University of Alberta

The Actin Cytoskeleton in Early Embryonic Development and Cytokinesis

by

Gene K. Wong

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Doctor of Philosophy

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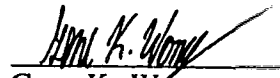

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ABSTRACT

This thesis examines the role of the actin cytoskeleton in early embryonic development and cytokinesis. Confocal laser scanning microscopy and fluorescently-labelled phallacidin have been used to investigate changes in the quantity and organization of filamentous actin (F-actin) in sea urchin eggs. Fluorescence quantification reveals cyclical increases and decreases in cortical F-actin content during early development, with a minimum occurring at cytokinesis. Accompanying these changes are the formation and disappearance of actin rootlets, filamentous structures extending from the bases of microvilli on the egg surface. Actin rootlet formation requires cytoplasmic alkalinization and is inhibited by cytochalasin D. Cytochalasin D washout experiments show that cortical actin cytoskeleton assembly can be blocked to within 5 minutes before cleavage and still permit normal cytokinesis. This suggests that the changes in cortical actin organization are not required for establishing the contractile apparatus for cytokinesis, but have other developmental functions. Spectrin, a component of the actin cytoskeleton, is implicated in many different processes during late embryogenesis, but little is known about its role in blastula formation. To investigate spectrin's role in early development, anti-spectrin antibodies have been microinjected into starfish embryos, inhibiting the activity of the maternal spectrin pool. Microinjecting anti-spectrin antibodies into one blastomere of two-cell embryos results in progressive increases in cell cycle time. Progeny of injected blastomeres are incapable of blastula epithelium formation, instead producing a loose aggregate of cells that stop dividing, extend surface protrusions, and become motile. At high doses of whole antibody or F_{ab} fragments, cells

initiate but fail to complete cytokinesis. Blastomeres injected with high doses of F_{ab} also fail to reform nuclei and arrest for up to 12 hours before reinitiating division. Injected embryos stained with fluorescently-labelled phallacidin show extensive disruption of the actin cytoskeleton. These results support previous studies implicating spectrin in stabilizing the cell surface and maintaining cortical cytoskeletal organization. They further demonstrate that spectrin is not required for initiation or contraction of the cleavage furrow, but functions in completing cytokinesis. Most surprising, however, is the involvement of spectrin in cell cycle regulation, which may involve monitoring actin cytoskeletal organization.

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Chapter 1

Introduction and Background

INTRODUCTION

Actin, microtubules and intermediate filaments are the principle filament systems comprising the cytoskeleton of cells. Together, these cytoskeletal elements serve four major functions in somatic cells: (1) the establishment and maintenance of cell shape, (2) the translocation of cellular components, (3) the binding of RNA and proteins to restrict their distribution, and (4) the control of cell motility (reviewed by Bement *et al.*, 1992). The actin cytoskeleton, a ubiquitous component of all eukaryotic cells (Bement *et al.*, 1992), performs these basic functions but is also involved in additional processes including exocytosis, cell-cell interactions, and cytokinesis.

Actin interacts with many other cytoskeletal proteins to perform its cellular functions. The myosins, a family of mechanoenzymes or molecular motors, mediate the sliding of actin filaments relative to one another, and use the filaments as tracks in the movement of intracellular components (Titus, 1993). Actin-binding proteins are involved in sequestering actin monomers, blocking the ends of actin filaments, regulating actin assembly/disassembly, and cross-linking actin filaments into higher organizational states (Stossel *et al.*, 1985). The interactions between actin and actin-binding proteins participate in the extensive actin rearrangements that occur during cell motility and through the cell cycle (Pollard and Craig, 1982).

Extensive actin rearrangements occur during embryonic development in sea urchin eggs, a popular model for cytoskeletal studies. Fertilization initiates a dramatic reorganization of the cortical actin cytoskeleton in sea urchin eggs, a change which suggests important roles in early embryonic development. During early development, the actin cytoskeleton appears to be involved in a variety of processes including the reorganization of the cell surface at fertilization, the incorporation of the sperm nucleus, the migration of intracellular vesicles to the cell cortex, and cytokinesis.

Although the changes in cortical actin organization at fertilization have been well documented, little is known about the subsequent dynamics of actin during early development and the role of cortical actin in cytokinesis. Studies on actin dynamics in the first cell cycle have produced conflicting results, suggesting either no significant change or a drop in the amount of cortical actin. To address these outstanding issues, I have used confocal laser scanning microscopy and fluorescently-labelled phallacidin to study the amount and dynamics of filamentous actin (F-actin) in the cortex through early embryonic development and cytokinesis. The results of this investigation illustrate that cortical F-actin content increases and decreases through the first two cell cycles of the sea urchin egg, with minimal amounts occurring during cytokinesis. Accompanying these changes are the assembly and disassembly of actin rootlets, filamentous structures which extend from the bases of microvilli on the egg surface. Shortly before cytokinesis, the actin rootlets disassemble and reappear at the completion of division. Actin rootlet formation requires cytoplasmic alkalinization and is inhibited by cytochalasin D. Cytochalasin D washout experiments show that the changes in cortical actin that occur

shortly after fertilization are not required for cytokinesis to occur, suggesting that this actin reorganization may serve other developmental functions.

First identified in erythrocytes, spectrin cross-links actin filaments and links actin to membranes (Bennett, 1990). These abilities permit spectrin to function in stabilizing the plasma membrane and maintain the normal organization of the actin cytoskeleton. Spectrin has also been examined in embryonic systems, where the distribution in early development and its functions in late embryogenesis have been studied in a variety of animals. In unfertilized sea urchin eggs, previous work has shown spectrin localization on the cytoplasmic face of the plasma membrane and intracellular vesicles, and its redistribution during early development (Fishkind *et al.*, 1990a, b). These studies have suggested important functions in early embryonic development.

My particular interest in spectrin began with contemplating what links the contractile ring to the plasma membrane during cytokinesis. During constriction of the contractile ring, the overlying plasma membrane is drawn into the cleavage furrow. As spectrin is able to attach actin filaments to membranes, it seems ideally suited for linking the contractile ring with the plasma membrane. The accumulation of spectrin in the cleavage furrows of mouse blastomeres (Schatten *et al.*, 1986), in the furrow canals of *Drosophila* embryos (Pesacreta *et al.*, 1989), and its biochemical identification in isolated cleavage furrows from sea urchin eggs (Yonemura *et al.*, 1990) places it in a position where it could serve this hypothesized linking function. At the time, however, no studies had demonstrated the involvement of spectrin in cytokinesis.

Many of spectrin's functions in late development have been determined using genetic approaches, techniques which cannot be applied in early developmental studies because of the large maternal pool of spectrin created during oogenesis. Thus, in order to examine the role of spectrin in early embryonic development and cytokinesis, I have microinjected antibodies against sea urchin egg spectrin into starfish blastomeres. This technique inhibits the activity of the maternal pool of spectrin so that the consequences on early development and cytokinesis can be assessed. The results show that spectrin is required for maintaining the stability of the plasma membrane and proper cell-cell contact for the formation of a normal blastula epithelium. The microinjection experiments also demonstrate that spectrin is not required for the initiation of cleavage, but is required for the successful completion of cytokinesis.

One of the most unexpected results in both of my studies is the finding of a link between the organization of the actin cytoskeleton and the normal progression of blastomeres through the embryonic cell cycle. Interestingly, a normal actin cytoskeletal organization is not required in the first cell cycle for cytokinesis and development to occur. Eggs can be incubated in cytochalasin D for up to 5 minutes before the onset of cleavage in control cells, and cytokinesis occurs normally. There is, however, a 15 minute extension of the first cell cycle when compared with controls, irrespective of the incubation time in cytochalasin D before washout. This link between actin cytoskeletal organization and cell cycle time also appears in the microinjection experiments. Microinjecting anti-spectrin antibodies into starfish blastomeres results in a progressive extension of the cell cycle. Examination with fluorescently-labelled phallacidin shows

extensive disruption of the actin cytoskeleton in microinjected embryos. These results suggest that the cell monitors the organizational state of the actin cytoskeleton as part of a mechanism which regulates progression through the cell cycle.

As the actin cytoskeleton is a central theme of my work, an overview of this filament system is presented here. The overview begins with a general consideration of actin structure and polymerization, followed by an examination of the various classes of actin-binding proteins, which are integral in regulating actin restructuring and organization. In reviewing the various classes of actin-binding proteins, selected examples are presented in each category. This background serves as the basis for introducing the actin cytoskeleton in sea urchin eggs. With the demonstrated link between the actin cytoskeleton and the cell cycle, a review of the embryonic cell cycle in the sea urchin egg is included for reference.

ACTIN

Observations of free-swimming nematodes in muscle tissue by W. Kühne in 1863 led to the conclusion that muscle contained "a concentrated solution of 'albumins'" rather than being solid as was thought during that time (Warrick and Spudich, 1987). Kühne (1864) later isolated a substance from muscle using solutions with high sal concentrations that he named "myosin" (Warrick and Spudich, 1987). In 1942, Albert Szent-Gyorgyi and colleagues created artificial fibrils from myosin and a protein which "activated" myosin, which they named "actin" (Warrick and Spudich, 1987). These artificial fibrils

contracted in the presence of ATP (Warrick and Spudich, 1987). In the same year, F.B. Straub (1942) identified actin as one of the two major proteins in muscle (Korn, 1982). Ten years later, A.G. Loewy (1952) extracted an actomyosin-like substance from *Physarum polycephalum* as part of his studies on "morphoplastic systems", defined as "a system capable of structural changes under the influence of ATP and related nucleotides". Partial purification of actin from *Physarum polycephalum* followed shortly afterward in 1956 by Ts'o *et al.*, with a highly purified preparation obtained in 1966 by Hatano and Oosawa. Since these initial isolations, actin has been identified in every eukaryotic cell examined, comprising as much as 15-20% of total cell protein (Korn, 1982).

Actin Structure

Actin Monomers (G-Actin)

Actin filaments assemble from protein subunits known as globular actin (G-actin), which is composed of a single 42 kDa polypeptide of 375 amino acids (Korn, 1982; Bremer and Aebi, 1992). G-actin appears to be a bi-lobed molecule consisting of a large and small domain, with approximately 150 amino-terminal amino acids confined to the small domain and almost 60% of the peptide, including the carboxy-terminal, in the large domain (Kabsch *et al.*, 1985; Pollard and Cooper, 1986; Bremer and Aebi, 1992). A small cleft separating the two domains is the presumed binding site for ATP or ADP. There is also a single high-affinity binding site for divalent cations, usually occupied by

Mg^{2+} in physiological systems, but which can also accommodate Ca^{2+} in a variety of experimental conditions (Korn *et al.*, 1987).

Although the amino acid sequence of G-actin appears to be highly conserved across different species (Korn, 1982), recent studies suggest that the actin family is much more complex than originally believed. This complexity is illustrated by the classification scheme of Herman (1993), which differentiates actin isoforms based on their amino terminal sequences. Class I actins include the β and γ isoforms which have a terminal N-acetyl aspartic or glutamic acid residue. Class II actins are primarily the muscle forms containing an amino-terminal methionine followed by cysteine and aspartic or glutamic acid.

The most recently identified isoforms of actin are the Class III group, which have been created with the discovery of Act2 in yeast, a homologue of conventional yeast actin Act1 (Lees-Miller *et al.*, 1992; Schwob and Martin, 1992; Herman, 1993). Proposals for the function of Class III actin include anchoring centrosomes or linking Class I actins to cytoplasmic microtubules (Herman, 1993). In interacting with cytoplasmic microtubules, Class III actins may participate in the regulation of intracellular vesicle movement through the activation of molecular motors like cytoplasmic dynein or kinesin (Herman, 1993).

Actin Filaments (F-Actin)

Actin filaments appear to be a two-start, double-stranded, right-handed helix with a filament diameter of about 6 nm (Korn, 1982; Pollard and Craig, 1982). The helices are staggered by 2.75 nm and cross over one another every 36 nm (Korn, 1982; Pollard and Cooper, 1986; Bremer and Aebi, 1992). Between each crossover point there are 13 actin subunits, which is the same number of G-actin molecules found in one complete turn of a single helix with a pitch of 72 nm (Korn, 1982; Pollard and Cooper, 1986; Bremer and Aebi, 1992).

Actin Polymerization

The polymerization of actin is one of the major processes by which changes in cell shape and motility occur (Pollard and Craig, 1982). Only Class I and II actins are capable of polymerizing to form 6 nm wide filaments (Herman, 1993). *In vitro* studies have divided actin polymerization into four steps, beginning with actin monomers (Pollard and Craig, 1982; Pollard and Cooper, 1986). G-actin can be maintained in its monomeric form in low protein concentration, low temperature, alkaline pH, and low salt concentration, but exposure to physiological concentrations of K^+ or Mg^{2+} results in the polymerization of pure actin into filaments (Pollard and Craig, 1982). The binding of Mg^{2+} to G-actin is thought to induce a conformation change in the monomer (Frieden *et*

al., 1980), activating it as the first step towards polymerization (Pollard and Craig, 1982; Pollard, 1990).

Nucleation is the second step in actin polymerization. This step is relatively slow and highly dependent on actin monomer concentration (Pollard and Craig, 1982). After activation, G-actin monomers dimerize, providing a basis for the formation of trimers (Korn, 1982; Pollard, 1990). Laboratory experiments using 10-20 μM actin concentrations suggest that the dimer dissociation rate is higher than the rate of dimerization or elongation (Pollard and Craig, 1982). The trimer is thought to be the first stable nucleus where the elongation rate is faster than the dissociation rate of the trimer. For this reason, and from kinetic modelling of the polymerization reaction, it is generally accepted that actin trimers are the nuclei for actin polymerization (Pollard and Craig, 1982; Pollard, 1990).

From the trimer nucleus the third step in the actin polymerization, elongation, can occur. Elongation refers to the association and dissociation of actin monomers at the ends of the filaments (Pollard and Cooper, 1986). These reactions involving G-actin are different at each end of the filament (Pollard and Craig, 1982; Pollard and Cooper, 1986), where a total of eight main reactions occur, entailing the association/dissociation of ATP/ADP-actin (Pollard, 1990). Decoration of actin filaments with heavy meromyosin (HMM) defines a polarity in actin filaments (Ishikawa *et al.*, 1969), where growth is faster at the "barbed" end than the "pointed" end (Woodrum *et al.*, 1975; Kondo and Ishiwata, 1976). Part of this difference in filament growth is attributed to the different rate constants at each end for ATP- and ADP-actin.

During polymerization, ATP-actin is hydrolyzed to ADP-actin, releasing energy which is dissipated and not utilized in the process (Korn, 1982; Korn *et al.*, 1987; Pollard, 1990). Although the function of this hydrolysis remains unknown, it has been suggested that the process permits the rapid depolymerization of F-actin at a later time (Carlier, 1989). Support for this suggestion is reflected in measurements of the rate constants during polymerization, which show that ATP-actin associates more rapidly than ADP-actin at both ends of filaments (Pollard, 1986; 1990). At the barbed end, ADP subunits dissociate more rapidly than ATP subunits, while the opposite is true at the pointed end (Pollard, 1986).

The *critical concentration* of actin monomers also affects the growth rate of filament ends. Before polymerization can occur, a minimum concentration of actin monomers must be present (Korn, 1982; Cooper and Pollard, 1986). This critical concentration may be the same or different at both ends of the actin filament, depending on solution conditions, affecting the elongation at either end (Kondo and Ishiwata, 1976; Pollard and Cooper, 1986). The critical concentration is also dependent on the nucleotide bound to actin, with ATP-actin having a lower critical concentration than ADP-actin (Korn, 1982). This difference appears to be related to the structure of ATP, as actin bound to adenylyl imidodiphosphate (AMP-PNP), a non-hydrolyzable homologue of ATP, has the same critical concentration as ATP-actin, even though AMP-PNP binds more weakly to actin than ADP (Cooke, 1975).

Annealing is the proposed last step in actin polymerization, where ends of actin filaments join one another (Cooper and Pollard, 1986). This process was first thought

to occur in experiments which fragmented actin filaments by sonication (Nakaoka and Kasai, 1969). A constant G-actin concentration during viscosity rise, along with the rates of viscosity increase, suggests that the main process in viscosity recovery is the "association between short polymers to form long F-actin" (Nakaoka and Kasai, 1969). *In vitro* experiments examining the unidirectional growth of actin filaments have produced electron micrographs showing the annealing of filaments (Kondo and Ishiwata, 1976). In these experiments, "seeds" of short actin filaments decorated with HMM, were observed on either end of undecorated actin filaments that formed by initiating actin polymerization *in vitro*. Annealing has also been demonstrated by mixing actin filaments with glutaraldehyde-fixed, HMM-decorated filaments, resulting in the creation of longer filaments composed of both unlabelled and labelled fragments (Murphy *et al.*, 1988).

Actin-Binding Proteins

Although it has been demonstrated that actin polymerization is affected by changing the solution condition, actin-binding proteins also play an important role in regulating actin polymerization and function, as illustrated in Figure 1.1. Many different types of actin-binding proteins have been identified, including actin monomer-binding, capping, severing, side-binding, cross-linking, and membrane attachment proteins (Korn, 1982; Pollard and Cooper, 1986). Definitive separation into these classifications is somewhat subjective, as many actin-binding proteins perform more than one function, permitting them to fit into more than one category. What follows is a general overview

of these groups of actin-binding proteins with selected examples which are summarized in Table 1.1.

Actin Monomer-Binding Proteins

Actin monomer-binding proteins share a number of common properties, including forming 1:1 complexes with G-actin; inhibiting nucleation and elongation rates; increasing the concentration of non-polymerized actin without raising the critical concentration required for polymerization; reducing the concentration and viscosity of F-actin; and possibly increasing the rate of F-actin depolymerization (Korn, 1982; Korn *et al.*, 1987). Members of this group include profilin, DNase I, vitamin D-binding protein (Korn, 1982), and thymosin β4 (Nachmias, 1993).

Profilin is an actin monomer-binding protein which has been studied fairly extensively and is thought to be involved in regulating actin polymerization. Profilin binds to G-actin in a 1:1 ratio to form a complex known as profilactin which prevents incorporation of bound actin monomers into filaments (Pollard and Cooper, 1986). This "buffering" of the actin monomer concentration in the cell is believed to play a role in regulating the polymerization of actin. Part of this regulation may involve phosphatidylinositol 4,5-bisphosphate (PIP_2), which appears able to promote the dissociation of profilactin into actin and profilin (Lassing and Lindberg, 1985). At the same time, profilin may interact with PIP_2, preventing its hydrolysis by phospholipase C (PLC) into inositol 1,3,4-triphosphate (IP_3) and diacylglycerol (DAG) (Goldschmidt-

Clermont *et al.*, 1990). These interactions with signalling pathways suggest some interesting mechanisms by which actin polymerization can be initiated from a concentration of sequestered monomers.

Actin Capping and Severing Proteins

Actin capping proteins bind to one end of actin filaments to affect the subunit reactions that take place there (Pollard and Cooper, 1986). Some common features of proteins in this group include the ability to accelerate nucleation, inhibit elongation, increase the critical concentration required for polymerization, and reduce the viscosity of filamentous actin by shortening filaments (Korn, 1982). The actin capping proteins can be divided into the gelsolin/villin, fragmin/severin, and "capping proteins" families (Pollard and Cooper, 1986).

The gelsolin/villin family consists of monomers with molecular weights between 90-95 kDa that are Ca^{2+}-dependent and nucleate, cap, and sever actin filaments (Korn, 1982; Pollard and Cooper, 1986). The fragmin/severin family includes polypeptides of approximately 45 kDa and also require Ca^{2+} to perform the same functions as gelsolin/villin proteins. The last family of capping proteins are heterodimers with subunits of 30-35 kDa and are Ca^{2+}-independent when functioning in nucleation or capping. Members of these capping proteins have been isolated from *Acanthamoeba* (Cooper *et al.*, 1984) and *Dictyostelium* (Schliecher *et al.*, 1984), and do not sever actin filaments.

The actin capping proteins described so far bind to the barbed end of actin filaments. Proteins which bind to the pointed end of actin filaments have also been identified. Acumentin is a 63.5 kDa monomer from macrophages which inhibits the elongation of actin filaments at the pointed end and may nucleate actin polymerization and shorten filaments (Southwick and Hartwig, 1982; Pollard and Cooper, 1986). β-actinin is a 35-37 kDa protein which also binds to the pointed end of actin filaments (Yokota and Maruyama, 1983). Although found in skeletal muscle in the region including the pointed end of actin filaments, β-actinin is able to inhibit elongation at both ends of actin filaments (Pollard and Cooper, 1986).

Actin Side-Binding Proteins

A number of actin-binding proteins bind to the sides of actin filaments to cross-link and bundle actin filaments. Tropomyosin and cofilin are two side-binding proteins that do not cross-link actin filaments. Tropomyosin is a dimer consisting of 35 kDa subunits which bind to the sides of actin filaments in striated muscle to regulate contraction (Stossel *et al.*, 1985; Pollard and Cooper, 1986). Several isoforms of tropomyosin have been found in different non-muscle cells. Cofilin, a 19 kDa monomer first isolated from bovine brain, also binds to the sides of actin filaments to inhibit actin polymerization, tropomyosin binding and actomyosin ATPase (Nishida *et al.*, 1984a, b). Cofilin also binds to G-actin in a 1:1 ratio and is also able to depolymerize actin filaments (Nishida *et al.*, 1984a, b).

Actin Cross-Linking and Bundling Proteins

Many proteins that bind to the sides of actin filaments are involved in the cross-linking and/or bundling of actin. The cross-linking protein α-actinin consits of two subunits between 90-110 kDa, and was first purified from skeletal muscle where it is a component of the Z-line (Pollard and Cooper, 1986). The bundling proteins include the fascin group, fimbrin, and villin (Pollard and Cooper, 1986). Fascin proteins are 57 kDa monomers which have been identified in protozoa, echinoderms, *Limulus*, and mammals. Fimbrin is a 68 kDa monomer found in the microvilli, microspikes, stereocilia, membrane ruffles, cell-substratum attachment sites (Bretscher, 1981) within a variety of vertebrate tissues (Pollard and Cooper, 1986). Villin also bundles actin filaments in microvilli as well as functioning as a capping protein.

Filamin or actin-binding protein 280 (ABP-280) is an actin cross-linking protein consisting of two identical 250-280 kDa polypeptide chains joined together at the carboxy-terminal end (Hartwig and Stossel, 1981; Gorlin *et al.*, 1990). The actin-binding site is at the amino-terminal end of each molecule. At high concentrations, some forms of filamin are able to create actin filament bundles (Hartwig and Stossel, 1981). The flexibility of the dimer suggests that filamin is involved in cross-linking actin filaments at large angles (Gorlin *et al.*, 1990). It has also been found that the filamin to actin ratio may regulate the ATPase activity of myosin II (Davies *et al.*, 1977; Sosiński *et al.*, 1984) and the contractility of acto-myosin gels (Janson *et al.*, 1992).

Actin Membrane Attachment Proteins

Many of the capping proteins are also thought to bind the ends of actin filaments to membranes (Pollard and Cooper, 1986). The capping protein gelsolin, for example, has binding sites for polyphosphoinositides which are constituents of the plasma membrane (Hartwig and Kwiatkowski, 1991; Weeds and Maciver, 1993). Gelsolin is also representative of a group of proteins involved in mediating the attachment of actin filament ends to membranes. Another group of proteins bind to the sides of actin filaments in order to attach them to membranes. One member of this group is ponticulin, a major 17 kDa integral membrane protein in *Dictyostelium* that binds to the sides of actin filaments and nucleates polymerization (Wuestehube and Luna, 1987; Luna *et al.*, 1990; Chia *et al.*, 1993; Hitt and Luna, 1994). Spectrin is also a member of this set of proteins.

Studies of erythrocyte spectrin have yielded most of the information on this protein, which cross-links actin filaments and anchors actin to membranes by binding to integral membrane proteins either directly or indirectly via ankyrin (Coleman *et al.*, 1989). Structurally, spectrins from different cells and organisms are very similar (Bennett, 1990). Spectrin is a flexible 200 nm molecule consisting of two distinct, extended rod-shaped subunits designated α and β (220-280 kDa). The α and β subunits are arranged laterally, anti-parallel to one another to form heterodimers. The heterodimers associate head-to-head to form tetramers. A calmodulin binding site is present on the α-subunit of nearly all spectrins. An ankyrin binding site, which helps

mediate the attachment of spectrin to the plasma membrane, is located on the β-subunit of most spectrins, roughly in the mid-region of the tetramers.

