colour change to red or purple is complete. The reaction time will increase proportionately to particle diameter size.

4. Store at 4°C for up to 6 months.
Appendix 8: Microtitration assay for determination of the correct protein concentration for gold sol stabilization

(Hodges et al., 1984)

Protocol:

1. Add 100 μl distilled water (dH₂O) to each of 10 1.5 ml Eppendorf microcentrifuge tubes.

2. Prepare a 1 mg/ml solution of protein to be assayed in dH₂O. Dialyze if necessary against 2 mM sodium borate, pH adjusted to just above the isoelectric point (pI) of the protein (almost all secondary antibodies are IgG class, pI 9.0).

3. To the first tube, add 100 μl of the protein solution. Serially dilute by removing 100 μl from the first tube, adding it to the second tube, and pipetting up and down three times. Then continue subsequent dilutions to the 9th tube, leaving the 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 (i.e. pH 9.05-9.10 for IgG) using 0.2 M K₂CO₃.

5. Add 500 μl gold sol to each well and stir with a pipet. Let stand for 15 min.

6. Assess the resistance of the mixture to salt-induced flocculation by adding 100 μl of 10% NaCl to each tube. Let stand for 5 min.

7. The last well to maintain a red colour represents the end point for protein stabilized gold.
8. Calculate protein stabilizing concentration per ml gold sol and double for experimental stabilizing concentration.
Appendix 9: Conjugation of gold sol to goat anti-rabbit IgG

(Hodges et al., 1984)

1. Dilute 6.87 μl stock goat anti-rabbit IgG (9.1 mg/ml) up to 1000 μl in 2 mM sodium borate-HCl, pH 9.0, to give a final concentration of 6.25 μg/ml as determined by the assay in appendix 8, and dialyze against borate buffer overnight. Note that when dialyzing, it is often useful to divide the protein solution into two dialysis bags in case one breaks.

2. Adjust the pH of 10 ml gold sol to 9.0 with 0.2 M K₂CO₃. Discard if acid is required.

3. Add 0.5 ml of the dialyzed 6.25 μg/ml protein all at once to stirring gold. Stir for 30 min.

4. Add 0.5 ml 10% bovine serum albumin in dH₂O, pH 9.0, to stirring gold conjugate and stir a further 5 min.

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

6. Resuspend in 10 mM tris with 50 mM NaCl and 0.1% bovine serum albumin, pH 8.2, and gradually increase the salt concentration to 150 mM NaCl by dialysis over 24 h.

7. Add sodium azide to a final concentration of 0.02%, and store at 4°C for 1 year.
Appendix 10: Biorad DC protein assay

(Biorad)

This assay is based on the reaction of proteins with an alkaline copper tartrate solution to Folin reagent, i.e. the reduction of Folin reagent by copper-treated proteins. Colour development occurs primarily through tryptophan and tyrosine residues. Several reduced species are produced with a characteristic blue colour with a maximum absorbance at 750 nm.

1. Make serial dilutions of the standard (bovine serum albumin) at concentrations between 0.2 and about 1.6 mg/ml (0.25, 0.5, 1.0, and 2.0 mg/ml were used) in the same buffer as the samples are in (homogenization buffer).

2. Make dilutions (e.g. 2%, 5%, and 10,%) of the sample in the same buffer in which the dilutions were produced.

3. Add 20 µl of reagent S to each ml of reagent A (an alkaline copper tartrate solution) to be used to make working reagent A'.

4. Add 5 µl of either the standard solution or the sample solution into wells of a clean, dry 96 well microplate (Fisher). The use of four wells for each sample and standard dilution, as well as for protein-free reference wells, reduces the amount of error in the assay.

5. Add 25 µl of reagent A' to each well.

6. Add 200 µl of reagent B (a dilute Folin reagent) to each well. Gently shake the plate for 5 s and pop any bubbles with a gel-loading micro-pipet tip.

7. After 15 min, the absorbances can be read at 750 nm (a Bio-Tek Microplate Autoreader EL 311 was used).
8. The standard absorbances can be plotted using a graphing program, such as Cricket Graph on an Apple Macintosh. Sample absorbances can then be used with the standard curve to determine sample protein concentrations.
Appendix 11: SDS-PAGE gradient gels
(After Laemmli, 1970; and Reimer, 1994)

Use Milli-Q filtered distilled water (ddH$_2$O) and electrophoresis grade reagents throughout. This protocol is for use with the Biorad Mini-Protean II gel electrophoresis system.

