The Association of Cell Cycle and Growth Related Protein Kinases with the Fibroblast Cytoskeleton

by

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I. ABSTRACT

In order for cells to divide, they pass through a regular sequence of growth and division phases, referred to as the cell cycle. These processes are under careful control of the cell's regulatory machinery. It is evident that some protein kinases are the master controllers of these events, since they regulate the activities of multiple proteins. This regulation involves phosphorylating the proteins at specific regulatory sites, activating some, and inhibiting others to coordinate their activities. The regulatory subunits are called cyclins and the catalytic subunits are called cyclin-dependent kinases (cdks). Each cdk catalytic subunit can associate with different cyclins, and the associated cyclin determines which proteins are phosphorylated by the cdk-cyclin complex. To determine the significance of kinases, two kinases (erk and cdc2) were studied in the rat muscle fibroblast. Detergent extractions of fibroblasts yielded an insoluble cytoskeleton fraction containing both erk and cdc2 kinases. Since these kinases are also found in sea urchin blastomere cells, the present study was undertaken to investigate the possibility that erk and cdc2 may help regulate the cell cycle in fibroblast cells. For this reason, this study was established to determine whether a link existed between a somatic cell, and studies previously performed on the sea urchin blastomere.

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II. Introduction

A cell is a complex structure with several components that are critical for its survival. Science has been able to define the various organelles associated with the cell and their unique functions. However, there are some roles that are still not completely understood. Regulation of organelles, like the cytoskeleton, is definitely an involved process. It has been shown in sea urchins that various protein kinases are associated with the cytoskeleton. Several proteins, like myosin and actin, have known roles and functions in supporting the cytoskeleton. Studies performed on sea urchin embryonic cells have also shown that specific kinases, like mapk and cdc2, are associated as well. It is clear that protein kinases are significant in cell cycle regulation, but whether this regulation affects somatic cells remains unanswered. For this reason, examination of the kinases on a somatic cell level will open new areas of study on a human level. Utilizing rat muscle fibroblasts, which are easy to grow in culture and commonly used for study, regulation of the cytoskeleton and the association of kinases can be examined. Thus, the answers to several of these ideas will be explored in greater detail throughout this study.

In order for cells to divide, they pass through a regular sequence of growth and division phases, referred to as the cell cycle. The cell cycle

consists of two major phases, which are interphase and mitosis. Interphase occupies a majority of the time required for the cell cycle, since it is the period where the cell begins its growth for division. Interphase is subdivided into three phases of which are G_1, S, and G_2 (Figure 1). The G_1 phase is a period of general growth and replication of cytoplasmic organelles. During the S phase, the chromosomal material is duplicated. In the G_2 phase, structures needed for mitosis are produced. Once the preparatory phases have been completed, mitosis occurs, followed by cytokinesis, which completes the cell cycle. In general, mitosis consists of two processes, karyokinesis (division of the nuclei) and cytokinesis (division of the cytoplasm). Karyokinesis is subdivided into four phases: prophase, metaphase, anaphase, and telophase. Each of these phases has their own defining characteristics that contribute to the completion of the cell cycle. These processes are under careful control of the cell's regulatory machinery.

To complete the cell cycle, cytokinesis must occur. Cytokinesis begins during telophase of mitosis and divides the cell into two daughter cells. The mitotic spindle does not seem to be directly involved in cytoplasmic division. However, cleavage always occurs at the midline of the spindle. It has been shown that microtubules, cytoskeletal subunits composed of tubulin, form the mitotic spindle. The mitotic spindle functions

Figure 1: A diagram of interphase and mitosis in the cell cycle. The top figure illustrates the typical somatic cell cycle, with the subphases of interphase shown as well. The bottom figure illustrates the embryonic cell cycle, with G_1 and G_2 being virtually non-existent or very minimal. Figure 1 is courtesy of Dr. Gary Walker.