Even though spectrin has been found in many cell types and is fairly conserved across species, its roles are not very clear. Spectrin is redistributed in a variety of different cellular processes. In lymphocytes, spectrin is concentrated cytoplasmically and is redistributed upon differentiation (Lee *et al.*, 1988). Spectrin redistribution also occurs after the initiation of cell-cell contact, leading to the formation of intracellular junctions (Nelson and Veshnock, 1987), and during secretion in chromaffin cells (Perrin and Aunis, 1985) and parietal cells (Mercier *et al.*, 1989). During oogenesis and embryogenesis, spectrin also undergoes an extensive redistribution in the mouse (Sobel and Alliegro, 1985; Damjanov *et al.*, 1986; Schatten *et al.*, 1986), *Xenopus* (Giebelhaus *et al.*, 1987; 1988), *Drosophila* (Pesacreta *et al.*, 1989), and sea urchin (Schatten *et al.*, 1986; Fishkind *et al.*, 1990a, b).

The use of antibodies against spectrin have provided some clues as to the function of spectrin in cells. The results of microinjecting anti-spectrin antibodies have yielded variable results. Injection of monoclonal antibodies to spectrin into tissue culture cells shows little effect (Mangeat and Burridge, 1984). In amoebas, however, microinjection of antibodies to spectrin has profound consequences on cell morphology, motility, and movement-related activities (Choi and Jeon, 1992). These results indicate that spectrin may have important structural roles in the maintenance of cell shape and stabilization of the plasma membrane.

Spectrin also appears to have a role in maintaining normal cell-cell contact and epithelial morphology. Initiation of cell-cell contact in MDCK cells results in a redistribution of spectrin (Nelson and Veshnock, 1987). It has been demonstrated that spectrin interacts with cell adhesion molecules (CAMs) which are involved in establishing cell-cell contact, such as E-cadherin (Nelson *et al.*, 1990) and the 180 kDa isoform of N-CAM (Pollerberg *et al.*, 1987). It is believed that by limiting the lateral mobility of CAMs and confining them to areas of cell-cell contact, cells are able to form proper epithelia. Genetic manipulations which disrupt normal spectrin structure and function in *Drosophila* embryos (Lee *et al.*, 1993) and cultured epithelial cells (Hu *et al.*, 1995) result in the loss of normal epithelial morphologies. These experimental manipulations along with the redistribution data suggest that spectrin is involved in the maintenance of cell-cell contact.

THE ACTIN CYTOSKELETON IN SEA URCHIN EGGS

The Unfertilized Sea Urchin Egg

The sea urchin egg cortex is a region which includes the plasma membrane and the underlying 1-5 μm of cytoplasm (Vacquier, 1981; Spudich *et al.*, 1982; Spudich, 1992). The cortical cytoplasm has been considered to possess different structural and functional properties from the deeper cytoplasm of eggs (Sardet and Chang, 1987). The cortical region of cells, in general, is also quite dynamic and important in the

determination of cell shape, directed cellular motility, the movement of materials in and out of cells, and cellular adhesion (Spudich, 1992). Many of these functions involve the actin cytoskeleton.

About 20% of total egg actin is contained in the cortex region (Spudich and Spudich, 1979). In isolated cortices from unfertilized sea urchin eggs, Vacquier and Moy (1980) isolated seven major proteins, including actin which constitutes about 12-27% of total cortical protein. Using the fungal metabolite phalloidin, which specifically binds to F-actin, and antibodies against actin, Spudich *et al.* (1988) and Bonder *et al.* (1989) have demonstrated the existence of two different organization states of actin in the sea urchin egg cortex, a polymerized and an unpolymerized state (Spudich, 1992).

DNase I inhibition assays in unfertilized eggs of the sea urchin *Paracentrotus lividus* indicate that filamentous actin accounts for 20% of total actin (Coffe *et al.*, 1982). Electron microscopy studies have observed a loose array of short actin filaments in the stubby microvilli of unfertilized eggs (Chandler and Heuser, 1981; Sardet, 1984; Henson and Begg, 1988; Spudich *et al.*, 1988) which appear to make end-on attachments to the plasma membrane (Henson and Begg, 1988). These actin filaments are also detected with fluorescently-labelled phalloidin as punctate staining in the cortex of whole eggs and isolated cortices which correspond to the distribution of microvilli (Cline and Schatten, 1986; Yonemura and Mabuchi, 1987; Bonder *et al.*, 1989). A network of longer actin filaments also exists on the cytoplasmic surface of the plasma membrane, often appearing to run between the microvilli (Sardet, 1984; Henson and Begg, 1988; Bonder *et al.*, 1989).

Biochemical experiments first pointed to the existence of actin in an unpolymerized state in the cortex of unfertilized sea urchin eggs (Spudich and Spudich, 1979; Spudich, 1992). Immunogold labelling suggests that the unpolymerized actin is distributed as a cortical shell about 1 μm thick (Spudich *et al.*, 1988). Fluorescent staining with anti-actin antibodies also shows that it surrounds the cortical granules in isolated cortices from unfertilized sea urchin eggs (Spudich *et al.*, 1988) and in cryosectioned specimens (Bonder *et al.*, 1989). This monomeric actin is bound to ADP (Spudich *et al.*, 1982) and is probably in some assembled state, similar to profilactin as described by Tilney (1976; 1978) in the *Thyone* sperm. This state may be involved in sequestering actin monomers and be responsible for the high critical concentration that is required for "crude" biochemical actin preparations to polymerize (Spudich *et al.*, 1979; Mabuchi and Spudich, 1980). "Purified" actin, which may be free of the actin-binding protein, has a lower critical concentration.

Spectrin is also a component of the actin cytoskeleton of sea urchin eggs. A large maternal pool of spectrin accumulates during sea urchin oogenesis, resulting in its distribution on the plasma membrane and the membranes of cortical granules, acidic vesicles, and yolk platelets (Fishkind *et al.*, 1990a). In the cortex, spectrin is believed to cross-link actin filaments, forming a network that stabilizes and organizes the plasma membrane, and provides a structure which allows spectrin-coated cytoplasmic vesicles to anchor (Bonder *et al.*, 1989; Fishkind *et al.*, 1990a). Spectrin may also contribute to maintaining the stability and physiological function of cytoplasmic vesicles through its interaction with ion pumps/channels in these membranes (Fishkind *et al.*, 1990a). Since

spectrin synthesis ceases after oogenesis and is not re-initiated until the late blastula stage (Wessel and Chen, 1993), development of the early embryo relies on the maternal pool of spectrin.

Actin in the Fertilized Sea Urchin Egg

Fertilization initiates a number of dramatic ionic and cytoskeletal changes in the sea urchin egg. Within 2 seconds of sperm-egg contact, ionic changes occur within the egg, beginning with the depolarization of the plasma membrane associated with a minor influx of Ca^{2+} and Na^{+} ions (Epel, 1980; Schuel, 1984). This membrane depolarization is the basis of the fast block to polyspermy (Jaffe, 1976). The influx of Na^{+} into the egg also results in the efflux of H^{+} which has the net effect of raising the intracellular pH. This cytoplasmic alkalinization results in the metabolic activation of the egg (Chambers, 1976; Epel, 1980).

Following the influx of Na^{+} is the release of intracellular Ca^{2+} beginning from the site of sperm entry and propagating as a wave through the entire egg (Gilkey *et al.*, 1978). This wave of Ca^{2+} results in the exocytosis of the cortical granules within 20 seconds after fertilization (Epel, 1980; reviewed by Vacquier, 1981). By releasing their contents into the perivitelline space, cortical granules contribute to the formation of the fertilization membrane.

The described ionic changes are also important in the reorganization of actin that occurs immediately following fertilization. Two of these major changes include the

formation of the fertilization cone and the elongation of microvilli. Both of these structures involve the process of actin polymerization in their formation.

The fertilization cone is an irregular and transient extension of the cortex at the site of sperm-egg fusion (Longo, 1980; Tilney and Jaffe, 1980). It is formed shortly after fertilization, engulfing the sperm nucleus and incorporating it into the egg (Longo, 1980). At its maximum complexity, transmission electron microscopy shows a large population of filament bundles which terminate at the plasma membrane and extend at least 5 μm into the cortical cytoplasm (Tilney and Jaffe, 1980). S1 decoration also reveals that these actin filaments are all oriented such that they "point" to the center of the egg (Tilney and Jaffe, 1980). These actin filaments are presumed to have an important role in sperm uptake, as eggs incubated in cytochalasin B shortly after fertilization do not form fertilization cones or incorporate sperm (Longo, 1980).

The polymerization of non-filamentous actin also contributes to the elongation of the short microvilli. After lengthening, each microvillus contains about 5-10 actin filaments (Burgess and Schroeder, 1977) cross-linked into a bundle by fascin (Spudich *et al.*, 1982). Electron microscope observations with heavy meromyosin (HMM) or myosin S1 decoration show that the filaments "point" away from the microvillus tip (Burgess and Schroeder, 1977; Begg *et al.*, 1978).

Corresponding to the release of Ca^{2+} and cortical granule exocytosis, actin polymerization begins at the point of sperm penetration and spreads over the entire cortex in a wave-like manner (Yonemura and Mabuchi, 1987). Polymerization is thought to be one of two steps in the restructuring of the egg cortex at fertilization, with the second

being actin filament organization (Tilney and Jaffe, 1980; Yonemura and Mabuchi, 1987). It appears that the release of Ca^{2+} permits the polymerization of actin and the elongation of microvilli (Begg *et al.*, 1982; Carron and Longo, 1982). However, the Na^{+}-dependent alkalinization of the cytoplasm is required for proper filament bundling to occur in the microvilli (Begg and Rebhun, 1979; Begg *et al.*, 1982; Carron and Longo, 1982).

The reorganization of the actin cytoskeleton at fertilization is believed to be important in other early embryonic events as well. The actin cytoskeleton seems to be involved in the migration and anchoring of pigment granules to the cell cortex (Allen *et al.*, 1992), and in the delivery of extracellular matrix materials, contained within intracellular vesicles, to the cell surface (Alliegro and McClay, 1988). It is also believed that the cortical cytoskeleton of the sea urchin egg participates in the formation of the contractile apparatus used in cytokinesis (Mabuchi *et al.*, 1980; Begg *et al.*, 1983; Cline and Schatten, 1986; Mabuchi, 1994).

Fertilization also initiates the redistribution of spectrin in the sea urchin egg (Fishkind *et al.*, 1990b). Exocytosis of the cortical granules may involve the participation of the actin cytoskeleton and spectrin, as this process is very similar to exocytosis in chromaffin cells, and may be blocked using anti-spectrin antibodies (Perrin *et al.*, 1987). Immediately following the exocytosis of cortical granules, spectrin accumulates underneath the plasma membrane and in the fertilization cone (Schatten *et al.*, 1986) and microvilli (Fishkind *et al.*, 1990a), suggesting an important role in forming and stabilizing these structures.

Within 20 minutes after fertilization, spectrin-coated acidic vesicles migrate radially outwards to the cortex where they become anchored (Fishkind *et al.*, 1990b). During the period of embryonic cellularization, spectrin is redistributed from the surfaces of cytoplasmic vesicles to the expanding plasma membrane (Fishkind *et al.*, 1990b). A similar recruitment of spectrin occurs during cellularization in *Drosophila* embryos; this is believed to help stabilize the new membrane produced during early development (Pesacreta *et al.*, 1989). The accumulation of spectrin at the plasma membrane may also function in maintaining the polarized distribution of integral membrane proteins and cell-cell contact during formation of the blastula epithelium (Fishkind *et al.*, 1990b; Wessel and Chen, 1993).

Actin in Cleavage and Cytokinesis

Fertilization initiates the start of rapid cell divisions in the sea urchin egg. Cytokinesis, the final process of cell division, produces two individual daughter cells by partitioning the cytoplasm. This partitioning first appears as an indentation in the cell surface at the plane of division, the start of the cleavage furrow. Determined by the mitotic spindle apparatus shortly before cytokinesis (Mabuchi, 1986; Rappaport, 1986; Rappaport, 1990), the cleavage furrow normally bisects the mitotic spindle in the equatorial plane, ensuring the equal segregation of chromosomes into two new cells (Salmon and Wolniak, 1990). Aster microtubules radiating outward from the

microtubule organizing centers are thought to be involved in cytokinesis (Salmon, 1989), but their precise role in determining the cleavage plane remains unclear.

Shortly before cytokinesis, cells become more rounded with an accompanying increase in cortical tension or *stiffness* (Wolpert, 1960; White and Borisy, 1983). Once the cells become rounded, microtubule asters from the mitotic apparatus somehow cause regional changes in the cortex, positioning and initiating the formation of the cleavage furrow (Rappaport, 1986). The nature of the signal involved in these regional stimulations is unknown, but it has been suggested to involve the microtubules themselves (Vallee *et al.*, 1990) and/or microtubule-associated proteins (Mabuchi, 1986). Where the signal may act in a dividing cell forms the basis of two theories on the mechanism of cytokinesis.

The Polar-Relaxation Theory of Cytokinesis

The polar-relaxation theory of cytokinesis proposes that microtubule asters relay a stimulatory signal to the poles of the cell, relaxing this region relative to the equator (Wolpert, 1960). One of the basic tenants of this theory is that mitotic asters exert their greatest influence on the closest area of the cell cortex, namely the polar regions (Wolpert, 1960). Computer simulations of cytokinesis, which assume that aster influence is spherical and additive, suggest that the elements of cortical tension (contractile filaments) are distributed uniformly in the cell cortex at random orientations (White and Borisy, 1983; White, 1990). As the polar regions relax, contractile filaments migrate

to the equator where they circumferentially align about the plane of division to produce the cleavage furrow.

Quick-freeze, deep etch preparations of cell cortices from the cleavage furrow of dividing sea urchin eggs suggest a mechanism by which the circumferential alignment of filaments can take place (Bonder *et al.*, 1988). In the cortical preparations, actin filaments decorated with myosin S1 fragments form an anastomosing network, even though aligned filaments are observed with fluorescent phalloidin staining. Bonder *et al.* (1988) suggest that cortical tension in the cell is regulated by the concentration of filaments in the cortex and the degree of actin cross-linking. During cleavage, a decrease in filament cross-linking occurs at the poles, reducing cortical tension in this region to allow a gradual *migration* of cortical actin to the equator, followed by the enlistment of myosin to provide the force for cytokinesis. The generated force is isotropic, resulting in a *collapse* of the actin network in the equatorial region. As this collapse proceeds, occasional filament fascicles are produced which could be interpreted as circumferentially aligned filaments in electron microscopy. This *collapsing baby gate* model takes into account the cortical tension development model of White and Borisy (1983), along with electron microscopic data collected from quick-freeze, deep-etch preparations (Bonder *et al.*, 1988).

The Equatorial Stimulation Theory of Cytokinesis

The second model of cytokinesis, originally advanced as the *Gel Contraction Theory of Cytokinesis* by Marsland and Landau (1954), proposes that cytokinesis occurs through contraction of the cortical gel in the plane of cell division. In this theory, it is proposed that microtubule asters exert additive effects in the cortex of the region closest to where the asters are confluent (Rappaport, 1986). The net effect is a high level of stimulation in the plane of cleavage which is responsible for the formation of the contractile apparatus.

In determining which of the two proposed mechanisms of cytokinesis is more probable, Rappaport (1986) reviewed and performed experiments which alter the geometric relationship between the mitotic apparatus and the cell surface. Shifting the mitotic apparatus after the beginning of cleavage initiates furrow formation relative to the new position. Moving asters apart from one another and closer to the poles results in the loss of furrow forming ability, an effect not predicted by the polar-relaxation hypothesis. Based on the polar-relaxation theory, artificially constricting the equatorial region of the cell, bringing the cortex of this area in closer proximity to the asters than the poles, should cause an equatorial relaxation and polar contraction. However, cells constricted in such a manner divide normally.

Rappaport's (1986) work suggests that asters exert an additive stimulatory effect on the equatorial cortex to initiate contraction. Sand dollar eggs confined in capillary tubes with single asters are able to cause furrow-like constrictions in the plane of the

astral center. Some of these furrows have been observed to bisect the aster. These results imply that asters exert a stimulus on the plasma membrane to produce the cleavage furrow, and that this stimulus is the additive result of the two asters in normal cell division (Rappaport, 1986). This theory of equatorial stimulation leading to contraction is consistent with the bulk of experimental data on the initiation of cytokinesis.

The Contractile Ring

A common feature in both theories of cytokinesis is the eventual process of active contraction that occurs in the cleavage furrow. Marsland and Landau (1954) recognized that fundamental similarities existed between cytokinesis and amoeboid movement, leading them to suspect the involvement of actin and myosin in the process. Electron microscopic investigations of cytokinesis by Schroeder (1968, 1970) found an annular structure in the cleavage furrow consisting of fine filaments that appear to associate with the overlying plasma membrane. This annular structure was termed the contractile ring, and was hypothesized by Schroeder (1970) to be the transient structure responsible for cytokinesis.

With the identification of the contractile ring, attention focused on elucidating how this structure functioned. The sliding-filament model of force generation for muscle contraction (Huxley and Niedergerke, 1954; Huxley and Hanson, 1954; Huxley, 1969) has been the basis of the proposal that a similar mechanism is employed by the

contractile ring. The two main proteins involved in muscle contraction are actin and the mechanoenzyme myosin. Studies on the contractile ring of HeLa cells (Schroeder, 1970) and *Arbacia* eggs (Schroeder, 1972) have found that cytochalasin B inhibits cytokinesis. Since cytochalasins bind to the barbed end of actin filaments to inhibit polymerization at this end (Cooper, 1987), these results suggest that actin is involved in cytokinesis. Electron microscopic studies have found that the filaments observed in the contractile ring bind HMM, providing further evidence that these filaments are actin (Schroeder, 1973).

According to the sliding-filament model, the localization of actin to the contractile ring also implies that myosin II participates in cytokinesis. Myosin II, or conventional myosin, is a hexamer consisting of two heavy chains, two essential light chains, and two regulatory light chains (Warrick and Spudich, 1987; Tan *et al.*, 1992). The molecular weight of the heavy chains is approximately 200 kDa, while light chains range between 14-20 kDa. The amino-terminal portion of the heavy chains associate with the light chains to form two enzymatically active heads. The remainder of the heavy chains form the tail region, allowing myosin II hexamers to associate with one another to form bipolar filaments (Pollard *et al.*, 1990). Bipolar myosin II filaments mediate the sliding of oppositely polarized actin filaments past one another. Thin-section electron microscopy has revealed the anti-parallel arrangement of actin filaments in the contractile ring of PtK2 cells (Sanger and Sanger, 1980; Mabuchi *et al.*, 1988), appearing to be circumferentially aligned with the plane of cell division (Schroeder, 1972; Sanger and

Sanger, 1980; Mabuchi *et al.*, 1988; Satterwhite and Pollard, 1992). This arrangement is consistent with the sliding-filament model of contractile force generation by myosin II.

Immunofluorescent studies have localized both actin and myosin to the cleavage furrow in a variety of cells, including HeLa cells (Fujiwara and Pollard, 1976), sea urchin eggs (Schroeder and Otto, 1988), *Dictyostelium* (Yumura *et al.*, 1984; Kitanishi-Yumura and Fukui, 1989), and rat kangaroo (PtK2) cells (Sanger *et al.*, 1989). Microinjection of fluorescently-labelled myosin light chains into live cultured cells also shows localization of myosin in the cleavage furrow as cytokinesis proceeds (Mittal *et al.*, 1987). The localization studies place both actin and myosin in a position to participate in cytokinesis.

Direct evidence indicating the involvement of myosin II in cytokinesis has been acquired using two different techniques. The first evidence came from Mabuchi and Okuno (1977) and Kiehart *et al.* (1982) through the microinjection of antibodies into starfish blastomeres to ablate myosin function. These microinjections inhibited cytokinesis without affecting nuclear divisions (karyokinesis), leading to the production of multinucleate cells. Further evidence has been provided from genetic manipulations in *Dictyostelium*, where cells expressing antisense myosin mRNA (Knecht and Loomis, 1987) and cells expressing a truncated heavy meromyosin gene (De Lozanne and Spudich, 1987) do not undergo cytokinesis, but continue karyokinesis to become multinucleate. Together, the localizations, antibody microinjections, and genetic mutants strongly implicate myosin as having an active role in cytokinesis.

Actin and myosin are not the only proteins found in the cleavage furrow. Other proteins, including actin-binding proteins, have been localized to the contractile ring (Satterwhite and Pollard, 1992). Since a dividing cell *pinches in*, the contractile ring must somehow be associated with the overlying plasma membrane. The intimate association of the contractile ring with the plasma membrane led Schroeder (1972) to suspect the requirement of a *substance* to adhere actin filaments to the plasma membrane. Thin-section electron microscopy of the contractile arc in newt eggs show the barbed end of actin filaments in the contractile arc attached to a granular mass that is associated with the plasma membrane (Mabuchi *et al.*, 1988). This furthers the likelihood of a complex, which could include actin-binding proteins, that may mediate the attachment of actin filaments to the plasma membrane.

Accumulation of spectrin in the cleavage furrow of dividing mouse blastomeres (Schatten *et al.*, 1986) and furrow canals of *Drosophila* embryos (Pesacreta *et al.*, 1989) suggests that spectrin may function in cytokinesis. Spectrin has been identified in isolated cleavage furrows from sea urchin eggs by two-dimensional SDS-PAGE (Yonemura *et al.*, 1990), but immunofluorescent studies do not reveal a large build up in the furrow region (Schatten *et al.*, 1986; Fishkind *et al.*, 1990b). As a mediator of cytoskeletal-membrane interactions, spectrin may link the contractile ring to the plasma membrane during furrowing.

The redistribution of spectrin during mitosis also suggests a possible role for spectrin in cytokinesis. During cell division, it is believed that mitotic kinases may phosphorylate the β-subunit of spectrin, resulting in its dissociation from the plasma

membrane (Fowler and Adam, 1992). This redistribution may be related to the restructuring of membrane-associated actin filament networks in preparation for cell division (Fowler and Adam, 1992). The production of multinucleate Caco-2 cells expressing truncated β-subunits provides further support for the involvement of spectrin in cytokinesis (Hu *et al.*, 1995).

Little is known regarding the assembly of the contractile ring. As studies by Rappaport (1986) seem to indicate, aster stimulation of the equatorial plane of division somehow results in the formation of the contractile ring. Although how the components of the contractile ring assemble in the cleavage furrow is unknown, a hypothesis has been formulated as to the mechanism of assembly. Fluorescent studies show that myosin II in a number of cells is concentrated to the contractile ring at the time of cytokinesis, but how this mechanoenzyme is recruited to the cleavage furrow remains unknown. It has been assumed that the interaction of myosin II with actin filaments is what keeps the mechanoenzyme in the contractile ring. However, evidence indicates that the association of myosin II with the contractile ring is independent of actin. When actin is removed from the cortex of isolated contractile rings with gelsolin in the presence or absence of ATP, myosin remains in the contractile ring (Schroeder and Otto, 1988; Otto and Schroeder, 1990). The results of these experiments have prompted Otto and Schroeder (1990) to suggest that another factor, independent of actin, may be involved in anchoring myosin II to the plasma membrane.

Assembly and disassembly of myosin II filaments in the contractile ring may be regulated via phosphorylation (Satterwhite and Pollard, 1992). In dividing sea urchin

eggs, myosin heavy chains are dephosphorylated during cytokinesis (Larochell and Epel, 1993). The phosphorylation of myosin heavy chains seems to determine their ability to self-assemble into filaments (Trybus, 1991; Tan *et al.*, 1992). The effects are variable, with self-assembly being inhibited in some cell types with heavy chain phosphorylation, and promoted in others. Isolation of cleavage furrows from sea urchin eggs suggests that myosin heavy chains in this region are selectively dephosphorylated when compared to the rest of the cell (Larochell and Epel, 1993). Other phosphorylation studies of the myosin II regulatory light chain imply that this process may also be important in regulating contraction during cytokinesis (Satterwhite *et al.*, 1992).

Actin has also been examined extensively in the cleavage furrow, and it is these studies which have provided the existing information regarding the assembly of the contractile ring. Sanger (1975) has studied the changing patterns of actin localization in chicken embryo fibroblasts. At interphase, cells show a filamentous actin pattern. As they enter division, cells round up to produce a diffuse actin pattern throughout the cytoplasm and some cortical localization. During cytokinesis, actin concentrates in the furrow region and then shifts to emerging pseudopodia at the end of the process.

In *Dictyostelium*, similar changes in actin distribution are observed (Kitanishi-Yumura and Fukui, 1989). At interphase, actin appears localized to the cell cortex. When the cell enters mitosis at prophase, endoplasmic actin staining increases while cortical actin staining decreases. At anaphase, the trend is reversed. Cortical actin staining is fairly uniform at cytokinesis, but a very ordered arrangement exists in the cleavage furrow, where parallel arrays of actin filaments are present. To account for the

different actin localization patterns in his study, Sanger (1975) has proposed that actin is recycled in the cell. Kitanishi-Yumura and Fukui (1989) are vague in accounting for their observations of translocation or relocalization of contractile proteins. The changes in actin distribution could either reflect processes of *de novo* polymerization of actin filaments in the cleavage furrow, or a reorganization of existing actin filaments in the cell.