Reagents:

**Sample Buffer**
For 5x stock, dissolve 42.78 g (0.5 M) sucrose, 37.5 g SDS (15%), 9.46 g Tris base (312.5 mM), and 0.925 Na$_2$ EDTA (10 mM disodium ethylenediaminetetraacetate). Make to 225 ml with ddH$_2$O with heat and stirring. Adjust pH to 6.9 with 1 N HCl and bring to 250 ml. Store in 10 ml aliquots at 4°C. For 2x reducing working solution, add 1.4 ml ddH$_2$O, 50 µl 2-mercaptoethanol, and 50 µl 0.5% bromophenol blue to 1 ml 5x stock sample buffer solution. For non-reducing 2x sample buffer, add 1.4 ml ddH$_2$O, 50 µl 0.5% bromophenol blue, and 4.6 mg (10 mM) iodoacetamide to 1 ml 5x stock sample buffer solution.

**Lower Tris (1.5 M)**
Dissolve 36.34 g Tris base and 0.8 g SDS in 150 ml ddH$_2$O and adjust pH to 8.8 with 10 N HCl. Add ddH$_2$O to a final volume of 200 ml.

**Upper Tris (0.5 M)**
Dissolve 12.11 g Tris base and 0.8 g SDS in 150 ml ddH$_2$O and adjust pH to 6.8 with 10 N HCl. Add ddH$_2$O to a final volume of 200 ml.
50% Acrylamide Dissolve 48.75 g acrylamide and 1.25 g bis-acrylamide in ddH$_2$O to a final volume of 100 ml.

10% Ammonium Persulfate Dissolve 50 mg in 500 μl ddH$_2$O. Store at 4°C and discard after 1 d.

Electrophoresis Buffer For 10x stock solution, dissolve 30.28 g Tris base, 144.13 g glycine, and 10.0 g SDS in 1 L dH$_2$O (needs not be Milli-Q filtered). Working (1x) buffer should have its pH adjusted to 8.3.

To pour 6 mini slab gradient running gels:

<table>
<thead>
<tr>
<th>Stock Solutions</th>
<th>4%</th>
<th>20%</th>
</tr>
</thead>
<tbody>
<tr>
<td>50 % Acrylamide</td>
<td>2.25 ml</td>
<td>10.80 ml</td>
</tr>
<tr>
<td>Lower Tris (1.5 M)</td>
<td>6.75 ml</td>
<td>6.75 ml</td>
</tr>
<tr>
<td>ddH$_2$O</td>
<td>18.00 ml</td>
<td>9.45 ml</td>
</tr>
<tr>
<td>TEMED</td>
<td>13.5 μl</td>
<td>13.5 μl</td>
</tr>
<tr>
<td>10% Ammonium persulfate</td>
<td>101.25 μl</td>
<td>101.25 μl</td>
</tr>
</tbody>
</table>

Degas for 5 min.

1. Prepare acrylamide solutions and degas for 5 min each.

2. To set up gradient gel apparatus, place the gradient maker on magnetic stirrer, and place a small circular stir bar in the column with the outflow tubing attached. Ensure that both the connecting port between the two columns and the outflow tubing are closed. For the later, either a valve or a hemostat may be used.
3. Add TEMED (N,N,N',N'-tetramethylethylenediamine) and ammonium persulfate to acrylamide solutions, swirl gently, and pour into the gradient maker. Ensure that the 4% acrylamide solution is poured into the side with the outflow tubing.

4. Attach outflow tubing to multi-gel apparatus (Biorad), turn on magnetic stirrer, open connecting port on gradient maker, and remove hemostat from outflow tubing. Allow the acrylamide to pour in slowly, so that all the solution has entered to about 2 cm from the top of the short glass plate within 2-3 min. Since this is a gravity-feed system, adjust the height of the multi-gel apparatus relative to the gradient maker to control flow rate.

5. Disconnect outflow tubing, and rinse out gradient apparatus immediately. Place 200 µl water-saturated isobutanol on each gel with a gel loading micropipet to ensure a flat, even surface during polymerization.