M

M

G2

G1

S

Interphase

M

M

S

Interphase
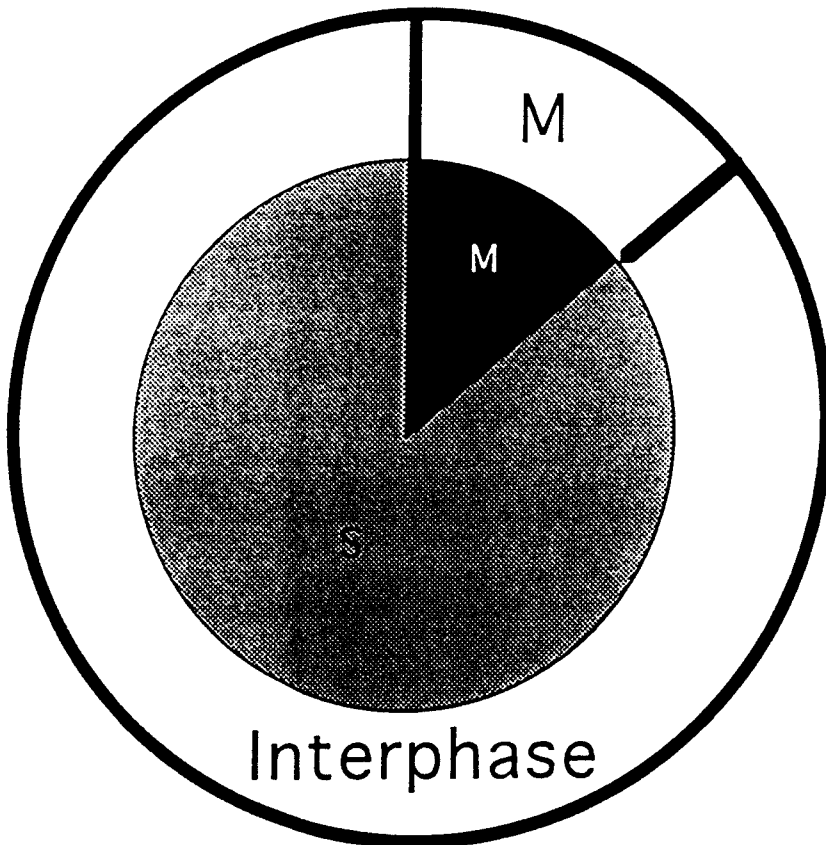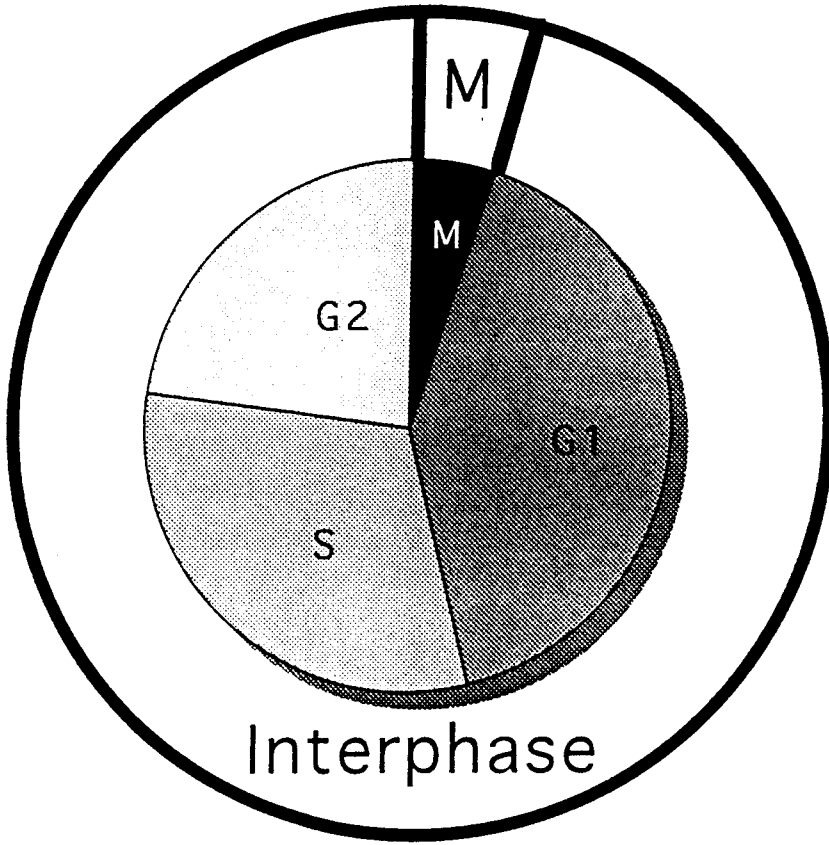
in the positioning and sorting of duplicated chromosomes during cell division. In animal cells during early telophase, the cell membrane begins to constrict around the circumference of the cell at the plane of the spindle equator, marking the onset of cytokinesis. This process starts as the developing cleavage furrow on the cell surface. This groove is generated by a contractile ring of actin and myosin filaments, which underlies the cell membrane at the plane of the spindle equator. As the ring contracts and shortens, the furrow in the cell membrane deepens until the connection between the two cell halves dwindles and then parts.