In an attempt to distinguish between the two possible mechanisms of actin recruitment in contractile ring formation, Cao and Wang (1990a) microinjected fluorescently-labelled G-actin and phalloidin at prometaphase or metaphase to label actin filaments in the cortex of the cell. The labelled filaments become concentrated in the cleavage furrow during cytokinesis, but total actin staining using fluorescein-phalloidin shows a less pronounced difference between the equatorial and polar regions of cells. Microinjection of rhodamine-actin shortly before cytokinesis, followed by fixation during mid-cytokinesis, reveals that actin is only slightly concentrated in the contractile ring when compared with the distribution of total actin filaments.

The Cao and Wang (1990a) study suggests that the contractile ring is formed primarily through the reorganization of existing actin filaments in the cell. Two possible mechanisms have been suggested by which existing actin filaments are concentrated in the cleavage furrow. The first method is through an increase in the number of actin filament binding sites at the equator of the cell during cytokinesis. Actin filaments continually undergoing *on-off* reactions on the plasma membrane may eventually be recruited to the areas of greater binding affinity from the cytoplasm. The second

proposed mechanism is through the process of cortical flow (Bray and White, 1988). Membrane-associated actin filaments may move from the polar regions towards the cleavage furrow. Both of these mechanisms may account for the observed decrease in rhodamine-phalloidin staining in the poles.

To further clarify which of the two proposed mechanisms is responsible for contractile ring formation, Cao and Wang (1990b) continued their study through the microinjection of rhodamine-phalloidin labelled F-actin into dividing cells. After associating with the cell cortex near the end of metaphase, labelled actin filaments migrate toward the cleavage furrow during anaphase and telophase. The injected fluorescent F-actin also diminishes in the polar regions of the cell. These results suggest that the contractile ring is formed from the recruitment of actin filaments in the cell cortex which flow toward the equator of the cell during cleavage.

In the Cao and Wang (1990b) model of contractile ring formation, actin filaments in the cytoplasm somehow become associated with the cell cortex (Figure 1.1). Actin-binding proteins, such as spectrin, may mediate this association. In the cortex, actin filaments then move toward the cleavage furrow where they can be reorganized to form the contractile ring. The filaments may then dissociate from the contractile ring and return to the cytoplasmic pool. Actin dissociation from the contractile ring during cleavage has been suggested by Schroeder (1972) to account for the volume changes that he has observed in this structure, and that have also been observed by Condeelis and Taylor (1977) during contraction of actomyosin gels prepared from *Dictyostelium* cell extracts. The mechanism of filament disassembly during contraction in these cases is

unknown. Once dissociated from the contractile ring and in the cytoplasm, actin may return to the polar regions of the cell to complete the cycle of actin movement.

THE EMBRYONIC CELL CYCLE

The somatic cell cycle consists of four discrete phases, G_1, S (DNA synthesis), G_2, and M (mitosis) (Pardee *et al.*, 1978; Murray and Hunt, 1993). G_1 is the gap between mitosis and DNA synthesis, while G_2 is the gap between DNA synthesis and mitosis. Cells may also exit the cell cycle and enter the G_0 phase, where they arrest early in G_1 as a result of nutrient starvation or growth factor deprivation (Murray and Hunt, 1993). The entire cell cycle itself lasts between 10-30 hours as measured in tissue culture cells (Pardee *et al.*, 1978) and 90 minutes in yeast (Murray and Hunt, 1993). Variability in the cell cycle times are mainly due to differences in the length of G_1, while the duration of S, G_2, and M phases are relatively constant (Pardee *et al.*, 1978).

Progression through the cell cycle is regulated by control points and checkpoints which ensure that events proceed in the correct order (Murray, 1994). The three main control points include START, mitosis ENTRY, and mitosis EXIT (Whitaker and Patel, 1990). START, or the restriction point, is a critical point in G_1 where cells pause if unfavourable conditions exist for growth (Murray and Kirschner, 1989; Murray and Hunt, 1993). Once past START, cells are committed to DNA replication in S phase (Murray and Hunt, 1993). ENTRY is triggered when DNA synthesis is complete and requires the duplication of centrosomes (Whitaker and Patel, 1990). Mitosis EXIT is

dependent upon the proper assembly of the mitotic spindle (Sluder, 1979; Whitaker and Patel, 1990).

Checkpoints monitor cell cycle events such as the replication of DNA and spindle assembly, generating a signal which arrests the cell cycle at a specific point when errors are detected (Murray, 1994). Checkpoints exist in G_1 to ensure that damaged DNA is not replicated. G_2 checkpoints prevent mitosis ENTRY if the replicated DNA has been damaged during this phase. The spindle assembly checkpoint ensures the proper segregation of chromosomes at mitosis. To some extent, cell cycle controls and checkpoints are intimately related with one another, with control points waiting for a signal to proceed and checkpoints sending a signal to stop.

The unfertilized egg sits in G_1 of the first mitotic cycle in a haploid and metabolically inactive state (Patel *et al.*, 1989; Whitaker and Patel, 1990; Zhang and Ruderman, 1993). Fertilization initiates the START of the cell cycle. START, and mitosis ENTRY and EXIT are control points in the sea urchin embryo that are shared with mammalian and yeast cell cycles (Whitaker and Patel, 1990). The early embryonic cleavages, which are initiated at fertilization, are rapid and synchronous, largely the result of suppressing G_1 and G_2 to essentially reduce the cell cycle to alternating S and M phases (Patel *et al.*, 1989; Murray and Hunt, 1993). G_1 is completely eliminated after the first cell cycle in sea urchin eggs (Hinegardner *et al.*, 1964). The increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) following fertilization initiates the cell cycle in sea urchin eggs (Patel *et al.*, 1989). S phase begins about 20-30 minutes after fertilization and lasts about 13 minutes (Hinegardner *et al.*, 1964; Hobart *et al.*, 1977),

followed by mitosis at about 70 minutes after fertilization (Hinegardner *et al.*, 1964; Whitaker and Patel, 1990; Zhang and Ruderman, 1993). Anaphase occurs about 90 minutes after fertilization, cytokinesis at 95 minutes after fertilization, and the nuclear envelope reforms by 110 minutes after fertilization (Whitaker and Patel, 1990; Zhang and Ruderman, 1993). In the sea urchin egg, however, S phase for the next cell cycle occurs in late anaphase or early telophase (Hinegardner *et al.*, 1964; Hobart *et al.*, 1977) corresponding with the formation of chromosome vesicles or karyomeres (Ito *et al.*, 1981). Following the replication of chromosomes, the embryonic cell cycle continues with G_2, M, and S phases. This progression is also dependent upon transient increases in $[Ca^{2+}]_i$ at the time of nuclear envelope breakdown and chromosome condensation (Patel *et al.*, 1989).

SUMMARY

This chapter has reviewed the actin cytoskeleton and the regulation of its functions by polymerization and actin-binding proteins including spectrin. The cortical actin cytoskeleton of the unfertilized sea urchin egg has also been introduced, with its dynamic changes during fertilization explored. These changes in the cortical cytoskeleton are believed to have important functions in early embryonic development, as well as in the process of cytokinesis, which is involved in the early cleavage divisions of the embryo. Fertilization also initiates the start of a number of different embryonic processes, including the entry into the cell cycle which consists of M, S, and G_2 phases.

The following two chapters of this thesis provide detailed accounts of my studies on the actin cytoskeleton and its role in early embryonic development and cytokinesis. Chapter 2 examines the dynamics of cortical F-actin in the first two cell cycles of the sea urchin egg using confocal laser-scanning microscopy and fluorescently-labelled phallacidin. The results illustrate that filamentous actin in the cortex of sea urchin blastomeres is much more dynamic than originally thought, with a minimum amount of actin in the cortex at the time of cell division. These observations raise interesting questions concerning the relative importance of the dramatic changes in actin organization that occur immediately following fertilization in early embryonic development. There are also equally important implications for the formation of the contractile ring during cytokinesis.

Chapter 3 reports on the role of spectrin in early embryonic development and cytokinesis. These sets of studies have been performed by microinjecting antibodies against spectrin into starfish blastomeres to ablate normal spectrin function. The results show that spectrin is required for maintaining the stability of the plasma membrane, actin cytoskeletal organization, normal cell-cell contact for blastula epithelium formation, and cytokinesis.

The unifying theme in both of these studies is discussed in Chapter 4, which considers the actin cytoskeleton and its dynamics during early embryonic development, and the involvement of the actin cytoskeleton in cytokinesis and cell cycle progression.

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Table 1.1. The different classes of actin-binding proteins with selected examples.

Protein	[a] ~MW (kDa)	Comments
Actin Monomer-Binding Proteins		
Profilin	12-15	
DNase I	31	
Vitamin D-Binding Protein	52-56	
Thymosin $\beta 4$	5	
Actin Capping and Severing Proteins		
Gelsolin	91	Also links actin to membranes
Villin	95	Also bundles actin filaments
Fragmin (*Phasarum*)	42	
Severin (*Dictyostelium*)	40	
Capping Protein		
Acantamoeba	30	
Dictyostelium	33	
Acumentin	63.5	Binds to pointed ends of filaments
β-actinin	35-37	Binds to pointed ends of filaments
Actin Side-Binding Proteins		
Tropomyosin	35	
Cofilin	19	Also binds to G-actin
Actin Cross-Linking and Bundling Proteins		
α-actinin	95-112	
Fascin	57	
Fimbrin	68	
Filamin	250-280	Also attaches actin to membranes
Actin Membrane Attachment Proteins		
Ponticulin	17	Integral membrane protein
Spectrin	220-280	Indirect linkage via ankyrin

[a]Approximate molecular weights are indicated as a range of the protein itself or its subunit components.

Figure 1.1. A schematic illustration of the various classes of actin-binding proteins. The dissociation of monomer-binding proteins allows actin to polymerize, which then permits other actin-binding proteins to cap, sever, cross-link, bundle, or attach filaments to the plasma membrane. The illustration also illustrates the multiple roles that many actin-binding proteins play.

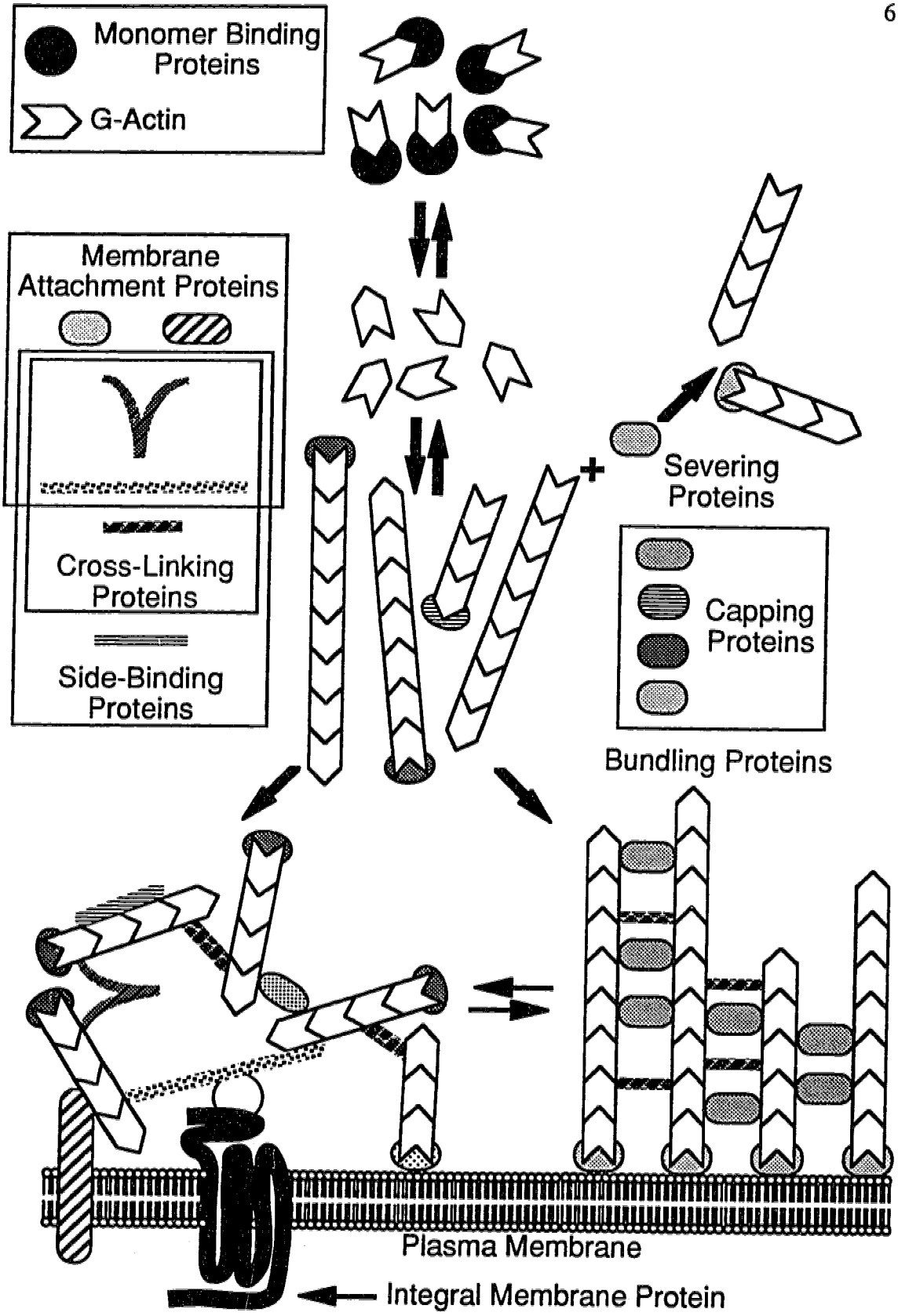
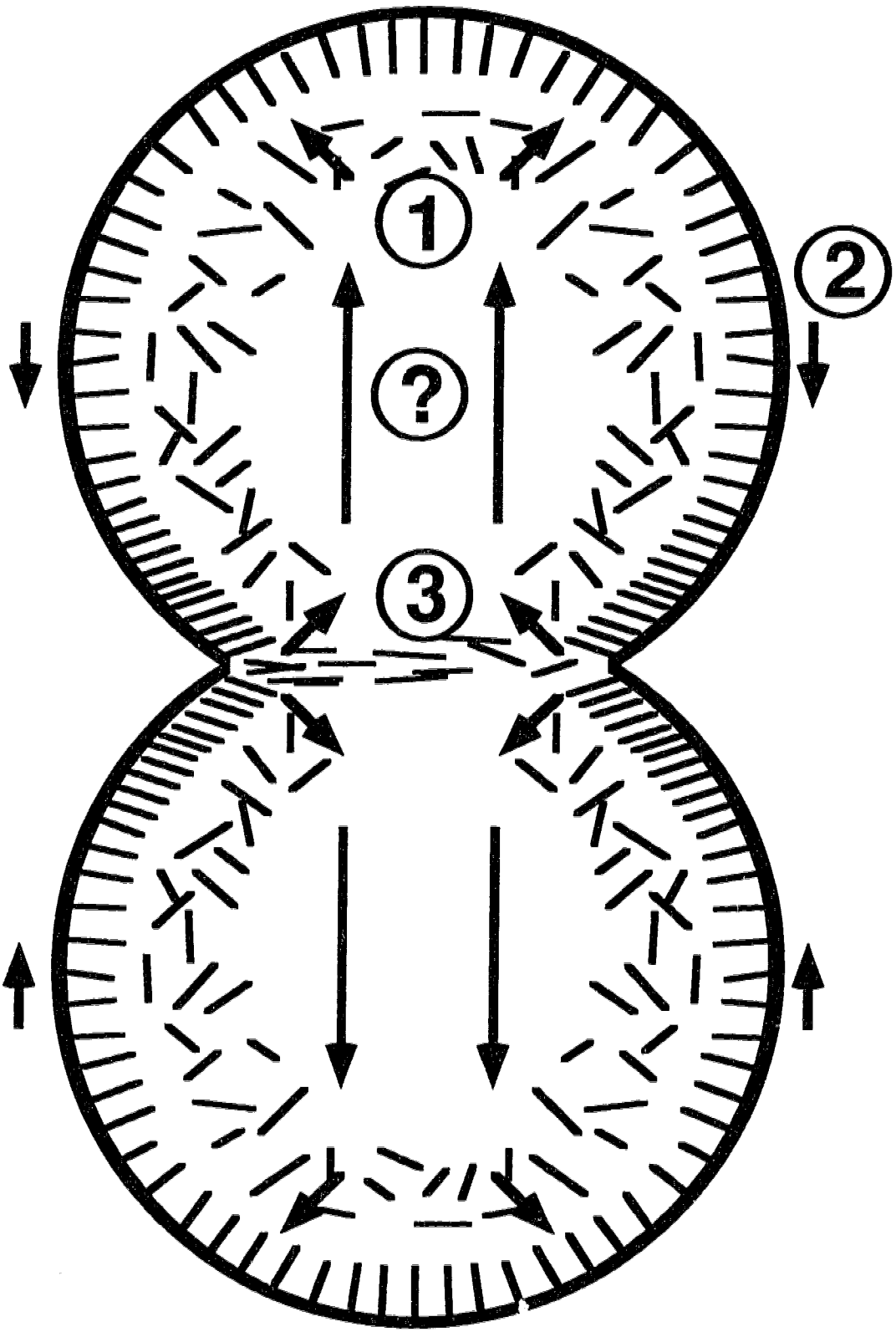

Monomer Binding Proteins
G-Actin
Membrane Attachment Proteins
Cross-Linking Proteins
Side-Binding Proteins
Severing Proteins
Capping Proteins
Bundling Proteins
Plasma Membrane
Integral Membrane Protein

Figure 1.2. The Cao and Wang (1990b) model of contractile ring formation. Arrows represent the movement of actin filaments, which are represented by the bars. Pre-existing actin filaments in the cytoplasm associate with the cell cortex (1). In the cortex, actin filaments move towards the plane of cell division (2), where they are recruited to form the contractile ring (3). As the contractile ring constricts, actin filaments dissociate and return to the cytoplasmic pool, where they may be utilized again at the next cell division (?).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Chapter 2

Dynamics of Filamentous Actin Organization in the Sea Urchin Egg Cortex During Early Cleavage Divisions: Implications for the Mechanism of Cytokinesis[1]

INTRODUCTION

Cytokinesis in animal cells results from the active constriction of the equator by the contractile ring, a transient structure composed of a circumferentially oriented band of actin filaments (reviews: Mabuchi, 1986; Rappaport, 1986; Salmon, 1989; Satterwhite and Pollard, 1992). The force for contraction is generated by the interaction of contractile ring actin filaments with myosin II (Mabuchi and Okuno, 1977; Kiehart *et al.*, 1982; DeLozanne and Spudich, 1987; Knecht and Loomis, 1987). In addition to actin and myosin, a number of different proteins have now been specifically localized to the cleavage furrow of dividing cells that may participate in the assembly and contraction of the cleavage apparatus (Satterwhite and Pollard, 1992; Fishkind and Wang, 1995). However, relatively little is known about the source of actin from which the contractile ring forms or the mechanism by which it is assembled. Two alternative mechanisms are currently considered for the assembly of the contractile ring for cytokinesis: recruitment of pre-existing cortical actin filaments or *de novo* assembly of filamentous actin in the equator of the cell (Mabuchi *et al.*, 1980; Usui and Yoneda, 1982; Mabuchi, 1986;

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[1] A version of this chapter has been submitted for publication. Wong, G.K., Allen, P.G., and Begg, D.A. 1996. *Cell Motil. Cytoskel.*

Mabuchi *et al.*, 1988; Satterwhite and Pollard, 1992). Existing evidence favours the recruitment of pre-existing actin filaments (Cao and Wang, 1990a, b; Mabuchi, 1994).

The sea urchin egg has been used extensively as a model system to study the mechanism of cytokinesis (Rappaport, 1986). One advantage of sea urchin eggs for such studies is that their cortex contains a highly organized array of actin filaments that assemble at fertilization and persist throughout early development (Schroeder, 1981a; Spudich, 1992). The cortex of the unfertilized sea urchin egg contains an extensive array of actin organized as a network of filaments within the cores of short microvilli and associated with the inner face of the plasma membrane (Henson and Begg, 1988; Bonder *et al.*, 1989), as well as a shell of non-filamentous actin in which the cortical granules are embedded (Spudich *et al.*, 1988; Bonder *et al.*, 1989). Upon fertilization, this cortical actin undergoes a dramatic reorganization associated with the exocytosis of cortical granules and the restructuring of the egg surface. A transient increase in intracellular free Ca^{2+} concentration and a Na^{+}-dependent alkalinization of the cytoplasm induce the polymerization of the shell of non-filamentous actin and its transformation into bundles of filaments that form the cores of numerous long microvilli (Tilney and Jaffe. 1980; Begg and Hyatt, 1982; Begg *et al.*, 1982; Carron and Longo, 1982). The cortical cytoskeleton of the fertilized egg is thought to participate in a number of events in early development, including the incorporation of the sperm nucleus by the egg (Tilney and Jaffe, 1980; Schatten and Schatten, 1981), the migration of intracellular vesicles to the cell cortex (Alliegro and McClay, 1988; Allen *et al.*, 1992), and the formation of the

contractile apparatus for cytokinesis (Mabuchi *et al.*, 1980; Begg *et al.*, 1983; Cline and Schatten, 1986; Mabuchi, 1994).

Although changes in the organization of cortical actin at fertilization have been well documented, relatively little is known about the subsequent dynamics of actin in the cortex during early development or its participation in the formation of the contractile ring. Biochemical studies have reported a large increase in total cortical actin after fertilization (Spudich and Spudich, 1979; Mabuchi *et al.*, 1980). However, these reports differ as to whether actin then decreases (Spudich and Spudich, 1979) or shows no significant change (Mabuchi *et al.*, 1980) up to first cleavage. Microinjection of fluorescently-labelled actin (Wang and Taylor, 1979; Hamaguchi and Mabuchi, 1988) or phallacidin (Hamaguchi and Mabuchi, 1982) into living echinoderm eggs have also demonstrated a substantial accumulation of actin in the cortex shortly after fertilization. Following this initial rise, a gradual reduction in cortical fluorescence has been observed (Wang and Taylor, 1979; Hamaguchi and Mabuchi, 1982), suggesting a decrease in cortical actin content. However, a similar decrease in cortical actin fluorescence over time has also been observed in unfertilized eggs (Hamaguchi and Mabuchi, 1982), raising the possibility that this decline may be an artifact. It is thus unclear how the organization of cortical actin changes as the embryo progresses through its early cleavage divisions.

We have used confocal fluorescence microscopy of fixed and BODIPY-phallacidin-stained embryos to investigate changes in the amount and organization of actin in the cortex during the first two cell cycles following fertilization. Quantification of fluorescence intensity in thin optical sections demonstrates a cyclical increase and

decrease in the amount of filamentous actin in the cortex, with minimum actin content occurring during cytokinesis. This variation in cortical actin content is accompanied by the growth and disappearance of rootlet-like bundles of actin filaments that extend radially from the bases of microvilli. Surprisingly, however, our results argue that the normal sequence of changes that occurs in cortical actin organization during the cell cycle is not required for the formation of the contractile apparatus for cell division.

MATERIALS AND METHODS

Materials

Choline chloride, Triton X-100, dimethyl sulfoxide (DMSO), A23187 Ca^{2+} ionophore, and cytochalasin D were obtained from Sigma Chemical Company (St. Louis, MO). Cytochalasin D was also purchased from Calbiochem Corporation (La Jolla, CA). BODIPY-phallacidin, a fluorescently labelled derivative of the fungal metabolite phalloidin which specifically binds to filamentous actin, was obtained from Molecular Probes, Inc. (Eugene, OR). Artificial sea water (ASW) was prepared from Tropic Marin® Sea Salt (Wartenberg, Germany). All other chemicals were of analytical reagent grade or better. *Strongylocentrotus purpuratus* sea urchins were obtained from Westwind Sea Labs, Inc. (Victoria, B.C., Canada).

Gamete Collection

Eggs were collected by injecting 0.5 M KCl into the coelomic cavity, de-jellied by three washings in divalent cation-free sea water (510 mM NaCl, 27 mM KCl, 2 mM HEPES, 1 mM EDTA, pH 8.0) followed by three washings in artificial sea water (ASW) or Na^+-free sea water (NaFSW), and stored on ice. Choline chloride for the NaFSW (424 mM choline chloride, 25 mM $MgSO_4 \cdot 7H_2O$, 23 mM $MgCl_2 \cdot 6H_2O$, 12 mM $CaCl_2 \cdot H_2O$, 9 mM KCl, 2 mM $KHCO_3$, pH 7.8-8.0) was recrystallized from a saturated

hot ethanol solution by adding acetone to the cloud point and then cooling to -20°C (Begg *et al.*, 1982). Testes were removed and stored "dry" on ice. Sperm were diluted in ASW immediately before use. Eggs were utilized in experiments only if the fertilization rate was $\geq$ 95%.