6. After 30-45 min, remove the gels from the multi-gel apparatus and rinse out the isobutanol with dH$_2$O. Remove any dH$_2$O touching the gel surface with a triangle of #1 filter paper. The gels are ready for use 2 h after begins, or they may be stored in plastic bags at 4°C for several days if wrapped in plastic film. If stored gels are used, allow them to warm to room temperature for 1 h prior to use.

7. Pour a stacking gel on top of the running gel.
Stacking gel: (makes enough for 2 gels)

Stock solutions

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>50% Acrylamide</td>
<td>0.36 ml</td>
</tr>
<tr>
<td>Upper Tris</td>
<td>1.50 ml</td>
</tr>
<tr>
<td>ddH₂O</td>
<td>4.14 ml</td>
</tr>
</tbody>
</table>

Degas 5 min

TEMED 6 µl
10% Ammonium Persulfate 45 µl

1. Prepare stacking gel by mixing the acrylamide, Upper Tris, and ddH₂O in a 100 ml vacuum Erlenmeyer flask and degas for 5 min.

2. Place the precast running gels into the plastic gel holders. The bottom of the gels/glass plates should be flush with the countertop and the bottom of the gel holders.

3. Place the gel holders onto the gel casting apparatus. A pipet full of running buffer placed onto the grey silicon gaskets prior to attaching the gel holders will ensure that the gels do not stick to the silicon.

4. Add the TEMED and ammonium persulfate to the degassed acrylamide solution, and swirl gently to mix.

Use a pipet to pour stacking gel acrylamide solution on top of running gel. Insert a Teflon well comb at a 45° angle to allow bubbles to escape. Allow stacking gel to polymerize for 20-30 min.
5. Remove comb and use 1x running (electrophoresis) buffer to wash out unpolymerized acrylamide and bubbles from the wells. Keep the wells full of buffer as this facilitates the loading of the samples. (The location of the wells may be premarked on the acrylic gel holder with permanent marker).

Sample preparation:

1. Add samples to an equal volume of 2x sample buffer. If S-S bonds need not be reduced, 2-mercaptoethanol may be excluded from the buffer, and the addition of 20 mM iodoacetamide will reduce protease action.

2. Heat reduced samples to 90°C for 10 min. Load samples and perform electrophoresis.

Running the gel:

1. Place the gel holders onto the electrode assembly. The small glass plate of the gel should fit into the lip of the grey silicon gasket on the assembly.

2. Fill the electrode well with electrophoresis buffer to above the level of the small glass plates.

3. Place at least 200 ml of electrophoresis buffer into the main well (for our tank with the leaky gasket, the buffer should come to about the same level as it is in the electrode well).

4. Run the gel at 95 V until the dye front reaches the top of the running gel.
5. Increase the power to 195 V until the dye front just runs off the running gel, or when required.

6. Disassemble the gel apparatus and remove the small plate. Using a plastic wash bottle containing dH₂O, rinse the gel into a glass wash dish.

7. Rinse in dH₂O for 2-3 min. The gel is now ready for further treatment.
Appendix 12: Immunoblotting

Immunoblots were performed with Biorad polyvinylidene difluoride (PVDF) membranes. Gloves should be worn when handling the membrane.

Membrane wetting:

1. Wet the membrane by slowly lowering it into methanol at a 45° angle over 1-3 seconds. The membrane will wet immediately and will change from an opaque white to a uniform translucent gray.

2. Immerse the membrane in dH$_2$O for 1-2 min to elute the methanol. Keep it submerged with forceps at first.

3. Equilibrate the membrane in transfer buffer without any changes for at least 15 min.

Note: Once the membrane has been wet with water, do not allow it to dry out until the proteins have been transferred to it. If the membrane dries out, even partially, repeat steps 1-3.

Transfer protocol:

- Following electrophoresis, gels should be equilibrated 15-20 min in transfer buffer (transfer buffer may contain up to 20% methanol; as a general rule, the transfer of high MW proteins works best without methanol).

1. Several hours prior to the transfer, prepare 2 L of transfer buffer (10mM CAPS, and 5% methanol, pH 11.0) and place in refrigerator (when immunoblots are
being performed frequently, it may be convenient to make up a 10x stock solution of transfer buffer without methanol). Fill the freezing unit with either the above buffer or with dH₂O and freeze at -70°C (this may be done the night before).