It has been shown with fluorescence microscopy that the actin and myosin of the contractile ring accumulate at the equator of the cell, midway between the poles of the spindle. Myosins are a family of motor proteins used for cellular movement. The myosin family has several proteins in this class, two of which are myosin I and II. A myosin-II molecule is composed of two heads and a long, rodlike tail. The head and tails are formed by two heavy chains and four light chains. Myosin II is an important motor protein. In the contractile ring, it is responsible for driving the membrane furrowing process during cell division. Myosin I is a smaller protein with a single head and is often membrane-bound. Myosin I provides a motive force for the movement of vesicles on filaments within a cell. In dividing cells, myosin II

is localized to the contractile ring while myosin I is concentrated at the cell poles. In addition, microinjection of anti-myosin II antibodies into dividing eggs abolishes cytokinesis (Mabuchi *et al.*, 1977). These findings suggest an important role for myosin II in cytokinesis and cell division.

The regulation of myosin II activity is regulated during the cell cycle. In addition, other cytoskeletal-associated proteins must be regulated during the cell cycle. Studies of myosin II light chain phosphorylation during cell cycle progression suggests that p34cdc2, phosporylates the regulatory light chain and inhibits myosin ATPase activity (Satterwhite & Pollard 1992). It has been proposed that p34cdc2 inhibits myosin activity until anaphase when cyclin B is destroyed, leading to a reduction of p34cdc2 activity. Thus, the regulation of p34cdc2 activity is hypothesized to provide the "timer" that regulates cortical contraction (Walker *et al.*, 1997). There is an increasing amount of experimental evidence to suggest that protein kinases coordinate the transition from mitosis to cytokinesis, and may also control the position and organization of the contractile ring (Walker *et al.*, 1997).

Many studies have examined the relationship of phosphorylation of myosin II and myosin II activity. Myosin regulatory light chain (MRLC) is phosphorylated by a number of protein kinases. Myosin light chain kinase (MLCK) is the predominant protein kinase responsible for stimulation of

myosin activity. Phosphorylation of MRLC at the protein kinase C target sites inhibits myosin ATPase activity. Inhibition is achieved in one of two ways. One, if these myosin light chain kinase sites are not phosphorylated, it reduces the subsequent phosphorylation at the MLCK sites by MLCK. Secondly, if the MLCK sites are already phosphorylated, it inhibits the actin-activated ATPase activity, their ability to hydrolyze ATP, by reducing the affinity of myosin for actin filaments (Mabuchi et al., 1996). It has been shown that cytokinesis is regulated by protein phosphorylation, and that the phosphorylation of MRLC might be a key event in this regulation.