F-Actin Dynamics in Normal Development

4 mL of settled eggs in 600 mL of ASW were fertilized and cultured at 12°C in glass beakers with gentle paddle stirring at 30 rpm. Fixation and processing of eggs for confocal microscopy was carried out at room temperature. Samples of eggs were taken at 5 minute intervals for the first 30 minutes after fertilization, and then every 10 minutes until 4 hours post-fertilization. Samples were fixed for 30 minutes in Millonig's phosphate-buffered fixative (3% formaldehyde, 0.20 M $NaH_2PO_4 \cdot H_2O$, 0.136 M NaCl, pH 7.0, modified from Cloney and Florey, 1968) and the eggs allowed to settle. The fixative was aspirated off and the eggs permeabilized for 10 minutes in wash buffer (50 mM HEPES, 50 mM PIPES, 0.6 M mannitol, 2 mM $MgCl_2$, 20 mM EGTA, pH 7.0) containing 0.1% Triton X-100. After permeabilization, eggs were washed three times in wash buffer without detergent and then stained with 10 μg/mL BODIPY-phallacidin in wash buffer for 30 minutes. Eggs were washed three times in wash buffer to remove excess phallacidin, and then placed in an anti-photobleaching mounting medium consisting of 2 μM n-propyl gallate and 60% glycerol in Tris-buffered saline (50 mM

Tris base, 150 mM NaCl). Eggs were mounted on glass slides, and the coverslips sealed with nail polish.

F-Actin Dynamics in Na^+-Free Sea Water

To investigate the pH-dependence of actin rootlet formation, 2 mL of settled eggs were cultured in 400 mL of NaFSW at 12°C with gentle stirring as described above. The same density of eggs were incubated in ASW to serve as controls. Eggs were parthenogenetically activated with 10 μM of the Ca^{2+} ionophore A23187. A23187 was stored at -20°C as a 1 mg/mL stock solution in DMSO. Samples were fixed at 5 minute intervals between 0 and 30 minutes post-activation for 30 minutes at room temperature in AC320 fixative (Bonder *et al.*, 1989). Eggs were cultured for an additional 30 minutes in NaFSW, and 1 M NaCl was added to a final concentration of 40 mM. This procedure results in the rapid, Na^+-dependent alkalinization of the egg cytoplasm (Begg *et al.*, 1982). Samples were then fixed at 5 minute intervals for the next 30 minutes (1 hour to 1 hour and 30 minutes post-activation). After fixation, eggs were permeabilized, stained, and mounted as described for the normal developmental series.

Cytochalasin D Experiments

Cytochalasin D was stored at -20°C as a stock solution of 1 mg/mL in DMSO. At 2 minutes post-fertilization, 4 μg/mL cytochalasin D was added to 4 mL of settled

eggs that were fertilized in 400 mL of ASW and cultured as described for the normal developmental series. After 30, 60, 90, or 120 minute incubations, eggs were washed 3 times with ASW to remove the cytochalasin and allowed to develop under normal conditions. Samples were fixed at 10 minute intervals following removal of the drug until the eggs completed first cleavage. Percent cleavage was determined by counting at least 100 eggs at each time point using a Reichert Polyvar 2 microscope with differential interference contrast (DIC) optics. Cells were counted as cleaving if they contained a detectable furrow. This definition included cells which had just initiated cleavage or completed first division. Eggs which were exposed to cytochalasin D for 60 minutes before washout were sampled at 10 minute intervals until 4 hours post-fertilization. These samples from 0 minutes to 4 hours post-fertilization were processed for confocal microscopy as described for the normal developmental series.

Confocal Microscopy

Eggs fluorescently stained for filamentous actin with BODIPY-phallacidin were viewed on a Leica Confocal Laser Scanning Microscope (CLSM) based on a Leica Aristoplan Microscope. Equatorial optical sections were collected with a 63x PL APO objective lens (NA 1.4) using 64 scans at a resolution of 512 x 512 pixels and transferred to a Power Macintosh 6100/66AV (Apple Computer, Inc., Cupertino, CA). Figures were composed using Adobe Photoshop 3.0.1 (Adobe Systems, Inc., Mountain view, CA) and hard copies produced by a Codonics NP-1600 Photographic Network Printer

(Middleburg Hts., OH). For quantification of cortical F-actin, images were collected (16 scans at a resolution of 512 x 512) using a 40x NPL Fluotar Flureszenz objective lens (NA 1.3) and transferred to a Power Macintosh 6100/66AV. All images were collected using the same slit size, offset and voltage settings, which were calibrated for the time point showing maximum fluorescence intensity.

Quantification of Cortical F-Actin.

At each time point studied, 10 embryos were analyzed using the software program NIH Image Version 1.56 for the Power PC (National Institute of Health). The pixel intensity range of cortical actin fluorescence was determined for the time point showing greatest fluorescence intensity using the density slice function of the NIH Image program. This point occurred at 20 minutes after fertilization with a pixel intensity range of 100 to 254. This density slice range was used to analyze all time points in the data set. Fluorescence intensity was integrated by multiplying the number of pixels within this range (the area) by the average pixel intensity. To control for differences in egg size, the data were normalized by dividing the integrated fluorescence by the cell perimeter. Statistical analysis was carried out using Microsoft Excel Version 3.00 (Seattle, WA) and StatWorks™.

RESULTS

Changes in Cortical F-Actin Content During Early Development

The intensity of fluorescent phallacidin staining in the egg cortex undergoes cyclical variations during the first two cleavage divisions (Figure 2.1), indicating changes in the content of filamentous actin. Unfertilized eggs show weak punctate staining corresponding to the distribution of F-actin in the short microvilli (Figure 2.1, 0:00). Cortical actin staining increases during the first portion of the cell cycle (Figure 2.1, 0:20, 2:50, & 4:00) and then decreases as the cell approaches cytokinesis (Figure 2.1, 2:20 & 3:40). During cytokinesis, F-actin staining appears greater in the cleavage furrow than in subfurrow or polar regions of the cortex (Figure 2.1, 2:20 & 3:40), in agreement with previous reports (Cline and Schatten, 1986; Mabuchi, 1994). In addition, the intensity of F-actin staining is greater in regions of blastomere contact, as reported previously by Wang and Taylor (1979). Changes in cortical F-actin content during the first two cell cycles was quantified by measuring the fluorescence intensity of BODIPY-phallacidin stained eggs (Figure 2.2). Unfertilized eggs show weak cortical F-actin staining. However, a dramatic increase in staining occurs after fertilization, peaking at 20 minutes post-fertilization. As development proceeds, there is a progressive decrease in the amount of cortical F-actin staining, reaching a minimum at the time of first cleavage (Figure 2.2A and 2.2B, arrows). At the completion of cell division, the amount of cortical staining increases significantly ($p < 0.005$). This same trend is

repeated at second cleavage, where cortical F-actin staining falls as the cell approaches division and rises again at the completion of cleavage (Figure 2.2B).

Dynamics of Cortical Actin Organization During Early Cleavage Divisions

Changes in the organization of cortical actin in *S. purpuratus* eggs during the first hour following fertilization are shown in Figure 2.3. By 10 minutes post-fertilization microvilli have elongated and short actin rootlets have begun to extend from the heavily stained bases of the microvilli (Figure 2.3, 0:10). These actin rootlets reach their maximum degree of development by 20 minutes post-fertilization, extending ~8 μm from the bases of the microvilli into the egg cytoplasm. Between 20 minutes and 1 hour, the number and degree of organization of rootlets decreases (Figure 2.3, 0:30 to 1:00), after which the rootlets remain essentially unchanged until first cleavage. Actin rootlets disappear during cleavage (Figure 2.4, 2:20) and reform following division, although they are less numerous and more weakly staining than those observed during the first cell cycle (Figure 2.4, 3:10). These cyclical changes in actin rootlet organization continue into the second and third cell cycles (Figure 2.4, 3:40, 4:00).

pH-Dependence of Actin Rootlet Formation

Previous studies have shown that the formation of actin bundles in elongated microvilli after fertilization is dependent on the Na^+-dependent alkalinization of the

cytoplasm (Begg *et al.*, 1982; Carron and Longo, 1982). Cytoplasmic alkalinization is achieved by Na^+/H^+ pumps in the plasma membrane, which results in a net influx of Na^+ and efflux of H^+ from the egg cytoplasm. In order to determine whether cytoplasmic alkalinization is also required for the development of actin rootlets, we parthenogenetically activated eggs with the Ca^{2+} ionophore A23187 in NaFSW to prevent cytoplasmic alkalinization (Begg *et al.*, 1982). Activation of eggs with A23187 in ASW resulted in normal rootlet formation (data not shown). Activation of eggs in NaFSW delayed the formation of microvilli, as reported previously (Begg *et al.*, 1982; Carron and Longo, 1982), and prevented the formation of actin rootlets (Figure 2.5, 0:00). Upon the addition of 40 mM NaCl, the staining of cortical actin filaments increased (Figure 2.5, 0:10), and actin rootlets formed (Figure 2.5, 0:15-0:25). The time course of actin rootlet development was 5 minutes longer than in normal development (Figure 2.5, 0:25), and rootlet organization subsequently decayed normally (Figure 2.5, 0:30). These results demonstrate that actin rootlets do not form in the absence of cytoplasmic alkalinization. However, upon an increase in cytoplasmic pH, rootlets develop over a time course similar to that of control eggs, even when the increase in cytoplasmic pH is delayed for 1 hour after activation.

Effects of Cytochalasin D on Cortical Actin Organization

The addition of 4 μg/mL cytochalasin D 2 minutes after fertilization completely inhibits the formation of actin rootlets (Figure 2.6, 0:30). This concentration of drug is

sufficient to block cytokinesis. Actin rootlets do not form during the first cell cycle when cytochalasin D is washed out after 30 minutes of incubation or longer (Figure 2.6, 2:00 and 2:30). However, rootlets do form after first cleavage in these cells (Figure 2.6, 3:00) and undergo the subsequent cycling observed in normal untreated eggs. Although rootlets did not appear in the first cell cycle, eggs were able to proceed through first cleavage after being exposed to cytochalasin D for 60, 90, or 120 minutes (Figure 2.7). The cleavage time in these eggs, however, was delayed by approximately 15 minutes, irrespective of the amount of time they were incubated in the drug. Under the culture conditions used, control eggs initiated cleavage at 125 minutes post-fertilization. Eggs held in the drug longer than 120 minutes showed progressively lower cleavage rates. Eggs in which cytochalasin treatment was reversed in time to allow first cleavage developed normally.

DISCUSSION

The results reported here illustrate the dynamic nature of cortical actin organization during early development of the sea urchin embryo and demonstrate that the amount of filamentous actin in the cortex undergoes cyclic increases and decreases as the embryo progresses through its early cleavage divisions. Cortical actin staining peaks near the beginning of the cell cycle and then gradually decreases to a minimum at cytokinesis. This result is surprising, since it has generally been assumed that the organization of the cortical cytoskeleton increases as the cell approaches cytokinesis. In addition, we find that cytokinesis can occur in embryos in which the normal developmental sequence of changes in cortical actin organization has been prevented by treatment with cytochalasin D. This result suggests that the normal sequence of changes observed in the organization of actin in the egg cortex is not involved in the establishment of the contractile apparatus for cytokinesis, but rather serves other developmental functions.

Changes in Cortical Actin Content During the Cell Cycle

The maximum cortical F-actin content occurs at 20 minutes after fertilization, corresponding to the point of greatest actin rootlet development. However, the progressive decrease in filamentous actin in the cortex during the latter portion of the cell cycle, and its subsequent increase following cytokinesis, are greater than can be

accounted for on the basis of changes in the amount of actin rootlets alone, and must therefore reflect changes in some other component of cortical F-actin as well. The observed increase in cortical F-actin following fertilization confirms previous studies using microinjection of fluorescently-labelled actin (Wang and Taylor, 1979; Hamaguchi and Mabuchi, 1988) or fluorescent phallacidin staining (Yonemura and Kinoshita, 1986; Yonemura and Mabuchi, 1987). Similarly, biochemical studies have demonstrated an increase in total actin in the sea urchin egg cortex following fertilization (Spudich and Spudich, 1979; Mabuchi *et al.*, 1980). However, these reports differ on whether the amount of cortical actin changes as the cell progresses through the cell cycle. Mabuchi *et al.* (1980) conclude that the amount of cortical actin remains constant between its increase at fertilization and cytokinesis. Spudich and Spudich (1979), on the other hand, report an increase in cortical actin by 60 minutes post-fertilization and a subsequent decrease by the time of cytokinesis. Our results demonstrate a statistically significant change in the amount of filamentous actin in the cortex during the cell cycle, with the minimum F-actin content occurring during cell division. The function of the increased level of cortical F-actin in the early part of the cell cycle is not clear, but may reflect a role for actin in various processes such as vesicle trafficking, cortical localization and surface stabilization. Disassembly of this extensive array of cortical actin filaments during cytokinesis may be required to modulate the mechanical properties of the cell surface for cell cleavage.

Actin Rootlets

Rootlet-like bundles of cortical actin filaments that extend from the bases of the microvilli undergo cycles of assembly and disassembly correlated with the early cleavage divisions of the embryo. Actin rootlets reach their maximum degree of development 20 minutes after fertilization, decrease in number and length between 20 and 60 minutes post-fertilization, and then remain essentially unchanged until first cleavage. Actin rootlets disappear during cytokinesis, but reassemble within 10 minutes after the completion of cleavage. This cyclic assembly and disassembly of actin rootlets continues through at least the third cell cycle. The rootlets observed during the first cell cycle appear to correspond to the cortical fibers described by Harris (1968) in thin section electron micrographs of fertilized *S. purpuratus* eggs.

The formation of cortical actin rootlets requires cytoplasmic alkalinization. Inhibition of cytoplasmic alkalinization at fertilization prevents the development of rootlets, but allows the formation of microvilli as well as a global increase in cortical F-actin staining. Previous studies demonstrated that fertilization of sea urchin eggs under conditions that block cytoplasmic alkalinization results in the formation of microvilli containing networks of actin filaments rather than the filament bundles normally found in microvillar cores (Begg *et al.*, 1982; Carron and Longo, 1982). Subsequent elevation of cytoplasmic pH induces the transformation of these filament networks into typical core filament bundles (Begg *et al.*, 1982; Carron and Longo, 1982). The results reported here demonstrate a similar dependence of cortical actin rootlet formation on cytoplasmic

alkalinization, suggesting that cytoplasmic pH may directly regulate actin rootlet assembly. However, since inhibition of cytoplasmic alkalinization delays the cell cycle in sea urchin eggs (Epel, 1980), the observed results may instead reflect a broader effect on the cell cycle. This second alternative seems more likely, since inhibition of rootlet assembly with cytochalasin D demonstrates the existence of a "window" during the initial 30 minutes of the first cell cycle during which rootlets can form. If rootlet assembly is inhibited with cytochalasin D beyond this point, the egg is unable to assemble rootlets until the beginning of the next cell cycle. In contrast to this result, actin rootlet formation can be inhibited for up to 1 hour by blocking cytoplasmic alkalinization. Subsequent induction of cytoplasmic alkalinization results in the assembly of cortical actin rootlets with a similar time course to that observed at fertilization.

While the function of cortical actin rootlets is not known, our results demonstrate that their formation during the first cell cycle is not required for normal development. Inhibition of actin rootlet formation by cytochalasin D does not prevent cleavage or subsequent development of the embryo following reversal of the drug. This observation suggests that either the extensive array of actin rootlets formed during the first cell cycle is not necessary for normal development, or that the function(s) carried out by these rootlets can be substituted by some alternative mechanism or by the shorter rootlets that develop in subsequent cell cycles.

A potential role for cortical actin rootlets in early development is the transport of intracellular vesicles to the egg cortex. A number of different types of vesicles migrate to the egg cortex following fertilization (Harris, 1968; Belanger and Rustad, 1972; Lee

and Epel, 1983; Wessel *et al.*, 1984; Alliegro and McClay, 1988). Alliegro and McClay (1988) have identified a group of extracellular matrix proteins which are transported to the cell surface by a cytochalasin-sensitive mechanism after fertilization. The transport and secretion of these proteins is complete within the first 30 minutes of development, a period which correlates with the maximum development of cortical actin rootlets. Similarly, Allen *et al.* (1992) have shown that pigment granules in *Arbacia punctulata* eggs migrate out to the cortex during this same time period along radially oriented paths, and that this migration is inhibited by treatment with cytochalasin B. Allen *et al.* (1992) hypothesized that the granules move on radially oriented bundles of actin filaments, and estimated their average length to be 6 μm, a value similar to the 8 μm actin rootlet length measured here.

Cortical Actin and Cell Division

Cells incubated in cytochalasin D to within 5 minutes of the onset of first cleavage are still able to undergo cytokinesis and develop normally. Since cytochalasin has been shown to disrupt cortical actin organization in sea urchin eggs (Banzhaf *et al.*, 1980; Usui and Yoneda, 1982; 1989; Cline and Schatten, 1986; Mabuchi, 1994), these results argue that the assembly of the contractile apparatus for cytokinesis does not require the maintenance of a normal cortical cytoskeleton throughout the cell cycle. However, although inhibition of cortical actin rootlet formation by cytochalasin D does not block cytokinesis, cleavage is delayed by 15 minutes, irrespective of the length of exposure to

the drug. This result suggests that cytochalasin D treatment blocks an actin-dependent event that influences the timing of the onset of cytokinesis, but is not required for cytokinesis to occur. Since embryos were exposed to cytochalasin D beginning 2 minutes after fertilization, and the same delay in cleavage onset was observed regardless of the length of exposure to the drug, I infer that the event occurs early in the cell cycle.

Studies of both sea urchin (Mitchison and Swann, 1955; Hiramoto, 1974; Yoneda, *et al.*, 1978; Schroeder, 1981b) and amphibian embryos (Sawai, 1979; Hara *et al.*, 1980) have demonstrated a dramatic increase in cortical stiffness immediately preceding the onset of cleavage, which is thought to function in the formation of the contractile ring. This increased cortical stiffness results from a global, isometric contraction of the cortex (Yoneda *et al.*, 1978; Hara *et al.*, 1980; Schroeder, 1981b), hypothesized to result from either an actomyosin-based contraction (Schroeder, 1981b; Usui and Yoneda, 1982) or an increase in actin filament cross-linking (Vacquier, 1981; Salmon, 1989). Surprisingly, however, our results demonstrate that rather than correlating with an increase in cortical actin, this cortical contraction occurs at the point of minimum cortical F-actin content, suggesting that cortical actin rootlets may act as a resistive element that is disassembled during periods of cortical contraction. Taken together, the results presented here suggest that the extensive array of cortical actin filaments that cyclically assemble and disassemble during early cleavage divisions of the sea urchin embryo do not participate in the generation of force for cortical contractions, but rather serve other developmental functions.

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Figure 2.1. Changes in cortical F-actin staining during early cleavage divisions. Time in hours and minutes after sperm addition is given in the upper right of each panel. Equatorial optical sections of BODIPY-phallacidin stained eggs viewed with confocal microscopy exhibit cyclical changes in the intensity of cortical F-actin staining. (0:00) The surface of the unfertilized egg shows weak punctate staining, corresponding to the distribution of filamentous actin in short microvilli. The unstained layer beneath the plasma membrane contains the zone of cortical granules. The diameter of unfertilized eggs routinely appears larger than that of fertilized embryos due to a greater degree of flattening during slide preparation. (0:20) Twenty minutes after fertilization the egg cortex stains intensely for filamentous actin. (2:20) During the first cleavage division, the overall intensity of cortical actin staining is decreased, but remains high in the cleavage furrow. (2:50) Following first cleavage, the intensity of cortical actin staining increases. Staining intensity is greatest at the region of blastomere contact. (3:40) Cortical staining again becomes reduced during second cleavage, with increased staining found in the cleavage furrow. (4:00) Following second cleavage, cortical actin staining again increases. Scale bar, 25 μm.

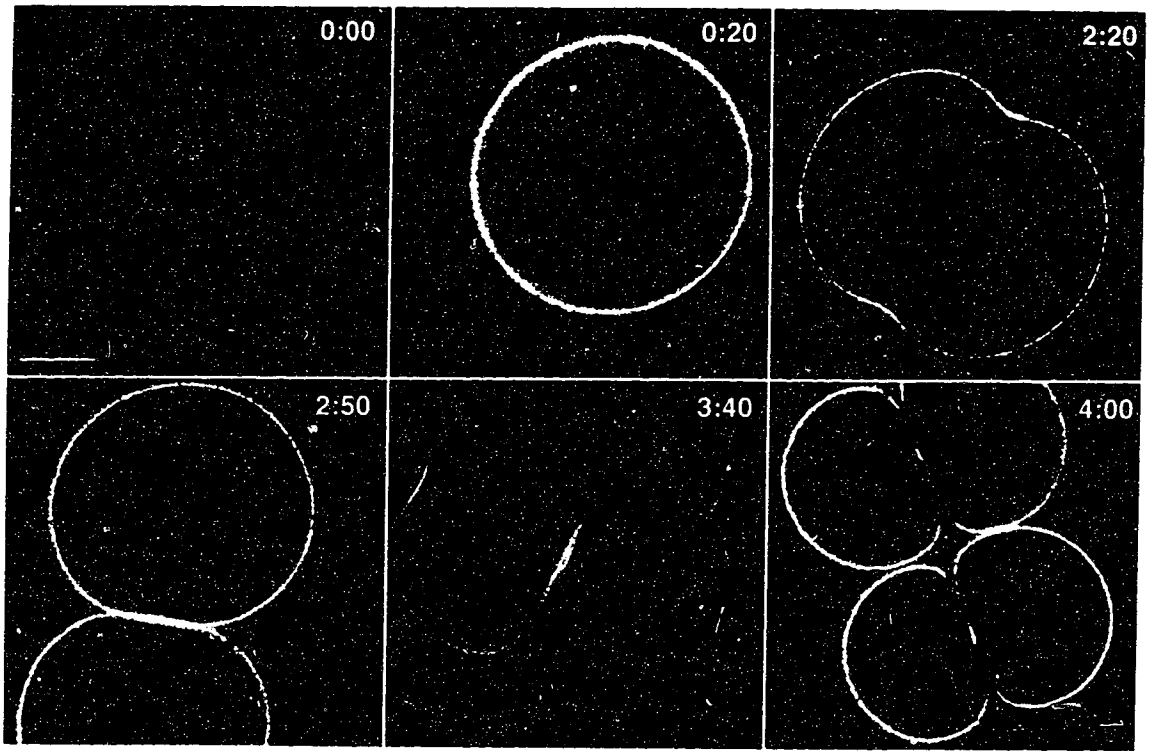
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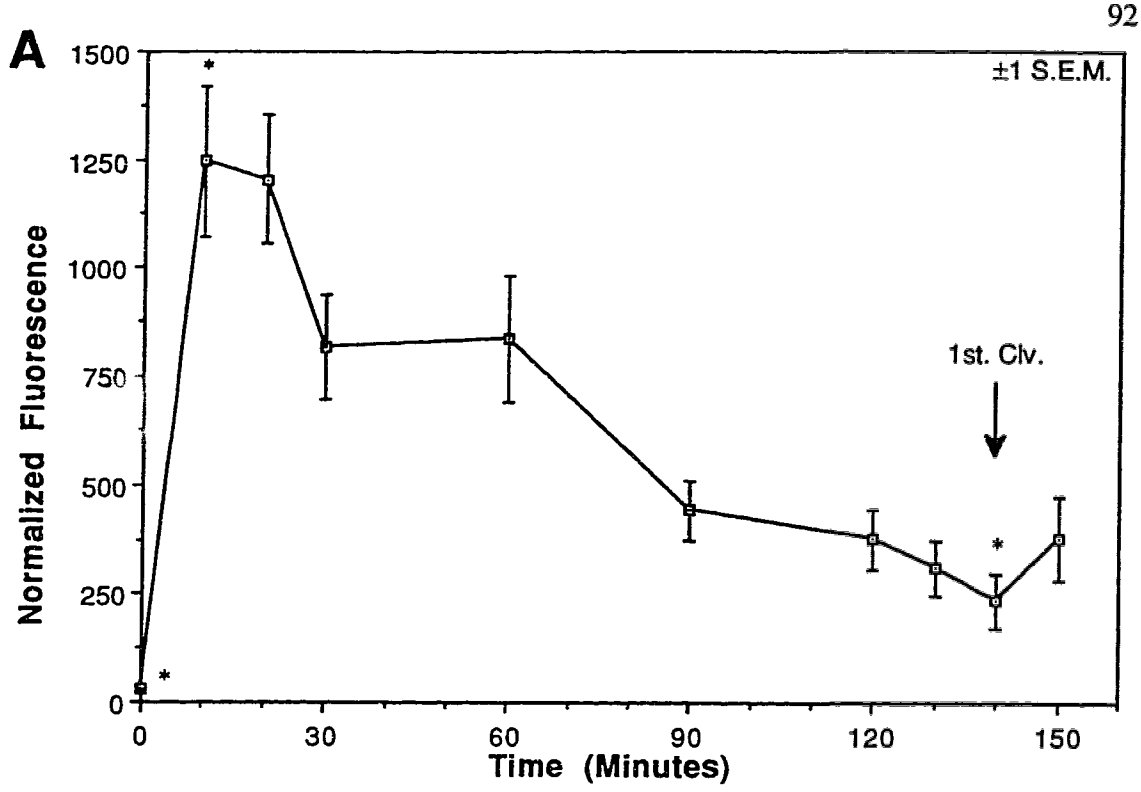
Figure 2.2. Quantification of changes in cortical fluorescence. (A) Shortly after fertilization the amount of F-actin staining increases sharply, peaking at 20 minutes. As development progresses, the amount of cortical staining decreases gradually, reaching a minimum at first cleavage (140 minutes post-fertilization). (B) In a separate experiment, cyclic changes in cortical F-actin staining can be observed. After an initial increase, cortical staining decreases as the egg approaches first cleavage (140 minutes post-fertilization) and then rises again in the second cell cycle. At second cleavage (220 minutes post-fertilization) this cycle is repeated. For each time point n = 10. Error bars = ± 1 standard error of the mean. Differences between successive time points indicated by asterisks are statistically significant ($p < 0.005$) as calculated by a paired t-test.