2. Assemble the Trans-Blot apparatus a few min before starting transfer:
   - install the electrode assembly into a well-washed buffer chamber
   - fill the buffer tank half full with transfer buffer (~400ml)
   - place a stir bar at bottom of unit
   - install the frozen cooling unit into buffer chamber
   - Place the tank into an ice bath to help maintain a low temperature.

3. Assemble the gel holder cassette. **Wear gloves!!** Stack on the black panel in order, flooding each component with transfer buffer before layering next component:
   - fiber pad
   - filter paper
   - gel
   - blotting membrane *
   - filter paper
   - fiber pad.
   * After this step, roll a glass pipet over top of membrane to exclude air bubbles and to establish good contact between membrane and gel.

4. Close cassette and place in buffer tank so the black panel of the holder faces the black cathode of electrode panel, and fill the tank with buffer to the top of upper row of circles. Turn on the magnetic stirrer, place on the lid and turn on the power supply to 100V for a 1 h transfer at room temperature.
5. Following transfer, rinse the membrane several times in dH₂O to remove any contaminants.

**Immunostaining:**

**TBST:** 20 mM tris, 0.15M NaCl, 0.1% Tween-20, and 0.1% NaN₃, pH 7.4

1. Rinse the membrane 2 x 5 min in TBST.

2. Place into the blocking solution (5% bovine serum albumin [BSA], 4% fetal bovine serum, and 2% normal goat serum in TBST) overnight with agitation at room temperature. Note: Dissolve BSA in TBST by layering BSA on TBST in a beaker. It will dissolve in about 1 h at 4°C without stirring.

3. Wash twice briefly, and then for 15, 10, and 5 min in TBST.

4. Incubate with 1° antibody (PJC-1) diluted 1:10,000 in TBST for 60-90 min.

5. Wash as in 3.

6. Incubate in 2° antibody (biotinylated goat anti-rabbit IgG) diluted 1:7,500 in TBST for 60 min.

7. Wash as in 3.

8. Incubate in streptavidin-HRP 1:15,000 in TBST for 60 min.
9. Wash as in 3 except use TBST without NaN₃ as azide interferes with the activity of horseradish peroxidase.

10. The blot is now ready for detection with the Amersham ECL kit.
Appendix 13: ECL protein detection
(Amersham Pharmacia Biotech)

This is an enhanced chemiluminescence detection system where luminol, a cyclic diacylhydrazide, is catalytically oxidized by horseradish peroxidase and hydrogen peroxide in alkaline conditions. Immediately following oxidation, the luminol is in an excited state. To return to the ground state, it releases photons. Phenols, which enhance the chemiluminescent emission of luminol, are also present in this system. The maximum light emission is at 428 nm (blue) and is detected by blue-sensitive film.

Prior to detection, the sample proteins must be electrophoresed on an SDS-PAGE gel, transferred to either a nitrocellulose or PVDF membrane, blocked and probed as detailed in appendix 12. The final antibody must have a peroxidase conjugate attached.

Notes:

- Wear powder-free gloves while working with the blot and reagents.
- Only a red safe light is safe for this film. Keep the film in a closed light-proof bag and cover the developing tray if a yellow safe light is used.

1. Mix an equal volume of detection reagent 1 with detection reagent 2. The final volume required is 0.125 ml/cm² of membrane.

2. Drain the excess buffer from the washed membranes and place them protein side up either in a lid from a rack of pipet tips or in a large plastic weigh boat. Add the detection reagent to the membrane, so that the reagents are held by surface tension to the membrane. Do not allow the membranes to become uncovered.
3. Incubate for precisely 1 min without agitation.

4. Drain off the excess detection reagent by touching the edge of the membrane to a piece of tissue paper. Wrap the membrane in a sheet of plastic film. Gently smooth out any air pockets.

5. Place the membrane, protein side up, in a film cassette. Work quickly to minimize the delay between incubation and detection. The signal strength is highest after about 10 min, and remains high for at least 1 h.

6. Switch off the lights and carefully place a piece of autoradiography film (Kodak X-OMAT-AR) on top of the membrane, close the cassette and expose for 15 s.

7. Remove film and develop immediately (see appendix 4). On the basis of the amount of signal detected by this first piece of film, adjust the exposure for the second and subsequent pieces of film.