A
1500
1250
1000
750
500
250
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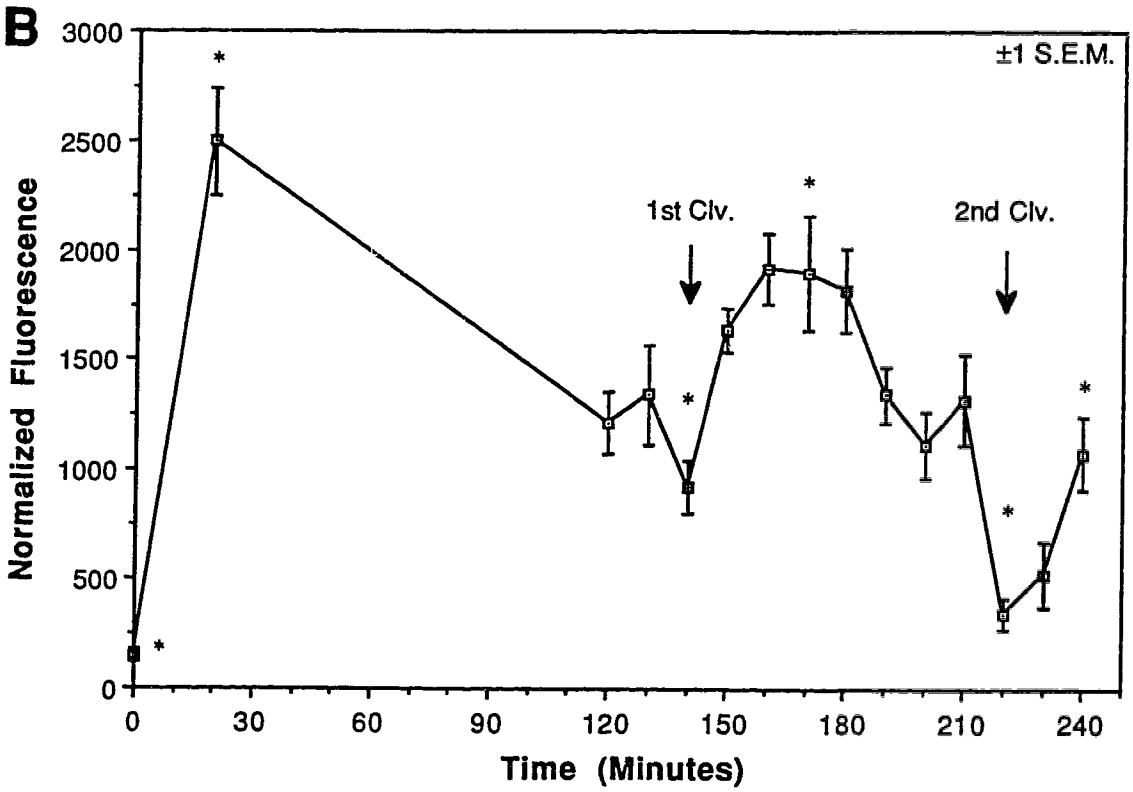
Normalized Fluorescence
±1 S.E.M.
1st. Clv.
0
30
60
90
120
150
Time (Minutes)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3000
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1st Clv.
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30
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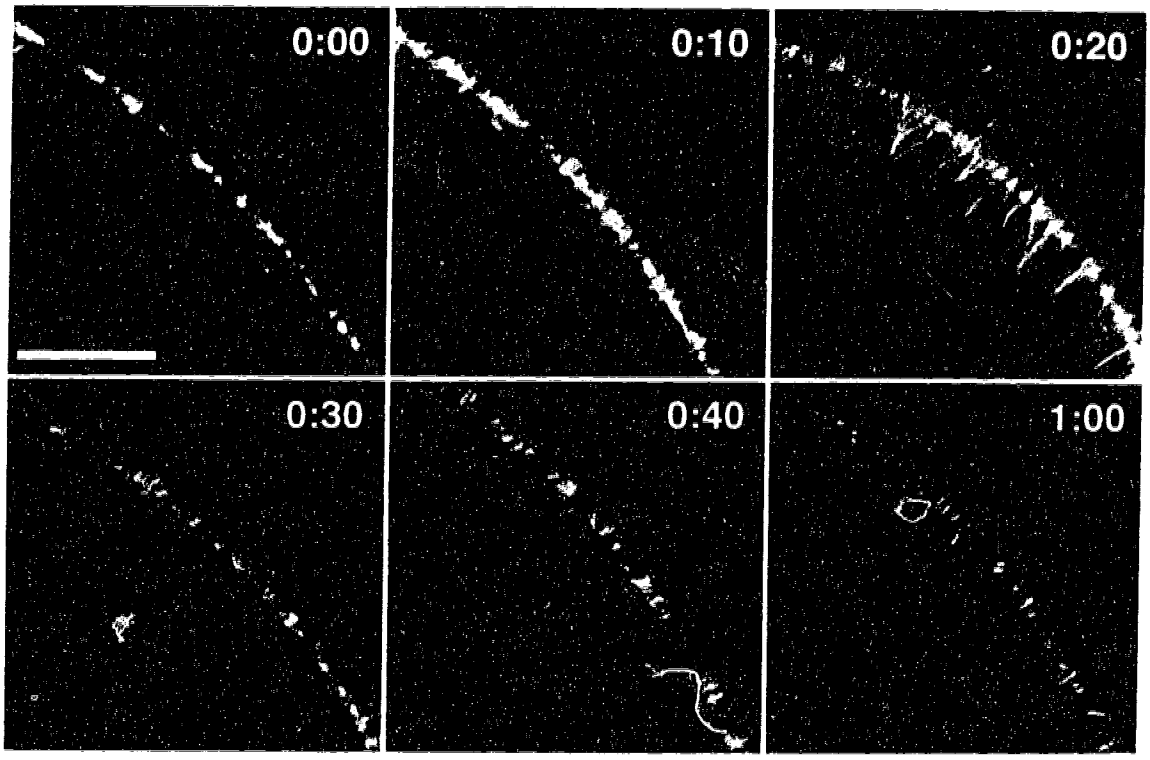
90
120
150
180
210
240
Time (Minutes)

Figure 2.3. Microvillar elongation and actin rootlet formation after fertilization. Time in hours and minutes after sperm addition is given in the upper right of each panel. The unfertilized egg (0:00) shows punctate staining of actin filaments within the short microvilli on the surface of the egg. Shortly after fertilization, microvilli elongate, and the beginnings of actin rootlet formation can be observed (0:10). By 20 minutes post-fertilization, actin rootlets extend radially from the bases of the microvilli (0:20). Rootlets reach their maximum length of about 8 μm and their maximum fluorescent staining intensity at this time. After reaching this peak, the actin rootlets begin to disassemble, becoming less numerous and more disorganized (0:30). This trend continues (0:40) until about 1 hour post-fertilization, when only a few rootlets remain (1:00). This organizational state is maintained until shortly before first cleavage. Scale bar, 10 μm.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1:00

Figure 2.4. Disappearance and reappearance of actin rootlets during cleavage. Time in hours and minutes after sperm addition is given in the upper right of each panel in the top row. A higher magnification view of each panel is shown in the lower row. Actin rootlets disappear within 10 minutes of first division (2:20). Following division, actin rootlets reassemble with an organizational state similar to that observed before first cleavage (3:10). Actin rootlets again disappear during second division (3:40) and reappear following the completion of cleavage (4:00). Scale bars, 10 μm.

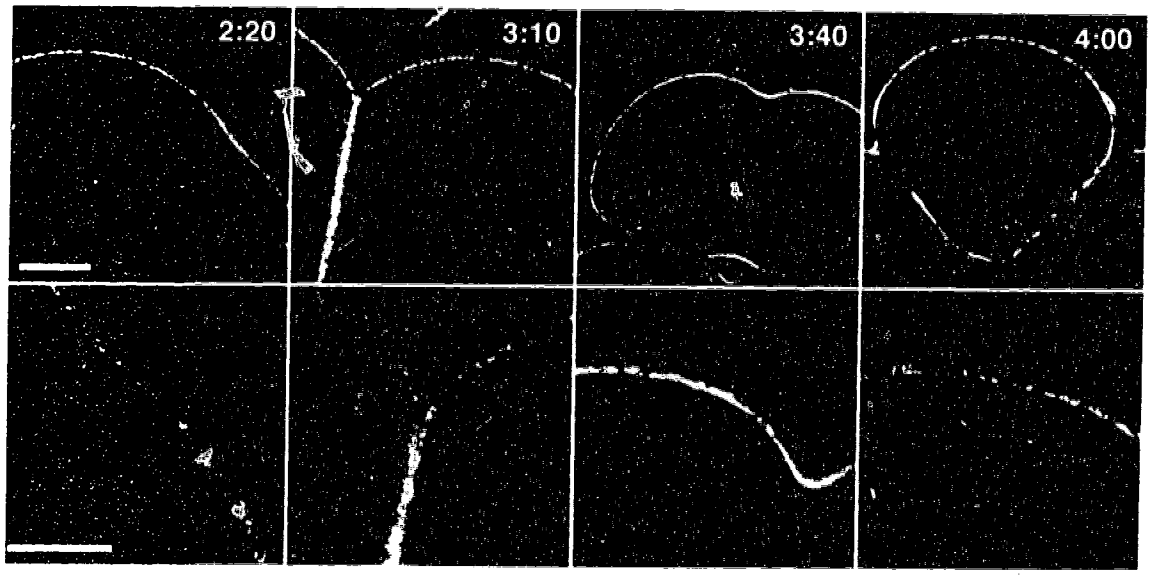
2:20
3:10
3:40
4:00

Figure 2.5. pH-dependence of actin rootlet formation. Eggs were activated in NaFSW with A23187 and incubated for 1 hour before the readdition of 40 mM NaCl. The time in hours and minutes after readdition of NaCl is given in the upper right corner of each panel. One hour after activation, eggs in NaFSW show elongated microvilli stained for F-actin, but no actin rootlets (0:00). Shortly after the readdition of NaCl, cortical fluorescence increases (0:10) before actin rootlets begin to form (0:15). Rootlets continue to elongate (0:20), reaching their maximum length by 25 minutes post-fertilization (0:25). The rootlets are not as highly organized as in normal development. By 30 minutes post-fertilization, the actin rootlets are in the process of disassembling and are reduced in number (0:30). Scale bar, 10 μm.

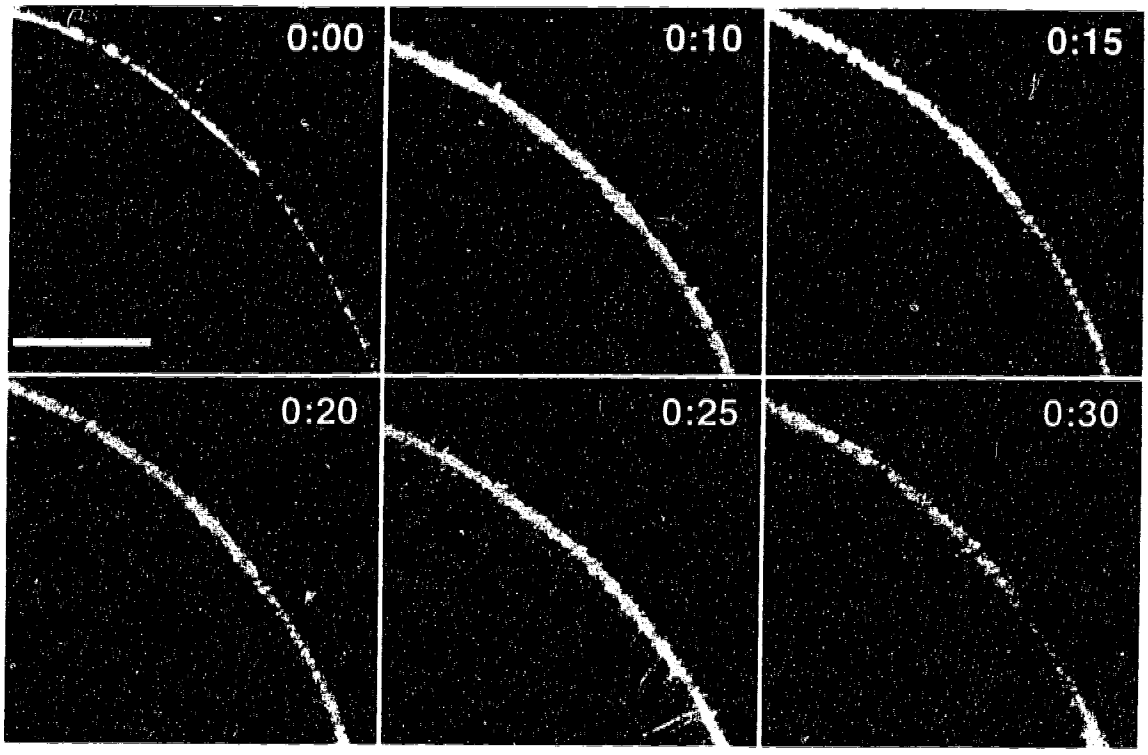
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Figure 2.6. Effects of cytochalasin D on actin rootlet formation. Time in hours and minutes after sperm addition is given in the upper right of each panel in the top row. A higher magnification view of each panel is shown in the lower row. The addition of 4 μg/mL cytochalasin D 2 minutes after fertilization prevents the formation of actin rootlets (0:30). After 1 hour of incubation in cytochalasin D, eggs were washed 3 times in ASW. Although actin rootlets do not develop following drug washout (2:00), eggs cleave (2:30) and form actin rootlets typical of the second cell cycle following cytokinesis (3:00). Scale bars, 20 μm top row, 10 μm bottom row.

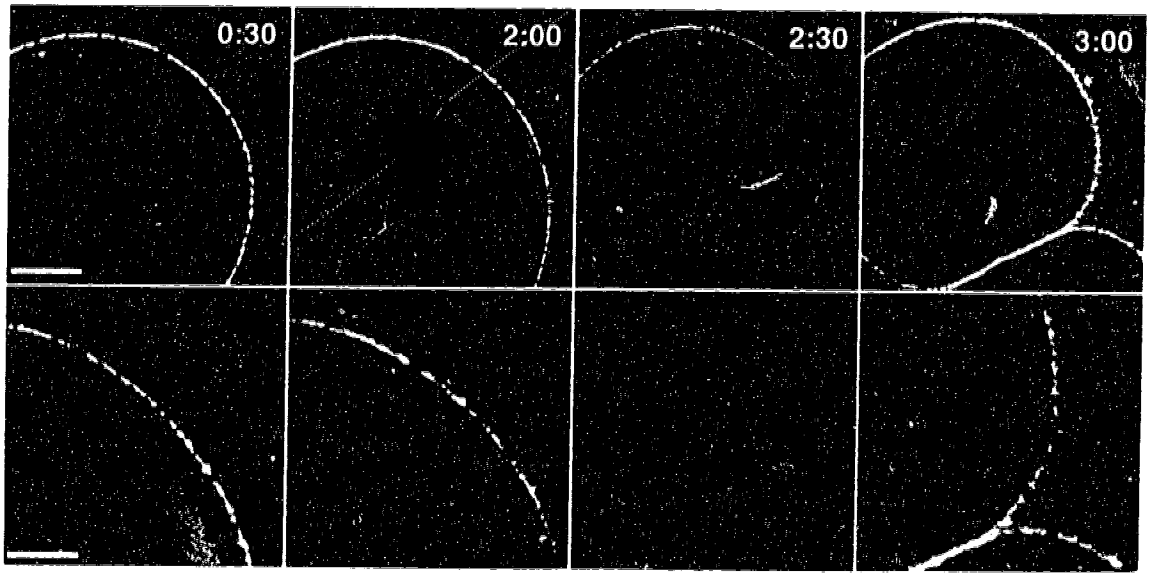
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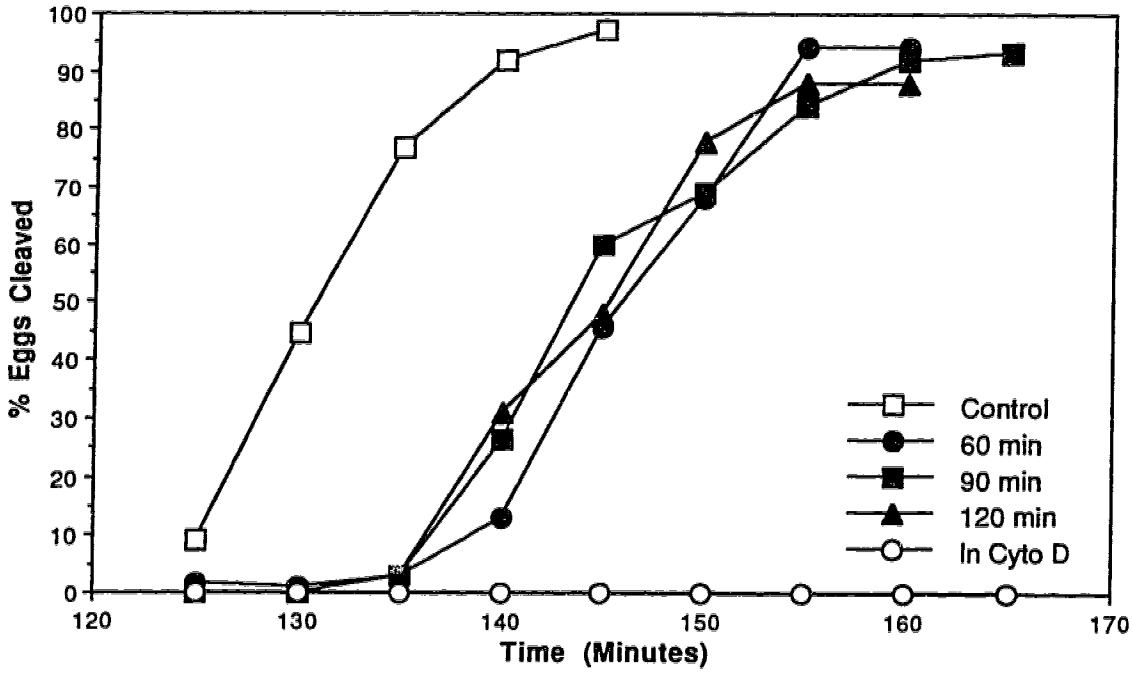
Figure 2.7. Washout of cytochalasin D permits eggs to divide. At 2 minutes post-fertilization, 4 μg/mL of cytochalasin D is added to eggs and incubated for 60, 90, or 120 minutes before being removed by three washings in ASW. Controls begin to cleave at 125 minutes after insemination. Eggs treated with cytochalasin D for various lengths of time divide 15 minutes later than controls, but with the same kinetics. Continuous incubation in this concentration of drug completely inhibits cytokinesis.


% Eggs Cleaved
100
90
80
70
60
50
40
30
20
10
0
120
130
140
150
160
170
Time (Minutes)
Control
60 min
90 min
120 min
In Cyto D

Chapter 3

Alteration of Cell Cycle Time and Induction of Surface Instability in Starfish Blastomeres Microinjected with Antibodies to Spectrin[1]

INTRODUCTION

Spectrin, the major structural component of the erythrocyte membrane skeleton, has been identified as a ubiquitous component of eukaryotic cells (Goodman *et al.*, 1988; Bennett, 1985; 1990; Bennett and Gilligan, 1993). The spectrin molecule is a tetramer composed of two heterodimers of α and β subunits linked head-to head, with an actin binding site at each end (Marchesi, 1985; Goodman *et al.*, 1988; Bennett, 1989). Spectrin tetramers both cross-link actin filaments into networks and bind to a number of integral membrane proteins, either directly or indirectly through ankyrin (Goodman *et al.*, 1988; Coleman *et al.*, 1989; Morrow, 1989; Bennett, 1990). This spectrin-mediated linkage of integral membrane proteins to the actin cytoskeleton functions in stabilizing the plasma membrane, establishing specialized subdomains of the membrane, and regulating cytoskeletal-membrane interactions (Goodman *et al.*, 1988; Coleman *et al.*, 1989). Spectrin also undergoes redistribution from cytoplasmic pools to sites of active membrane dynamics during activation of lymphocytes (Lee *et al.*, 1988), stimulation of actin secretion by gastric parietal cells (Mercier *et al.*, 1989) and cell-cell contact leading

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[1]A version of this chapter has been submitted for publication. Wong, G.K., Hoyle, D.H.R, and Begg, D.A. 1996. *Dev. Biol.*

to the formation of intercellular junctions (Nelson and Veshnock, 1987). In addition, spectrin undergoes phosphorylation-dependent cycles of redistribution between the actin cytoskeleton and soluble cytoplasmic pools during mitosis in tissue culture cells (Fowler and Adam, 1992), suggesting that it participates in the reorganization of the actin cytoskeleton associated with cell division.

Spectrin undergoes extensive redistribution during oogenesis and embryogenesis in the mouse (Sobel and Alliegro, 1985; Reima and Lehtonen, 1985; Damjanov *et al.*, 1986; Schatten *et al.*, 1986), *Xenopus* (Giebelhaus *et al.*, 1987; 1988), *Drosophila* (Pesacreta *et al.*, 1989) and sea urchin (Schatten *et al.*, 1986; Fishkind *et al.*, 1990a, b). Embryos contain a maternal pool of spectrin that redistributes from the cytoplasm to newly formed plasma membrane during cellularization of the embryo (Sobel and Alliegro, 1985; Reima and Lehtonen, 1985; Giebelhaus *et al.*, 1987; Pesacreta *et al.*, 1989; Fishkind *et al.*, 1990b). Zygotic expression of spectrin does not appear to occur until the mid-blastula transition (Giebelhaus *et al.*, 1987; Wessel and Chen, 1993), except in the mouse, where spectrin transcription has been reported to begin at fertilization (Sobel and Goldstein, 1988). *Drosophila* embryos containing mutations in the α-spectrin gene are able to develop to the first or second larval instar, presumably by utilizing this maternal pool of spectrin, but exhibit loss of epithelial cell-cell interactions and cell substrate interactions at the larval stage (Lee *et al.*, 1993). Mutations in *Drosophila* spectrin that disrupt head-end interchain binding prevent actin network formation and inhibit oogenesis and larval development, even though the mutant spectrin remains stably associated with membranes (Deng *et al.*, 1995), indicating that the cross-linking of actin

into networks is required for development. Because of the large maternal pool of spectrin, however, genetic approaches have not been able to address the role of spectrin during the early cleavage divisions that lead to the cellularization of the embryo.

We previously purified spectrin from sea urchin eggs (Fishkind *et al.*, 1987) and demonstrated its distribution on the plasma membrane and the membranes of cortical granules, acidic vesicles, and yolk platelets (Bonder *et al.*, 1989; Fishkind *et al.*, 1990a). Exocytosis of the cortical granules at fertilization causes a redistribution of spectrin from the cortical granule membranes to the plasma membrane and to bundles of actin filaments that develop within microvilli following fertilization (Fishkind *et al.*, 1990a, b). This redistribution suggests that spectrin functions in the stabilization of the plasma membrane and the assembly of the cortical actin cytoskeleton. Within 20 minutes after fertilization, spectrin-coated acidic vesicles migrate to the cortex where they become anchored and remain throughout development (Fishkind *et al.*, 1990b). The function of these acidic vesicles is not known. The amount of spectrin associated with yolk platelet membranes gradually decreases during embryogenesis, suggesting that it functions as the major pool of spectrin that redistributes to the new plasma membrane generated during the cellularization of the embryo (Fishkind *et al.*, 1990b).

The potential role of spectrin in cytokinesis is unclear. Caco-2 cells that express truncated β-spectrin subunits become multinucleated (Hu *et al.*, 1995), suggesting that spectrin is required for normal cytokinesis. Spectrin has been reported to accumulate in the cleavage furrows of dividing mouse blastomeres (Schatten *et al.*, 1986) and the furrow canals of *Drosophila* embryos (Pesacreta *et al.*, 1989). While spectrin has been

identified in isolated cleavage furrows from sea urchin eggs by two-dimensional SDS-PAGE (Yonemura *et al.*, 1990), immunofluorescent localization studies do not reveal a significant accumulation in the cleavage furrow compared with other regions of the egg surface (Schatten *et al.*, 1986; Fishkind *et al.*, 1990b).

To investigate the function of spectrin during early cleavage divisions, we microinjected affinity-purified antibodies against sea urchin egg spectrin into developing starfish embryos to inactivate the pool of maternal spectrin. The results of these studies implicate spectrin in the regulation of cytoskeletal-membrane interactions that stabilize the cell surface, participate in the completion of cytokinesis, and mediate cell-cell interactions during the formation of the blastula epithelium. Unexpectedly, the results also demonstrate a dose-dependent increase in cell cycle transit time, suggesting that spectrin functions in the regulation of cell cycle timing.

MATERIAL AND METHODS

Materials

Patiria miniata starfish were obtained from Marinus Inc. (Long Beach, CA) and Westwind Sea Labs, Inc. (Victoria, B.C., Canada). Artificial sea water (ASW) was prepared from Tropic Marin® Sea Salt (Wartenberg, Germany). For the immunoblotting protocol, nitrocellulose was obtained from Schleicher & Schuell (Keene, NH), Carnation Instant Skim Milk Powder from Nestle (Don Mills, ON, Canada), goat-anti-rabbit IgG conjugated to alkaline phosphatase from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA), and 50 mg/mL nitro blue tetrazolium (NBT) and 50 mg/mL bromochloroindolyl phosphate (BCIP) stock solutions from Fisher/Promega (Madison, WI). Ponceau S Red solution, Protein A Sepharose beads, cytochalasin D, HEPES, 1-methyladenine, Triton X-100, and the proteolytic inhibitors aprotinin, leupeptin, and phenylmethylsulfonyl fluoride (PMSF), were obtained from Sigma Chemical Company (St. Louis, MO). DEAE ion-exchange resin was purchased from Whatman (Maidstone, England). CNBr-Activated Sepharose 4B was obtained from Pharmacia Biotech (Uppsala, Sweden). An Affi-Prep Protein A MAPS II Kit and acrylamide were purchased from Bio-Rad Laboratories (Hercules, CA). Papain was purchased from Boehringer-Mannheim GmbH (Mannheim, Germany). BODIPY-phallacidin and the DNA-binding dye 4,6-diamidino-2-phenylindole (DAPI) were obtained from Molecular Probes, Inc. (Eugene, OR). All other chemicals were of analytical reagent grade or better.

Collection and Handling of Gametes

A small portion of ovary or testis was removed from the arm of a starfish through a slit made at the junction between two arms. Testes were stored "dry" in a Petri dish at 4°C. Ovaries were placed in a finger bowl containing 50 mL of ASW at 14°C. To initiate the release and maturation of oocytes, 1-methyladenine was added to a concentration of 10 μM for 1.5 hours (Chaet, 1960; Kanatani, 1969). Mature oocytes were washed 3 times in ASW, fertilized and processed for microinjection as described below.

Preparation of Antibodies

The immunoglobulin fraction of pre-immune rabbit serum was precipitated with ammonium sulfate (Harlow and Lane, 1988), resuspended in 5 mM sodium phosphate-buffer with 0.02% NaN_3 at pH 7.4 and dialyzed overnight at 4°C against three changes of the same buffer. IgGs were further purified using DEAE ion-exchange resin following the batch protocol of Harlow and Lane (1988) and vacuum concentrated/dialyzed against microinjection buffer (Zavortink *et al.*, 1983) to a final concentration of 24 mg/mL.

The production of rabbit polyclonal antibodies against sea urchin egg spectrin is described elsewhere (Bonder *et al.*, 1989). Polyclonal antibodies to sea urchin egg spectrin were affinity-purified against human erythrocyte spectrin, isolated by the method of Cohen and Foley (1984) and coupled to CN-Br Activated Sepharose 4B beads, as

described previously (Bonder *et al.*, 1989). Affinity-purified antibodies were vacuum concentrated/dialyzed against microinjection buffer to a final concentration of 8 mg/mL for microinjection.

Preparation of F_{ab} Fragments

IgGs were purified from pre-immune rabbit serum using an Affi-Gel Protein A MAPS II Kit according to the manufacturer's instructions. Isolated IgGs were vacuum concentrated/dialyzed against 100 mM sodium acetate at pH 5.5 to 5 mg/mL and digested overnight with 10 mg of papain/mg of IgG (Harlow and Lane, 1988). F_{ab} fragments were recovered using the Affi-Gel Protein A MAPS II Kit and vacuum concentrated/dialyzed against microinjection buffer to a final concentration of 9.8 mg/mL for microinjection.

F_{ab} fragments from immune rabbit serum were prepared by the same procedure as above and affinity-purified as described for whole antibodies to generate monospecific antibody fragments. The affinity-purified F_{ab} fragments were vacuum concentrated/dialyzed against microinjection buffer to a concentration of 2.3 mg/mL, and then further concentrated to 3.3 mg/mL using a Centricon-10 microconcentrator (Amicon Division, W.R. Grace & Co., Beverly, MA).

Immunoprecipitation of Native Spectrin

Oocytes were washed once in wash buffer (0.9 M glycerol, 100 mM HEPES, 5 mM EGTA, pH 7.5) at 14°C and resuspended in 10 volumes of homogenization buffer (wash buffer with 1 mM ATP, 1 mM DTT, 0.5 mM PMSF, 50 μg/mL aprotinin, 20 μg/mL leupeptin, 0.02% NaN_3, pH 7.5) containing 4 μg/mL cytochalasin D to inhibit actin polymerization. Oocytes were homogenized in a Dounce homogenizer on ice using 10 passes with a tight B pestle, and the homogenate was centrifuged at 250,000 g for 1 hour at 4°C. The clear supernatant was transferred to a 15 mL centrifuge tube, incubated overnight at 4°C to allow the formation of a precipitate, and then centrifuged at 10,000 g to recover the supernatant. If this step was omitted, a non-specific precipitate formed during subsequent incubations.

A 10% v/v suspension of Protein A Sepharose beads was prepared in homogenization buffer. 2.5 mL of the recovered supernatant was placed in each of three 15 mL centrifuge tubes with 50 μL of the bead suspension alone, or 50 μL of the bead suspension and either 80 μL of pre-immune or immune rabbit serum. The tubes were tumbled at 4°C for 4 hours, and the beads collected by centrifuging at maximum speed in an IEC Clinical Centrifuge (International Equipment Company, Needham Hts., MA) for 10 minutes. The beads were resuspended in 1 mL of homogenization buffer containing 0.5 M NaCl, transferred to 1.5 mL microcentrifuge tubes, and incubated on ice for 30 minutes to disrupt any non-specific binding. The beads were recovered by centrifuging at 10,000 rpm in a benchtop microcentrifuge for 1 minute, washed three

times in homogenization buffer, and boiled for 5 minutes in 50 μL of SDS-PAGE sample buffer (Laemmli, 1970).

Electrophoresis and Immunoblotting

SDS-PAGE was performed using 5-15% gradient mini-gels, made from a 30%:0.8% acrylamide:bis-acrylamide stock solution, and transferred to nitrocellulose in transfer buffer (25 mM Tris-base, 192 mM glycine, 20% methanol, 0.01% SDS) with a Genie Blotter apparatus (Idea Scientific, Corvallis, OR) at 0.5 A for 3 hours at 4°C. Nitrocellulose sheets were stained with ponceau S red as described by Harlow and Lane (1988) to visualize proteins, and incubated in blocking buffer (5% skim milk powder, 0.01% antifoam A, 0.0001% thimerosal, 100 mM boric acid, 25 mM sodium borate, 75 mM NaCl, pH 7.4) at 4°C overnight on a rocking platform. Pre-immune and affinity-purified antibodies were diluted to 5 μg/mL and incubated with the nitrocellulose sheets as described by Fishkind *et al.* (1990a). Antibodies were detected using the procedure described by Harlow and Lane (1988) for alkaline phosphatase.

Microinjection

Oocytes were fertilized and allowed to develop at 14°C to the two-cell stage (~3.5 hours) before being placed in a microinjection chamber (Kiehart, 1982). One blastomere of a two-cell stage embryo was microinjected using the method of Kiehart

(1982), leaving the other uninjected sister cell to serve as a control. A small drop of Wesson Oil (Hunt-Wesson Foods, Inc., Fullerton, CA) was coinjected with the antibody solution to mark the injected cell (Kiehart, 1982). Injected embryos were followed through hatching using differential-interference contrast (DIC) optics on a Zeiss microscope with a 16x objective lens (NA 0.35) or a Reichert Polyvar 2 microscope with a 25x objective lens (NA 0.55) or a 40x objective lens (NA 1.00). Video records were collected using a Dage-MTI Newvicon NC-67M video camera (Michigan City, IN) and a JVC BR-9000U time-lapse video recorder (Victor Company of Japan, Ltd., Japan). Still images were captured from the video tape records by a Power Macintosh 6100/66AV computer (Apple Computer, Inc., Cupertino, CA), and figures composed with Adobe Photoshop 3.0.1 (Adobe Systems, Inc., Mountain View, CA). The figures were printed on a Codonics NP-1600 Photographic Network Printer (Middleburg Hts., OH) with a grayscale cartridge.

Fluorescent Nuclear Staining

Embryos were microinjected as described above, allowed to develop for 2, 6, or 10 hours, and fixed (3% formaldehyde, 0.20 M Millonig's phosphate buffer, 0.136 M NaCl, pH 7.0; Cloney and Florey, 1968) overnight at 4°C. All subsequent steps were carried out at room temperature. Embryos were permeabilized in fixative containing 0.1% Triton X-100 for 30 minutes, and washed three times in PBS before being stained with 1 μg/mL DAPI in PBS for 30 minutes. Embryos were washed three times in PBS

to remove excess fluorescent stain, and mounted in an anti-photobleaching medium (2 mM n-propyl gallate, 60% glycerol, 50 mM Tris-base, 150 mM NaCl, pH 7.5) on glass slides. Cover slips were sealed to the slide with nail polish. Embryos were observed by DIC and fluorescence microscopy on a Reichert Polyvar 2 microscope using a 40x objective (NA 1.00). Photomicrographs were taken on Ilford FP4 Plus film and developed with full-strength Kodak D-76 as recommended by the film manufacturer.

Fluorescent Actin Staining

Embryos were microinjected and allowed to develop for approximately 14 hours before being fixed, permeabilized, and washed as described above. Embryos were stained with 2 U/mL BODIPY-phallacidin in PBS for 30 minutes, followed by three washes in PBS to remove excess phallacidin. The stained embryos were mounted in anti-photobleaching medium, as described above, and observed using a Leica Confocal Laser Scanning Microscope based on a Leica Aristoplan Microscope. Images were collected with a 63x PL APO objective (NA 1.4) using 64 scans at a resolution of 512 x 512 pixels and transferred to a Power Macintosh 6100/66AV. Figures were composed using Adobe Photoshop 3.0.1 and printed on a Codonics NP-1600 Photographic Network Printer with a grayscale cartridge.

RESULTS

Antibody Specificity

Immunoblots of affinity-purified anti-sea urchin egg spectrin antibodies against whole starfish eggs showed monospecific staining of a closely spaced doublet with apparent molecular weights of 242-245 kDa (Figure 3.1 a, lane 2). The antibody recognized both the 240 and 220 kDa α- and β-subunits of human erythrocyte spectrin (Figure 3.1 a, lane 3). Pre-immune IgG showed no staining of either starfish eggs or human erythrocyte spectrin (Figure 3.1 a, lanes 4 and 5). Immunoblots using immune and pre-immune F_{ab} fragments gave identical results (data not shown). To determine the specificity of anti-spectrin antibody for native spectrin in *Patiria* eggs, immunoprecipitation of native spectrin from egg homogenates was performed using immune rabbit serum. Figure 3.1 b is an immunoblot which demonstrates that immune serum immunoprecipitated native spectrin from starfish egg homogenates.

Microinjection of Antibodies Against Spectrin

Microinjection of 0.2-1.0 ng of pre-immune antibodies showed no detectable effect on normal development in a total of 15 embryos (Figure 3.2 a-d). Both injected and uninjected blastomeres divided synchronously with identical cell cycle times (Figure 3.5 a), measured as the interval between cleavage furrow initiations, and participated in

the formation of a normal blastula (Figure 3.2 d). Injections of 0.3-2.3 ng of pre-immune F_{ab} fragments (33 embryos) produced identical results (data not shown).

Injection of 0.2 ng of affinity-purified anti-sea urchin egg spectrin antibody disrupted normal blastula formation (Figure 3.2 e-h). Progeny of the injected blastomere produced a loose aggregate of cells instead of a blastula epithelium (Figure 3.2 h). The injected cells eventually stopped dividing, exhibited increased surface activity, extended pseudopodia, and became motile. Injected blastomeres exhibited a progressive increase in the length of the cell cycle compared to their uninjected sister blastomeres (Figure 3.5 b), causing the injected cells to fall behind one cell cycle by 4 hours after injection. Effects on the cell cycle timing were dose-dependent, with higher antibody doses producing progressively greater cell cycle delays. Injection of 0.5 ng of antibody caused a greater increase in the length of the cell cycle. Control blastomeres showed a consistent average cell cycle time of 54 minutes, while injected blastomeres started with a cell cycle time of 1 hour that gradually increased to 1.5 hours by the eighth cell cycle (Figure 3.5 c). These data are representative of 9 embryos followed through the blastula stage.

Injection of 0.8 ng of anti-spectrin antibody caused a dramatic alteration in cell cycle timing (Figure 3.5 d). Injected blastomere completed the second cell cycle in 2.5 hours and the third cycle in 4.5 hours, while the control cells maintained an average cell cycle time of 48 minutes. Many cells initiated a cleavage furrow that subsequently regressed (Figure 3.3 b-d). When the injected blastomeres stopped dividing, they became motile and produced numerous surface blebs that eventually led to cell

fragmentation (Figure 3.3 f). Similar results were observed in 17 other injected embryos followed through development.

In order to determine whether the effects described above might be due to a side effect of spectrin immunoprecipitation by the intact antibody, we microinjected F_{ab} fragments. The injection of 0.1-0.4 ng of immune F_{ab} fragments into blastomeres produced cleavage delays and morphological changes similar to those observed in whole antibody-injected cells. The injection of higher doses of immune F_{ab} fragments, however, had an even more pronounced effect on development. Microinjection of 0.6 ng of immune F_{ab} caused blastomeres to arrest at the second or third cell cycle (two- or four-cell stage) (Figure 3.4) for extended periods of time before resuming division. The majority of arrested blastomeres exhibited extreme alterations in their morphology, including distortion of cell shape, the clumping of granular cytoplasm, and the formation of clear areas within the cytoplasm in which small particles exhibited Brownian motion (Figure 3.4 b and c). After a variable period of time, these cells regained a normal appearance and resumed division (Figure 3.4 d and e). The progeny of these cells showed similar morphologies to blastomeres injected with 0.8 ng of IgG. These included failure to complete cytokinesis, the cessation of cell division, and increased surface blebbing which led to cell fragmentation (Figure 3.4 f). An example of this cell cycle arrest is illustrated in Figure 3.5 e, where the injected embryo remained in the second cell cycle for over 9 hours before resuming cell divisions at average intervals of 1.4 hours, compared to 37 minutes for normal cells at the same developmental stage. The measured cell cycle time of 15 embryos showed that injected blastomeres arrested for

between 3-12 hours at the second or third cell cycle before resuming division with extended cell cycle times. Similar results were obtained in 62 other embryos followed through development.

To determine which portion of the cell cycle was affected by the microinjection of anti-spectrin antibodies, the mitotic time was measured as the interval between nuclear envelope breakdown and cleavage furrow initiation. No differences were observed in the mitotic times of injected blastomeres and uninjected controls (Figure 3.5 f), indicating that the non-mitotic portion of the cell cycle was extended.

Nuclear Morphology

Blastomeres injected with high doses of IgGs contained larger nuclei than control cells at the same developmental stage (Figure 3.4 e and Figure 3.6 a-c). In order to examine whether nuclear enlargement reflected an increase in the chromatin content, embryos were stained with the DNA specific dye DAPI. No detectable differences in DAPI fluorescent staining were observed between injected and control blastomeres at similar developmental stages (Figure 3.6 d-f), indicating that nuclear enlargement was not the result of endoreplication. Chromatin strands were uniformly dispersed in the nuclei of control cells. In the injected blastomeres, various chromatin distribution patterns were observed. These ranged from dispersed dots and strands within enlarged nuclei, to a very condensed single bright spot without any discernible structure (Figure 3.6 e and f).

F_{ab}-injected blastomeres that exhibited cell cycle arrest did not reform normal nuclei until division resumed (Figure 3.7 a and c) . These injected cells frequently contained a clear area, devoid of any discernible nuclear envelope, occupying the expected location of the nucleus. Staining with DAPI revealed numerous small fluorescent dots (Figure 3.7 b and d), either dispersed or grouped together to form the clear area. These dots may represent karyomeres, individual chromosomal vesicles (Ito *et al.*, 1981; Montag *et al.*, 1988) that failed to fuse and form a normal nucleus. A range of stages in the fusion of these fluorescent spots and partial assembly of nuclei was also observed.

Effects of Anti-Spectrin Antibodies on the Actin Cytoskeleton

The increased surface activity of injected cells and failure to complete cytokinesis suggest that the anti-spectrin antibodies alter normal cortical actin cytoskeleton-membrane interactions. To determine what effect spectrin antibodies had on the actin cytoskeleton, injected embryos were fixed and stained with BODIPY-phallacidin and examined with confocal scanning laser microscopy. Control embryos at the two-cell stage showed a similar accumulation of filamentous actin in the cell cortex and microvilli (Figure 3.8 a) to what has been previously observed in sea urchin eggs (Hamaguchi and Mabuchi, 1988; Bonder *et al.*, 1988; Wong *et al.*, submitted). As development progressed, the microvilli became less prominent, and cortical F-actin staining increased dramatically, especially in areas of cell-cell contact (Figure 3.8 b).

Blastomeres injected with 0.1-0.2 ng of F_{ab} fragments exhibited dramatic changes in the distribution of filamentous actin. At early times after injection, microvillar F-actin staining was absent, small surface blebs appeared, and cytoplasmic fluorescence was more diffuse compared to controls at the same stage (Figure 3.8 c). The amount of F-actin in the cortex decreased as the injected blastomeres progressed through development (Figure 3.8 d and e). In cells that began exhibiting surface protrusions, filamentous actin was concentrated within the blebs (Figure 3.8 f).

DISCUSSION

The results reported here implicate spectrin in regulating cytoskeletal-membrane interactions during early development that stabilize the cell surface and mediate cell-cell interactions required for the formation of the blastula epithelium. They also suggest that spectrin is not required for the formation of the cleavage furrow, but functions in stabilizing the furrow during the completion of cytokinesis. Surprisingly, however, the results demonstrate that the microinjection of anti-spectrin antibodies causes a dose-dependent increase in the length of the cell cycle, suggesting that spectrin functions in regulating the timing of the cell cycle.

Spectrin and the Cell Cycle

The microinjection of anti-spectrin antibody may alter cell cycle timing either directly, by some as yet unidentified molecular mechanism, or indirectly, by disrupting the organization of the actin cytoskeleton. Actin cytoskeletal organization may be monitored as part of a mechanism which regulates the rate of progression through the cell cycle, with the increase in the length of the cell cycle being proportional to the degree of cytoskeletal disruption. Such a mechanism is consistent with the observation that the increase in cell cycle length is proportional to antibody dose, and that antibody microinjection causes a reduction in the amount and organization of filamentous actin in the cortex of injected blastomeres. It has previously been demonstrated that spindle

assembly functions as a cell cycle checkpoint (Murray and Hunt, 1993; Murray, 1994), and that reduction in the quantity of spindle microtubules or changes in their spatial organization increases the length of the mitotic phase of the cell cycle in sea urchin blastomeres (Sluder, 1979; Sluder and Begg, 1983). Similarly, a decrease in the amount of cortical actin or disruption of its normal organization may delay or block cell cycle progression.

Previous studies have demonstrated that alteration of actin cytoskeletal organization causes cell cycle arrest in yeast and tissue culture cells. In the fission yeast *S. pombe*, mutations in the tropomyosin or profilin gene block cytokinesis and cause cell cycle arrest (Balasubramanian *et al.*, 1992; 1994). In addition, mutations in *rae1*, a *r*ibonucleic *a*cid *e*xport defect in *Schizosaccharomyces pombe*, disrupt both the actin and tubulin cytoskeleton and arrest the cell cycle (Brown *et al.*, 1995). Treatment of tissue culture cells with cytochalasin D or latrunculin, a compound produced by the Red Sea sponge, disrupts the actin cytoskeleton and blocks S phase (Takasuka *et al.*, 1987; Iwig *et al.*, 1995; Böhmer *et al.*, 1996), presumably by preventing progression through the checkpoint known as START (Murray and Kirschner, 1989; Murray and Hunt, 1993). Although embryonic cells exhibit fewer checkpoints than somatic cells (Hartwell and Weinert, 1989; Whitaker and Patel, 1990) due to the suppression of G_1 and G_2 (Murray and Hunt, 1994), key checkpoints including START and mitosis ENTRY and EXIT are retained in the early sea urchin embryo (Whitaker and Patel, 1990). It is possible that inhibition of spectrin function may act through START or mitosis ENTRY, but may not affect EXIT from mitosis, since the length of the mitotic period is not altered.

Fowler and Adam (1992) demonstrated a redistribution of spectrin from the plasma membrane to the cytoplasm of cultured CHO and HeLa cells during mitosis that may be regulated by the phosphorylation of spectrin by mitotic kinases. The observation that high doses of anti-spectrin F_{ab} arrest the cell cycle between cleavage and nuclear reformation suggests that anti-spectrin antibody may inhibit cortical actin reorganization associated with cell division. This inhibition may be exerted directly by blocking the ability of spectrin to form actin filament networks and/or to mediate actin network-membrane interactions, or indirectly by altering spectrin phosphorylation-dephosphorylation.

The mechanism by which cell cycle arrested blastomeres re-enter the cell cycle is not known. Selective degradation of F_{ab} fragments in injected blastomeres may release a sufficient pool of spectrin to allow the cell to re-enter the cell cycle. Alternatively, activation of spectrin transcription may allow sufficient spectrin synthesis to compensate for the spectrin deficit. The sea urchin egg contains low levels of spectrin RNA, and transcription is not normally activated until the late blastula stage (Wessel and Chen, 1993). However, depletion of the spectrin pool may activate a feedback mechanism that initiates spectrin transcription prematurely to re-establish a sufficient pool of spectrin to escape the cell cycle block.

While microinjection of low doses of F_{ab} fragments into blastomeres produce the same progressive increase in the length of the cell cycle observed with the injection of intact anti-spectrin antibody, high doses of F_{ab} fragments causes arrest of the cell cycle at the next cleavage division. This difference may be due to the fact that a given dose

of F_{ab} fragments contains more antibody binding sites than an equivalent dose of intact antibody due to the removal of the F_c region of the antibody molecule, which constitutes ~20% of total IgG mass (Harlow and Lane, 1988). Alternatively, the smaller size of the F_{ab} fragment may permit binding to a greater number of sites on a spectrin molecule because of reduced steric hindrance, making F_{ab} fragments more effective than intact antibody in inhibiting spectrin function.

Spectrin and Nuclear Reformation

Nuclear reformation following mitosis during the early development of echinoderm embryos occurs by the fusion of karyomeres, vesicles formed by the deposition of nuclear envelope around individual chromosomes during late anaphase (Ito *et al.*, 1981; Montag *et al.*, 1988). The dispersed DAPI-stained vesicles found in cell cycle-arrested blastomeres may represent unfused karyomeres. Blastomeres are observed in various stages of nuclear reformation, but no normal nuclei appeared during cell cycle arrest. Inhibition of nuclear reformation may result from the arrest of the cell cycle at a time that is non-permissive for nuclear reformation, or may reflect a direct role for spectrin in nuclear reformation. Localization of spectrin to the nuclear membrane has been reported in neurons (Zagon *et al.*, 1986), amoebae (Choi and Jeon, 1989), and amphibian oocytes (Campanella *et al.*, 1990). Although spectrin has not been localized to the nucleus of echinoderm eggs (Schatten *et al.*, 1986; Fishkind *et al.*, 1990a, b), intense anti-spectrin staining of cytoplasmic vesicles might mask more subtle nuclear

staining. In addition, the formation of a transient, perinuclear array of actin filaments has been reported during nuclear reformation following mitosis in tissue culture cells (Clubb and Locke, 1996). If a similar structure participates in the process of nuclear reformation in starfish embryos, inhibition of spectrin function might prevent normal nuclear assembly.

Spectrin and the Cytoskeleton

The cortical actin cytoskeleton becomes disrupted in blastomeres injected with anti-spectrin antibodies, suggesting that spectrin functions in maintaining the organization of actin in the egg cortex. This result is consistent with ultrastructural localization studies demonstrating the cross-linking of cortical actin filaments by spectrin in the cortex of fertilized sea urchin eggs (Fishkind *et al.*, 1990a). Microinjection of anti-spectrin antibodies into amoeba also causes dose-dependent changes in cell morphology and motility consistent with the disruption of the actin cytoskeleton (Choi and Jeon, 1992). However, in contrast to these results, Mangeat and Burridge (1984) report that microinjection of anti-spectrin antibodies into tissue culture cells causes the immunoprecipitation of spectrin and aggregation of intermediate filaments, but no other detectable changes to the cytoskeleton or cell morphology. However, the cellular consequences of inhibiting spectrin function may be less apparent in tissue culture cells. *Drosophila* mutants with defects in spectrin interchain binding that inhibit tetramer formation exhibit first larval instar lethality (Deng *et al.*, 1995). Although this mutant

spectrin remains stably associated with the plasma membrane, it is unable to cross-link actin filaments into networks (Deng *et al.*, 1995), supporting the hypothesis that the cross-linking of actin filaments by spectrin is necessary for development.

The progeny of injected blastomeres eventually stop dividing and form actin-containing surface blebs that lead to the fragmentation of the cell. I hypothesize that the induction of surface blebbing results from the destabilization of the cortical actin cytoskeleton, caused by the inhibition of spectrin's ability to cross-link actin filaments into networks and/or mediate the attachment of actin filament networks to the plasma membrane. Human melanoma cell lines deficient in the actin binding protein ABP-280 also display increased surface blebbing (Cunningham *et al.*, 1992; Cunningham, 1995). Like spectrin, ABP-280 cross-links actin filaments into networks and binds to integral membrane glycoproteins (Brotschi *et al.*, 1978; Ezzell *et al.*, 1988; Cunningham *et al.*, 1992; Sharma *et al.*, 1995). Cunningham *et al.* (1992) demonstrate that transfection of ABP-280 deficient cells with functional ABP-280 suppresses blebbing and restores normal cell motility, suggesting that this protein is required for membrane stability and the coordinated formation of surface protrusions. Our results suggest that the inhibition of spectrin function by microinjected anti-spectrin antibodies causes a similar destabilization of the cortical cytoskeleton, leading to the formation of surface blebs and subsequent cell fragmentation.

Surface blebbing and cell fragmentation are characteristics of apoptosis (Wyllie *et al.*, 1980; Earnshaw, 1995) which can be induced if the cell cycle is perturbed (Earnshaw, 1995; Evan *et al.*, 1995). It is interesting to note that the arrest of cell

division and induction of cell fragmentation occurs in injected blastomeres at the time the controls reach the mid-blastula stage, the point where defective cells are most likely to be eliminated during early embryogenesis (Hartwell and Weinert, 1989). Thus the observed fragmentation of cells caused by surface blebbing may represent the elimination of defective cells by apoptosis.

Spectrin and Formation of the Blastula Epithelium

The progeny of injected blastomeres are unable to participate in the formation of a blastula epithelium, suggesting that spectrin may function in the polarization of blastula epithelial cells and in establishing cell-cell contacts required for junction formation. Previous studies have implicated spectrin in the polarization of MDCK epithelial cells in culture (Nelson and Veshnock, 1987; Nelson *et al.*, 1990). In addition, spectrin has been shown to interact with cell adhesion molecules involved in the establishment of cell-cell contact such as E-cadherin (Nelson *et al.*, 1990) and the 180 kDa isoform of N-CAM (Pollerberg *et al.*, 1987), presumably limiting their lateral mobility in the plasma membrane and confining them to areas of cell-cell contact (Pollerberg *et al.*, 1987; Goodman *et al.*, 1988; Bennett, 1990). Anti-spectrin antibodies may inhibit the association of spectrin with cell adhesion molecules, preventing the progeny of injected cells from forming normal intercellular contacts required for the development of the blastula epithelium. This interpretation is consistent with recent reports that *Drosophila* containing mutations in the α-spectrin gene are unable to form a normal gut epithelium

(Lee *et al.*, 1993), and that transfection of cultured Caco-2 cells with truncated β_G-spectrin results in the loss of epithelial morphology (Hu *et al.*, 1995).

Spectrin in Cytokinesis

Blastomeres injected with high doses of anti-spectrin antibody or F_{ab} fragments initiate, but fail to complete cytokinesis. Cleavage furrows in these cells appear to form and contract normally, but regress during later stages of cytokinesis, suggesting that spectrin functions in the completion of cytokinesis, but is not required for the initiation or contraction of the cleavage furrow. This interpretation is consistent with the observation that the redistribution of spectrin to furrow canals during cellularization of the *Drosophila* embryo lags behind that of actin and myosin, suggesting that spectrin functions in stabilizing the furrow canals once they have formed (Pesacreta *et al.*, 1989). Previous studies have identified spectrin as a component of isolated cleavage furrows from sea urchin eggs (Yonemura, *et al.*, 1990); however, immunofluorescent staining does not show any detectable accumulation of spectrin in the cleavage furrow compared with other regions of the egg cortex (Schatten *et al.*, 1986; Fishkind *et al.*, 1990b). These results suggest that spectrin functions in stabilizing the cleavage furrow during contraction, and may be required for the completion of cytokinesis.

The results of the studies reported here implicate spectrin in the organization of actin cytoskeleton and in cytoskeletal-membrane interactions required for the stabilization of the cell surface and maintenance of cell-cell contact. Further, the results suggest that

spectrin is required for the stabilization of the cleavage furrow, but is not necessary for the formation or contraction of the contractile ring. Surprisingly, however, the results demonstrate that inhibition of spectrin function causes a dose-dependent increase in the length of the cell cycle which correlates with the disruption of the cortical actin cytoskeleton, suggesting that a mechanism exists which monitors the organization of the actin cytoskeleton to regulate progression through the cell cycle.

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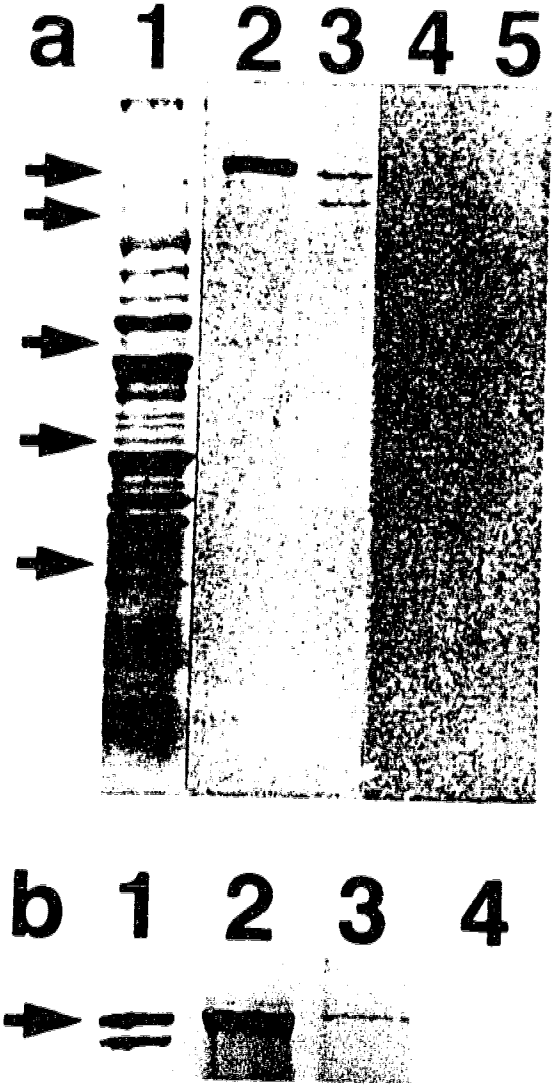
Figure 3.1. (a) Affinity-purified antibodies to sea urchin egg spectrin immunoblotted against eggs of the starfish *Patiria miniata* and purified human erythrocyte spectrin. Lane 1, Coomassie blue stained SDS-PAGE of total starfish egg protein. Immunoblot of affinity-purified anti-spectrin antibodies showed monospecific staining of a closely spaced polypeptide doublet in *Patiria* eggs that ran in the range of 242-245 kDa (lane 2), and of human erythrocyte α- and β-spectrin at 240 and 220 kDa (lane 3). Pre-immune antibodies showed no specific binding to *Patiria* egg proteins (lane 4) or purified human erythrocyte spectrin (lane 5). Arrows indicate molecular weights of (starting from the top) 240 kDa (human erythrocyte α-spectrin), 194 kDa (myosin), 116 kDa (β-galactosidase), 85 kDa (bovine serum albumin), and 49 kDa (ovalbumin). (b) Immunoblot of native spectrin immunoprecipitation from *Patiria miniata* egg homogenates. Under native conditions, immune serum antibodies precipitated the closely spaced α- and β-spectrin doublet (lane 2). Pre-immune serum (lane 3) and protein A-Sepharose beads alone (lane 4) show a small amount of non-specific spectrin binding. Lane 1 shows a purified human erythrocyte spectrin control. Arrow indicates the 240 kDa α-subunit.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b 1 2 3 4

Figure 3.2. Injection of pre-immune immunoglobulin fraction and affinity-purified anti-spectrin antibodies into *Patiria miniata*. The time after microinjection (hours:minutes) is indicated in the lower right of each panel. Scale bars = 50 μm. (a-d) *Patiria miniata* embryos were unaffected by the microinjection of pre-immune immunoglobulins. (a) A two-cell stage *Patiria miniata* embryo was injected with 0.5 ng of pre-immune antibodies. An oil droplet (*asterisk*) marks the injected cell. Both the injected and uninjected control blastomere underwent synchronous cell divisions (b and c), eventually forming a normal blastula with an intact epithelium (d). Injections of 0.2-1.0 ng of pre-immune IgGs or 0.3-2.3 ng of pre-immune F_{ab} fragments produced identical results. (e-h) Microinjection of anti-spectrin antibody caused cell cycle delays and resulted in the production of abnormal embryos. (e) An embryo at the two-cell stage was injected with 0.2 ng of antibody. An oil droplet (*asterisk*) marks the injected cell. (f & g) The progeny of the injected blastomere are larger than those of the uninjected blastomeres, due to an increase in cell cycle time. (h) The progeny of the uninjected control cells formed a normal blastula epithelium (right side of panel), but cells derived from the injected blastomere produced a loose aggregate. Some of the cells became motile, breaking away from the aggregate and entering the blastocoel (*arrow*).

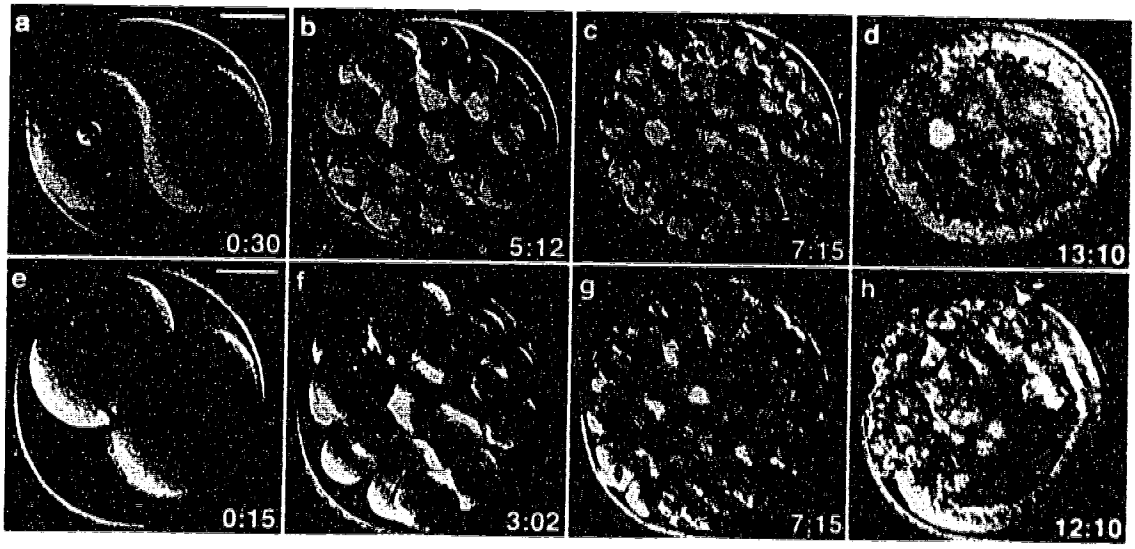
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Figure 3.3. Microinjection of 0.8 ng of anti-spectrin antibody resulted in lengthy cell cycle delays and profound morphological alterations in injected blastomeres. The time after microinjection (hours:minutes) is indicated in the lower right of each panel. Scale bar = 50 μm. (a) A two-cell stage embryo was injected with 0.8 ng of anti-spectrin IgG. (b) By 3.5 hours, the injected cell only completed 2 divisions. Attempts at cleavage continued, but furrow regressions caused failures in cytokinesis (compare c and d). The progeny of the injected blastula, many containing enlarged nuclei (e), completed fewer divisions than blastomeres injected with lower antibody doses. When division stopped, many of the injected cells extended surface protrusions (f, arrow), and became motile. The surface blebbing increased in intensity, leading to cell fragmentation.

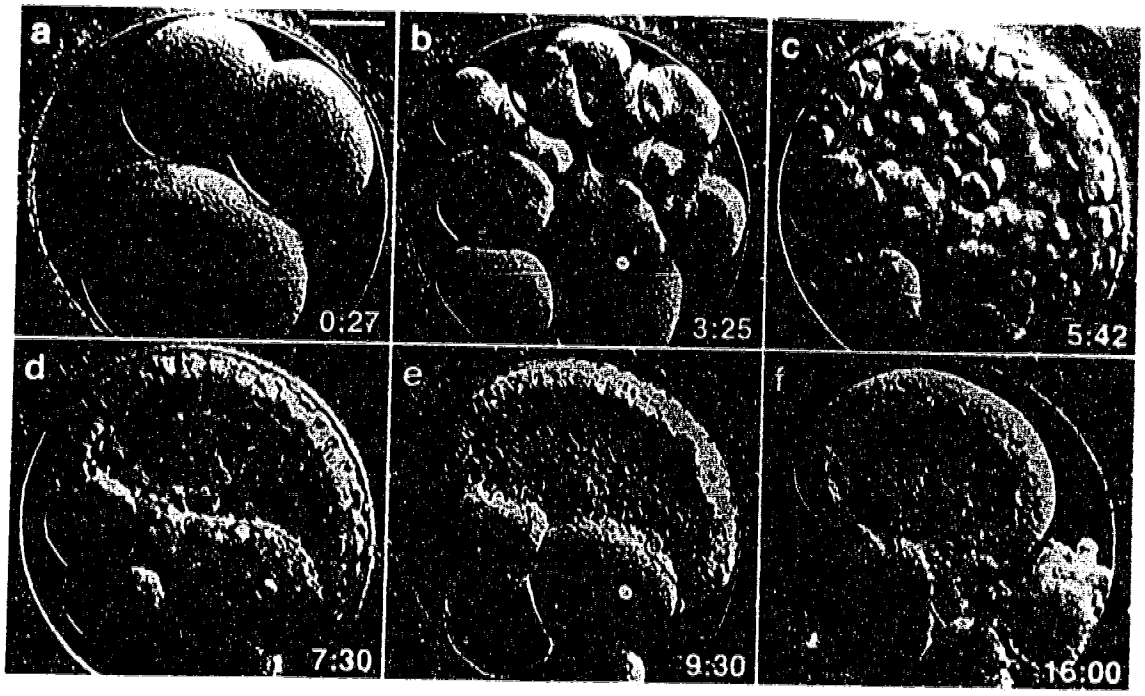
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Figure 3.4. Microinjection of 0.6 ng of anti-spectrin F_{ab} fragments arrested the cell cycle. Time after microinjection (hours:minutes) is indicated in the lower right of each panel. Scale bar = 50 μm. (a) A two-cell stage embryo was microinjected with 0.6 ng of anti-spectrin F_{ab} fragments. The injected blastomere arrested at the second cell cycle (two-cell stage), became irregular in shape and developed clear areas at the cell poles (b and c, *arrows*). By 12 hours after injection, the blastomere regained a normal appearance (d) before resuming division (e) and formed a loose aggregate of cells that exhibited pronounced surface activity and became motile (f). Progeny of the uninjected blastomere formed an independent blastula which hatched normally (right side of d and e).

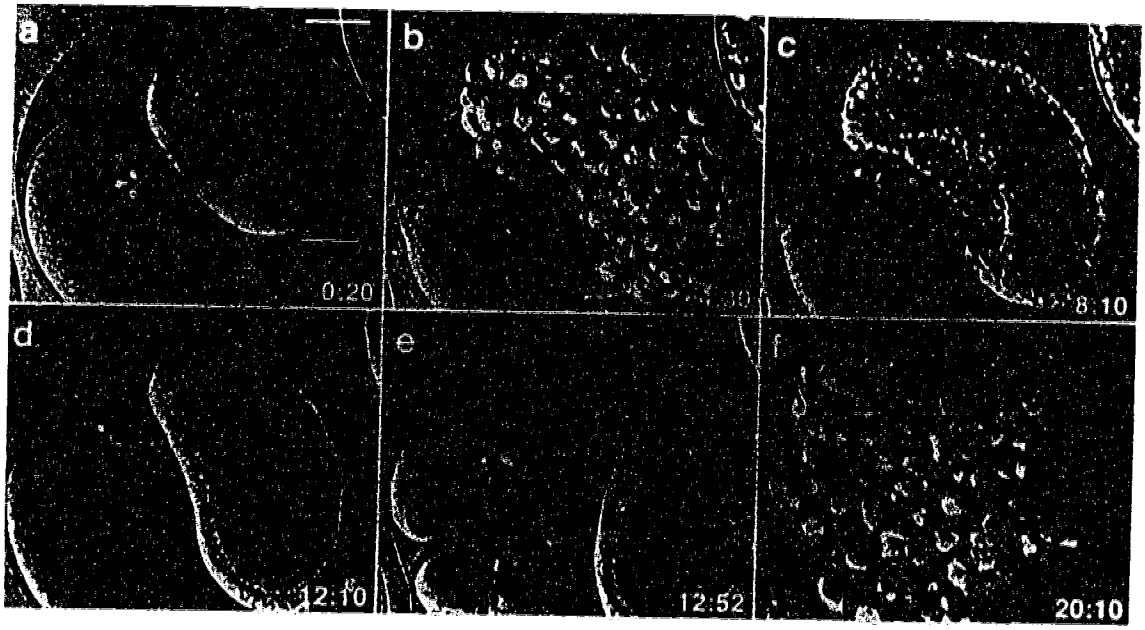
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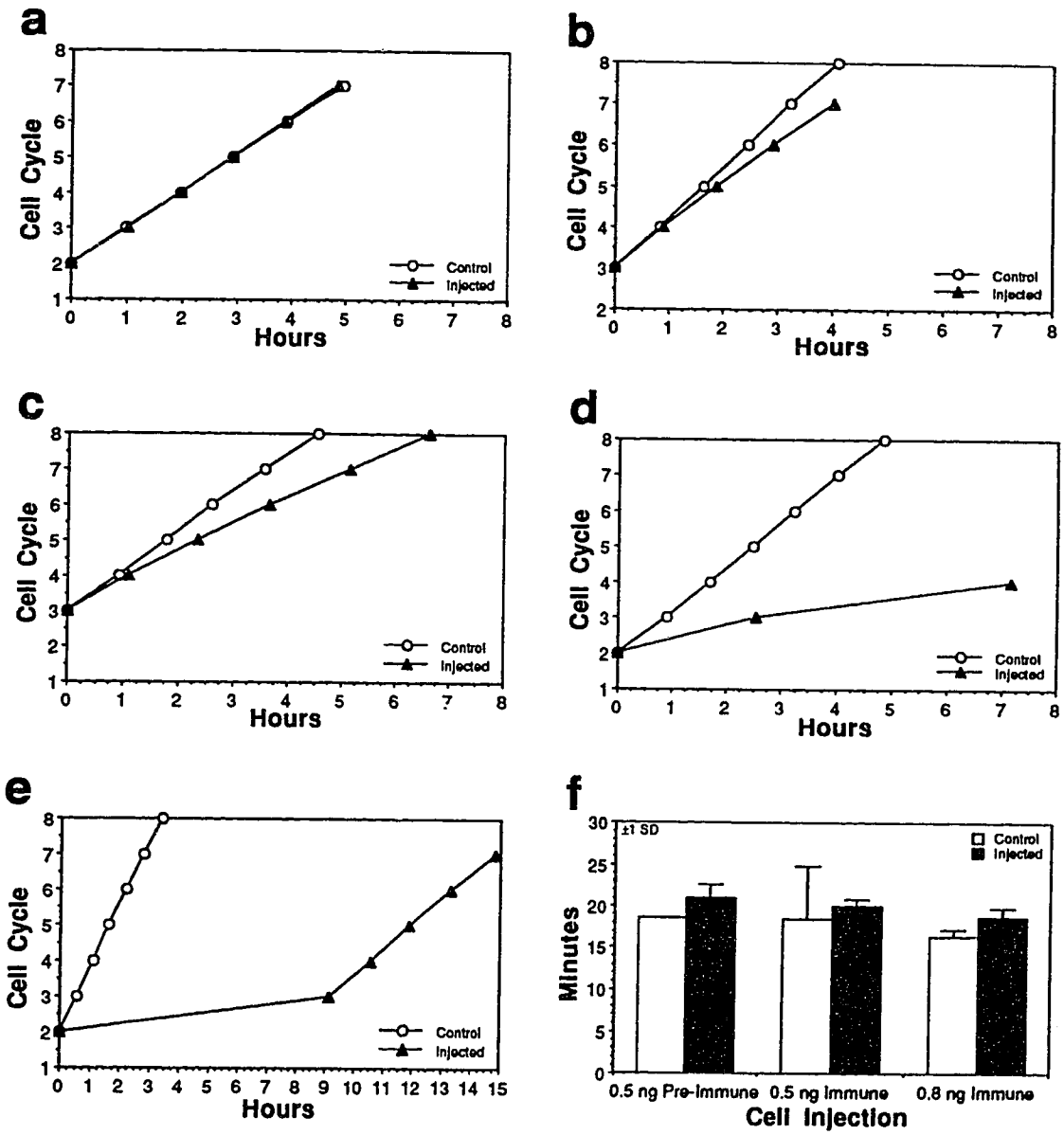
Figure 3.5. Comparison of cell cycle times of injected blastomeres and their uninjected sister controls. (a) The cell cycle time of a blastomere injected with 0.5 ng of pre-immune antibodies (s) was identical to the control (m). (b) Injection of a blastomere with 0.2 ng of affinity-purified anti-spectrin antibody (s) caused a progressive increase in cell cycle time compared to the uninjected sister blastomere (m). After 5 division cycles, the injected blastomere was 1 cell cycle behind the uninjected control. (c) Injection of 0.5 ng of anti-spectrin antibody resulted in a greater increase in cell cycle time (s). By the eighth division, the cell cycle time of the injected blastomere had increased to approximately 1.5 hours compared to 54 minutes in the control (m). (d) A blastomere injected with 0.8 ng of affinity-purified anti-spectrin IgG (s) required 2.5 hours to complete the second cell cycle and 4.5 hours to complete the third cell cycle, compared with an average cell cycle time of 48 minutes in the uninjected sister cell (m). (e) Injection of 0.6 ng of affinity-purified anti-spectrin F_{ab} fragments (s) arrested the blastomere in the second cell cycle for over 9 hours. Upon resuming division, the average cell cycle time was extended to 1.4 hours compared to 37 minutes in the uninjected sister cell (m). (f) Comparison of the mitotic timing of injected blastomeres with uninjected controls. No significant difference was observed between the mitotic timing of antibody-injected cells (solid bars) and uninjected sister cells (open bars). Hence, delays in cleavage resulted from an extension of the non-mitotic portion of the cell cycle. Error bars represent one standard deviation above and below the mean.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Control
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Hours
Control
Injected
e
Cell Cycle
Hours
Control
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f
±1 SD
Minutes
Control
Injected
0.5 ng Pre-Immune
0.5 ng Immune
0.8 ng Immune
Cell Injection

Figure 3.6. The enlarged nuclei of blastomeres injected with 0.1 ng of anti-spectrin F_{ab} fragments do not show increased DAPI fluorescent staining for DNA. Scale bar = 50 μm. (a-c) Differential interference contrast (DIC) photomicrographs. (d-e) corresponding fluorescence micrographs. (a) Control 8-cell stage embryo showing normal-sized nuclei (arrows). (d) Fluorescence micrograph showing distribution of DAPI staining in control nuclei. (b & c) Progeny of blastomeres injected with anti-spectrin antibody show enlarged nuclei (arrows). Normal blastula epithelia formed by the progeny of the uninjected blastomere appear on the left side of the photomicrographs. (e and f) DAPI staining of embryos shown in (b) and (c) respectively shows no increase in the nuclear staining of injected blastomeres. Chromatin distribution within the enlarged nuclei is variable, ranging from highly aggregated to dispersed. Control nuclei show more uniform, intense staining. The majority of blastula epithelial cells in (f) are in mitosis and show condensed chromosomes.

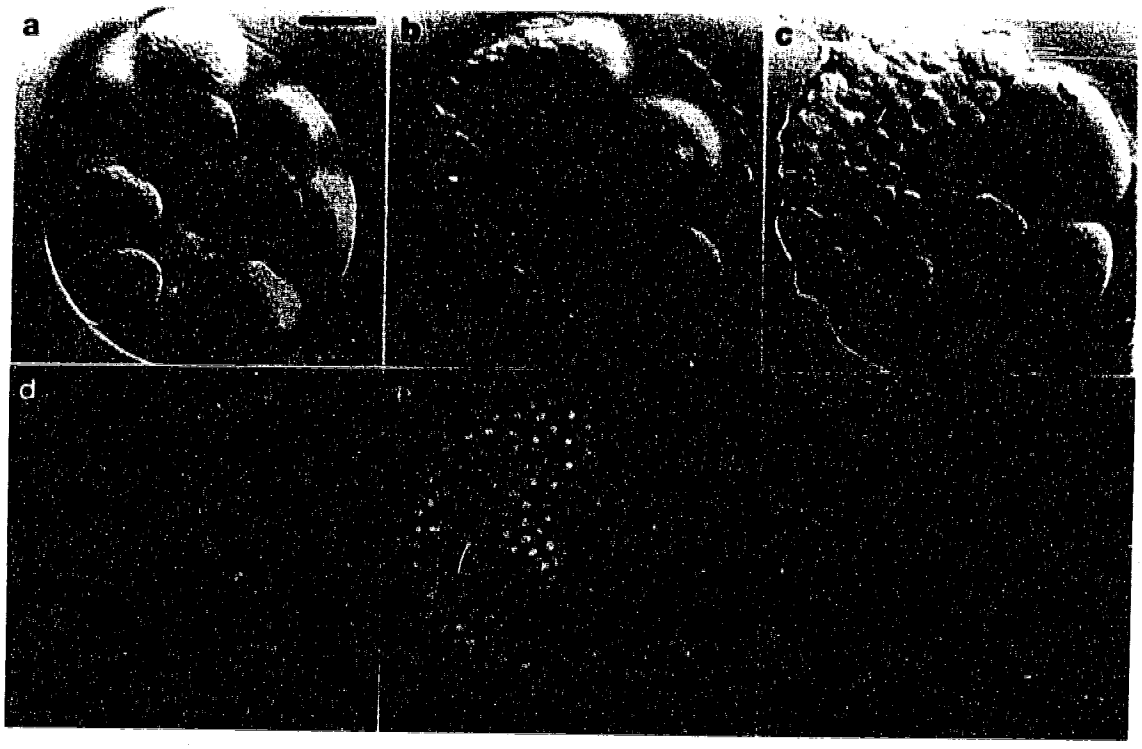
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Figure 3.7. Failure of normal nuclear assembly in embryos injected with 0.6 ng of anti-spectrin F_{ab} fragments viewed by DAPI staining. Scale bar = 50 μm. (a & c) DIC photomicrographs of F_{ab}-injected embryos. The progeny of the uninjected blastomeres formed normal blastula epithelia (upper part of micrographs). Injected blastomeres are marked with an oil drop (asterisks). The injected blastomere in (a) arrested at the second cell cycle (two-cell stage). No nucleus or nuclear envelope was detected. (b) DAPI fluorescence photomicrograph of (a) in the same optical plane. The injected cell contains dispersed fluorescent dots (*arrow*), which may represent karyomeres that failed to fuse to form a normal nucleus. Round to elliptical-shaped normal nuclei can be seen in the progeny of the control cell. The injected blastomere in (c) completed second cleavage and arrested at the third cell cycle (four-cell stage). Again, no nucleus or nuclear envelope was detected in either daughter cell of the injected blastomere. (d) DAPI fluorescence photomicrograph of (c) in the same optical plane. The two daughter cells from the injected blastomere show dispersed dots (arrows), presumably karyomeres, which failed to fuse to form a normal nucleus.

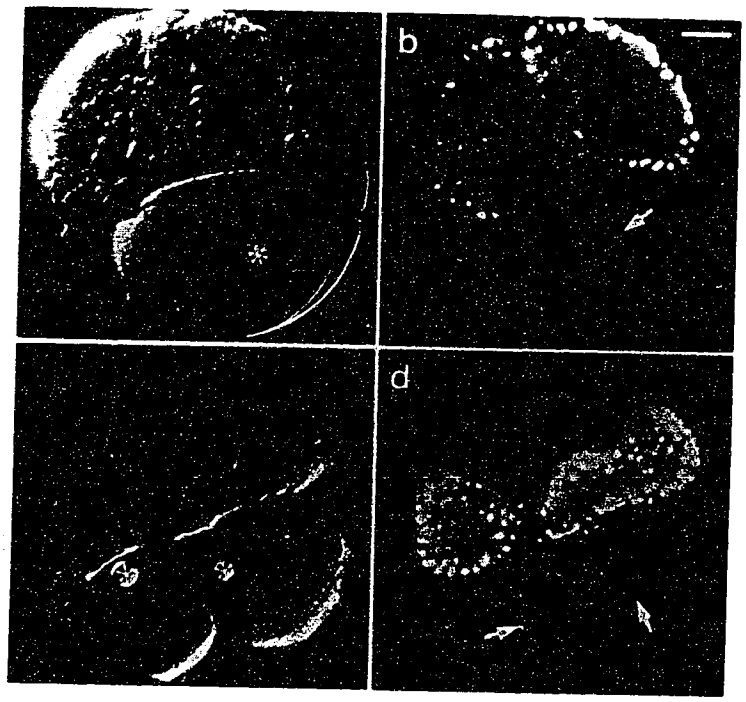
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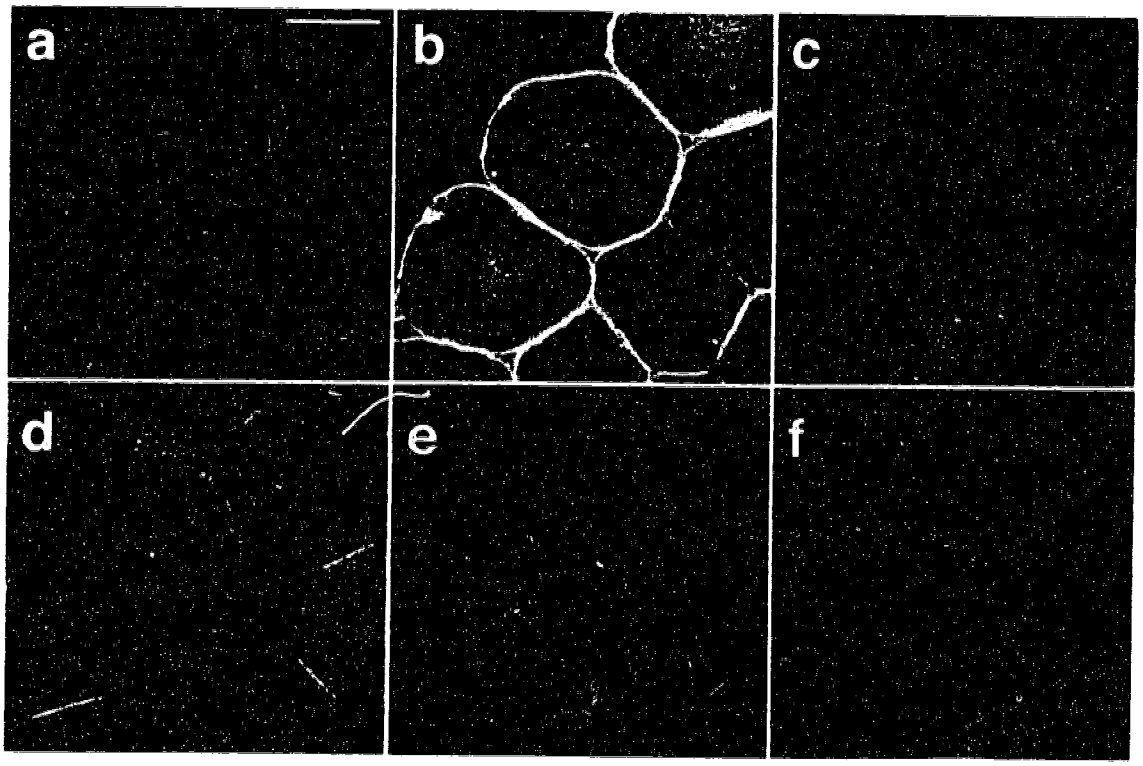
Figure 3.8. The distribution of filamentous actin in control and injected starfish embryos. Scale bar shown in panel a = 20 μm in a, c & f and 40 μm in the other panels. (a) Normal starfish blastomeres at the two-cell stage stained with BODIPY-phallacidin showed filamentous actin in the microvilli and along the plasma membrane. (b) As development progressed, the microvilli became less prominent and the amount of F-actin in the cortex increased. Actin appears concentrated at regions of blastomere contact. (c) Blastomere at the two cell stage injected with anti-spectrin antibody. F-actin staining appears homogeneous throughout the cell. Microvilli are absent, and have been replaced by small surface blebs. (d) The progeny of injected cells exhibited significantly reduced cortical F-actin staining, but still maintained a concentration in regions of cell-cell contact. (e) As development proceeded, the amount of cortical and cytoplasmic F-actin decreased. (f) When injected cells stopped dividing, filamentous actin became concentrated within surface blebs.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Chapter 4

Conclusions and Summary

CONCLUSIONS

The studies presented in this thesis have examined some of the roles of the actin cytoskeleton in early embryonic development and cytokinesis. Fertilization initiates the start of the cell cycle in sea urchin eggs and a dramatic reorganization of the actin cytoskeleton. I have found that the amount of cortical F-actin increases and decreases through the first two cell cycles, reaching a minimum content at the time of cytokinesis. These changes are also accompanied by the assembly and disassembly of actin rootlets, which disappear shortly before cytokinesis. Actin rootlet formation requires cytoplasmic alkalinization and is inhibited with cytochalasin D. Cytochalasin D washout experiments show that the cytoskeletal changes that occur after fertilization are not required for normal cytokinesis and embryonic development, suggesting that the reorganization of actin serves other developmental functions.

My studies have also shown that spectrin is required for early embryonic development. Ablating spectrin function demonstrates that it participates in maintaining the stability of the plasma membrane, actin cytoskeletal organization, and proper cell-cell contact for blastula epithelium formation. Surprisingly, inhibiting spectrin function also results in a progressive increase in cell cycle time. This change in cell cycle time is likely related to the disruption of actin cytoskeletal organization in the injected

blastomeres. Disruption of normal cytoskeletal organization by incubating eggs in cytochalasin D followed by washout, also extends the first cell cycle by 15 minutes. Together, the results from both of my studies suggest a link between the organization of the actin cytoskeleton and progression through the cell cycle.

Actin Filament Dynamics

Confocal laser scanning microscopy of sea urchin eggs stained with fluorescently-labelled phallacidin clearly shows a dramatic increase in the amount of cortical F-actin shortly after fertilization. By quantifying the fluorescence, it is also shown that there is a significant decrease in the amount of cortical F-actin as the egg approaches first cleavage. These changes are repeated in the second cell cycle, where the amount of cortical F-actin increases shortly after first cleavage and again drops to a minimum at cytokinesis. This minimization is surprising, considering the general assumption that actin cytoskeletal organization increases as the cell approaches division. As discussed in Chapter 2, the increase in cortical stiffness that occurs at cytokinesis does not involve an increase in the amount of F-actin in the cortex. Instead, the reduction in F-actin content may remove a resistive element in the cortex to facilitate cytokinesis.

A recent study by Heil-Chapdelaine and Otto (1996), employing fluorescence quantification of F-actin labelled with rhodamine-phalloidin, suggests that no significant change occurs in the amount of cortical F-actin after fertilization. This conclusion is contrary to my findings and the majority of other studies on cortical actin. Heil-

Chapdelaine and Otto (1996) report that the total amount of F-actin in fertilized eggs drops to 30% of the amount in unfertilized eggs by the time of cytokinesis. This result and the fluorescent images presented by Heil-Chapdelaine and Otto (1996) is inconsistent with my confocal images and photomicrographs from other works, including Wang and Taylor (1979) and Hamaguchi and Mabuchi (1982; 1988).

My confocal images and fluorescence quantification clearly show much more cortical F-actin in fertilized than in unfertilized eggs throughout two cell cycles. The persistence of microvilli with their cores of F-actin bundles as observed through the first two cell cycles partially accounts for there being more F-actin in fertilized than in unfertilized eggs. Considering all the available data, it is difficult to imagine that total F-actin could drop below that of unfertilized eggs, given the dramatic changes and amounts contained within the cell cortex after fertilization and throughout development.

Part of the differences between our results could be the inordinate 8 hour fixation time that was employed by Heil-Chapdelaine and Otto (1996) before staining with phalloidin for an additional 8 hours. This extended period may have resulted in the extraction of much of the egg actin. When Heil-Chapdelaine and Otto (1996) isolate egg cortices, they employ a fixation time of 15 minutes followed by a 5-8 hour staining, producing radically different results in their photomicrographs which show very intense F-actin staining when compared with whole egg preparations. Results from the isolated cortices are more consistent with my results, which have also been obtained from eggs with comparatively shorter fixation and staining times.

Another important difference is my use of confocal laser scanning microscopy versus conventional epifluorescence microscopy which Heil-Chapdelaine and Otto (1996) employ. Confocal microscopy offers the advantage of taking an optical section through whole eggs, providing much clearer images with more detail of cortical F-actin than viewing whole eggs or isolated cortices using a conventional microscope. Using confocal microscopy to collect the images for fluorescence quantification eliminates much of the background and overlapping or additive fluorescence that occurs with conventional epifluorescence microscopy. Hence, evaluating the amount of cortical F-actin and following its dynamics using optical sections through the equatorial plane of whole eggs, is a more accurate reflection of cortical F-actin content than measurements made with a conventional microscope.

In their quantification of fluorescence, Heil-Chapdelaine and Otto (1996) claim a less than 10% variation between equatorial focal planes and those grazing the surface of eggs. Again this seems unlikely, given the numerous microvilli and amount of cortical F-actin as shown in my confocal images. A surface view of the egg with a confocal microscope appears very similar to the fluorescence micrographs of isolated cortices that Heil-Chapdelaine and Otto (1996) present in their work. This conflicting data within their own paper also makes their fluorescence quantification somewhat unreliable.

My results demonstrate that the actin cytoskeleton is very dynamic through the first two cell cycles. Following the peak of F-actin organization after fertilization, there is a steady decrease in the amount of F-actin up to the time of cytokinesis. Confocal

imaging of live sea urchin eggs injected with rhodamine-phalloidin shows a similar accumulation and decrease in the amount of F-actin in the cortex (Terasaki, 1996). After the dramatic increase in the amount of F-actin in the egg cortex, actin filaments detach and translocate away from the egg surface (Terasaki, 1996). These observations suggest that the movement of actin filaments into the cytoplasm may be partially responsible for the decrease in the amount of cortical F-actin that occurs as the cell approaches first division.

If the process described by Terasaki (1996) occurs *in vivo*, then actin filaments should have also been observed in the cytoplasm of fixed sea urchin eggs. However, the extraction of actin filaments during permeabilization may account for the lack of any actin filaments being observed in the cytoplasm of fixed embryos using confocal microscopy. Alternatively, Terasaki's (1996) results may be an artifact, where the egg is employing an alternate method to reduce the amount of F-actin in the cortex as it approaches first cleavage. Instead of normally reducing the amount of cortical F-actin through depolymerization, for example, the egg may resort to an alternate method where phalloidin-stabilized actin filaments are severed and translocated away from the cortex. To determine if the translocations are an artifact of phalloidin stabilization, fluorescently-labelled G-actin can be microinjected into live sea urchin eggs, and its dynamics followed with confocal microscopy. These experiments would also further clarify how the amount of cortical F-actin is reduced through the first cell cycle.

The Actin Cytoskeleton and the Cell Cycle

Both of the studies that I have completed suggest that the actin cytoskeleton is involved in regulating the cell cycle. Disrupting the normal reorganization of the actin cytoskeleton that takes place after fertilization with cytochalasin D extends the first cell cycle by 15 minutes. Continued disruption of the actin cytoskeleton in starfish blastulas through early development, by ablating normal spectrin function, results in a progressive lengthening of the cell cycle.

Incubating eggs in cytochalasin D prevents the reorganization of actin that takes place shortly after fertilization. Fertilized eggs incubated in cytochalasin D for up to 120 minutes, 5 minutes before the normal onset of cleavage, followed by washout divide 15 minutes later than controls, irrespective of the length of time spent in the drug. This suggests that a brief window exists early in the cell cycle that allows actin reorganization and the formation of actin rootlets to occur. Beyond this point, the egg is either unable or not required to reorganize its cortical cytoskeleton, as there are no detectable changes in cortical F-actin after removing cytochalasin D until cytokinesis, the stage when the cortex may receive a signal to prepare for cell division. This signal may initiate the assembly of the cortical actin structures that are required for cytokinesis. However, with its normal cytoskeletal organization disrupted with cytochalasin D, the egg may require an additional 15 minutes to properly construct these cortical actin structures before proceeding with cytokinesis.

By ablating spectrin function in starfish blastomeres, an extension of the cell cycle results. Spectrin has been shown to be phosphorylated during mitosis in tissue culture cells, resulting in its redistribution, presumably to facilitate the restructuring of the actin cytoskeleton in preparation for cytokinesis (Fowler and Adam, 1992). The microinjection of antibodies against spectrin into starfish blastomeres may hinder this mitotic phosphorylation/dephosphorylation, impeding actin reorganization before or after cytokinesis. This impediment result in the cell taking longer to restructure its actin cytoskeleton, producing a lengthening of the cell cycle time.

Together, the cytochalasin D washout and the microinjection experiments seem to suggest the existence of a link between the organization of the actin cytoskeleton and cell cycle timing. The egg may monitor the state of actin cytoskeletal organization as part of its mechanism in regulating progression through the cell cycle. A disrupted cytoskeleton may result in delays in restructuring actin for key cell cycle events like cytokinesis. Only when a proper actin organization is established will the egg continue through the cell cycle.

Cytokinesis

Actin and myosin II are key proteins involved in the active constriction of the contractile ring during cytokinesis. The Cao and Wang (1990a, b) studies on rat kidney cells propose that pre-existing actin filaments associate with the cell cortex and migrate to the plane of division where they are recruited to form the contractile ring. Terasaki's

(1996) experiments demonstrate that mechanisms exist in the sea urchin egg which can translocate actin filaments. The existence of such a system in sea urchin eggs suggests that the contractile ring could be partly formed by the translocation of pre-existing actin filaments (Terasaki, 1996).

Although not directly addressing the mechanism of contractile ring formation, my results suggest that it is unlikely that actin filaments in the rootlets of sea urchin eggs participate in contractile ring assembly. The disappearance of actin rootlets shortly before cytokinesis, the minimization of the total amount of F-actin in the cortex, and the cytochalasin D washout experiments showing that actin rootlet formation is not required for cell division to occur, tends to point toward another source for F-actin, leaving actin rootlets to serve other functions in development. Even though actin rootlets may not provide the actin for contractile ring assembly, there still is a tremendous amount of F-actin in the cortex that is not associated with rootlets and may be utilized for cytokinesis. Although Terasaki (1996) did not elaborate on the fate of translocated actin filaments in the cytoplasm of eggs, it is possible that these filaments are recycled and used in contractile ring assembly at cytokinesis. As eggs injected with low concentrations of phalloidin divide normally (Hamaguchi and Mabuchi, 1982; Terasaki, 1996), it would be worthwhile to follow the fate of translocated actin filaments in live sea urchin eggs to determine if they participate in the assembly of the contractile ring.

Many actin-binding proteins have been implicated in having a role in cytokinesis (Satterwhite and Pollard, 1992). Mutations in tropomyosin and profilin block cell division (Balasubramanian *et al.*, 1992; 1994), suggesting that actin-binding proteins have

regulatory as well as mechanical functions in cytokinesis. The identification of spectrin in cleavage furrows suggests an involvement in cytokinesis, which I have demonstrated by microinjecting spectrin antibodies into blastomeres. Inhibiting spectrin function does not impede the ability of blastomeres to initiate a cleavage furrow, but does result in their failure to complete cytokinesis. Although I have shown that spectrin is necessary for the successful completion of cytokinesis, its actual role in this process remains somewhat unclear. In *Drosophila*, spectrin localizes to the furrow canals after actin and myosin, suggesting that it functions in stabilizing this structure after its initiation (Pesacreta *et al.*, 1989). Applying this proposed function to my results, spectrin may stabilize the cleavage furrow of blastomeres after its initiation. If spectrin is prevented from performing this function during cytokinesis, then a regression in the cleavage furrow can result, an effect which has been observed in injected blastomeres.

The Actin Cytoskeleton in Early Embryonic Development

One of the functions of the reorganization of the actin cytoskeleton after fertilization is in the transport of intracellular vesicles. During early embryonic development, pigment granules (Allen *et al.*, 1992) and intracellular vesicles containing materials for the extracellular matrix (Alliegro and McClay, 1988) migrate out to the cell cortex. This migration is cytochalasin sensitive, implicating the involvement of actin filaments in the process. The actin rootlets in the egg cortex, which are also inhibited

from forming by cytochalasin D, are probably the structures which provide the tracks for the migration of intracellular vesicles.

Interestingly, a normal actin cytoskeletal organization is not required in the first cell cycle for early embryonic development. Preventing the reorganization of actin that occurs after fertilization with cytochalasin D does not affect normal embryonic development if washed out up to 5 minutes before first cleavage. The recovery of a normal actin cytoskeletal organization after first cleavage may compensate for functions that are impeded during the first cell cycle, allowing for normal development to occur. The reappearance of actin rootlets after first cleavage, for example, may permit the intracellular vesicles that are destined for the cell cortex in the first cell cycle to reach this final destination later in development.

My studies have also provided results which illustrate the function of spectrin in early embryonic development. Without proper spectrin function, blastomeres are unable to form a normal blastula epithelium. This result is likely a consequence of blastomeres being unable to maintain the cell-cell contact that is necessary for establishing a blastula epithelium. Spectrin is probably directly involved in this process, linking cell adhesion molecules, which participate in cell-cell interactions, directly to the actin cytoskeleton. The net result of this deficiency is the formation of a loose aggregate of cells that eventually fragment apart. Before fragmenting, however, injected cells also show increased surface activity, reflecting spectrin's role in stabilizing the plasma membrane.

As a disrupted actin cytoskeletal organization seems to underlie the developmental defects that I have observed, it would be of great interest to determine if ablating the

function of other actin-binding proteins results in similar developmental defects. For example, by knocking out filamin function, F-actin cross-linking may be inhibited, disrupting the normal actin cytoskeletal organization and producing similar developmental defects that have been observed with spectrin.

Another interesting result from the microinjection experiments is the possibility that spectrin participates in the reformation of the nucleus after mitosis. Further work will have to be completed to determine if spectrin does serve a role in this process. Studies in the sea urchin egg using immunogold labelling techniques and transmission electron microscopy, for example, will help to determine if spectrin is localized to the nuclear membrane and karyomeres during reformation of the nucleus. Such a localization would place spectrin in a position to participate in proper nuclear assembly at the end of mitosis. Alternatively, if spectrin is not found on nuclear membranes, then the results may suggest that the high dose F_{ab} injections arrest the blastomeres at a point that does not permit normal nuclear reformation.

SUMMARY

This thesis has examined the role of the actin cytoskeleton in early embryonic development and cytokinesis. It has shown that the cortical actin cytoskeleton is very dynamic, displaying cycles of filament assembly and disassembly while progressing through the cell cycle, minimizing the amount of F-actin in the cortex at the time of cytokinesis. It has also been discovered that a normal actin cytoskeletal organization is

not required during the first cell cycle for cleavage and subsequent development to occur. It was also demonstrated that ablating spectrin function results in a longer cell cycle. This extension in cell cycle time may occur from disrupting normal actin cytoskeletal organization. This result and the cytochalasin D experiments demonstrate a link between the actin cytoskeleton and the cell cycle. Normal blastula formation requires proper maintenance of actin cytoskeletal organization throughout early development, and this organization requires spectrin. By ablating its normal function, it has been shown that spectrin participates in cytokinesis, maintains normal cell-cell contact, and stabilizes the plasma membrane. These results have provided a new insight into the dynamics and role of the actin cytoskeleton in early embryonic development and cytokinesis.

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