The regulatory machinery directs passage of the cell through the cell cycle. This regulation involves a variety of stimulatory and inhibitory growth factors, some of which may be synthesized by the cell itself, and some of which may be synthesized and released into the surrounding medium by neighboring cells. Studies have revealed that cell replication is primarily controlled by regulating the timing of two critical events in the cell cycle: the initiation of DNA synthesis and mitosis. A family of cycle dependent protein kinases, maturation promoting factor (MPF), are the master controllers of these events. They regulate the activities of multiple proteins that play a role in cell cycle progression. This regulation involves phosphorylating the proteins at specific activating sites, as well as inhibitory

sites. The regulatory subunit of MPF is cyclin. Cyclins concentration cycles in phase with the cell cycle. Cyclins are responsible for the activation of the catalytic subunit of MPF. The catalytic subunits are called cyclin-dependent kinases (cdks). They have no kinase activity unless they are complexed with cyclins. Each cdk catalytic subunit can associate with different cyclins, and the associated cyclin determines which target proteins are phosphorylated by the cdk-cyclin complex (Alberts *et al.*,1994).

There are hundreds of protein kinases in a cell and they are organized into a network of signaling pathways. In addition to MPF, other protein kinases help coordinate the cell's activities, drive the cell cycle, and relay signals into the cell. Two protein kinases of great importance that have been well studied are erk (mapk) and cdc2(cdc1). These kinases are key agents that contribute to the orchestration of cell cycle activities. Mitogen activated protein kinase erk1 (extracellular signal-regulated protein kinase 1) has an impact on processes in the cytoplasm, the nucleus, the cytoskeleton, and the membrane (Cobb *et al.*, 1994). This family of protein kinases play a significant role in hormonal signal transduction. Activation of map kinases requires two phosphorylations. Increased activity results from phosphorylation of a Tyr and Thr residue that lie one residue apart on each enzyme (Cobb *et al.*, 1994). Inactivation occurs following

dephosphorylation by a phosphotyrosine phosphatase, a phosphoserine/threonine phosphatase, or a dual specificity phosphatase (Cobb *et al.*, 1994). Map kinases have significant regulatory power, which is evident from their range of substrates. These substrates include other protein kinases, cytoskeletal proteins, transcription factors, membrane enzymes, and other enzymes (Cobb *et al.*, 1994).

Along with map kinases like erk1, cdc2 also has a significant function in cell cycle regulation by helping to orchestrate cell cycle dependent changes in cell structure and function (Pockwinse *et al.*, 1997). Cdc2, a cell cycle regulator, is a catalytic subunit of a protein kinase complex called the M-phase promoting factor which induces entry into mitosis and is universal among eukaryotes (Draetta *et al.*, 1988). Cdc2 can be turned on and off in response to specific cellular signals. Phosphorylation of threonine in cdc2 is required for strong cyclin binding and kinase activity. Its dephosphorylation is necessary for cells to exit mitosis (Figure 2)

The mitotic apparatus plays a critical role during the process of karyokinesis. The mitotic apparatus attaches to and captures chromosome pairs, aligns the chromosomes, and then separates them. With the help of microtubule motor proteins and the microtubule assembly, the process of mitosis rarely fails. Evidence also suggests that protein kinases play a large

role in regulation of the cytoskeleton. A study by Walker *et al.* (1997), tested whether the isolated cell cortex, composed of actin filaments and actin-binding proteins, was subject to regulation by kinases. Studies of the cortex demonstrated that three major polypeptides were phosphorylated by kinases associated with the cortex. Two cortical kinases, P34cdc2 and a Map kinase were found to be associated with the cortical actin cytoskeleton and their activity was cell cycle dependent, in that they were varied in levels association at different phases in the cell cycle.

Numerous studies have been performed over the past several years to examine the role of the cytoskeleton during cytokinesis and its regulation throughout the cell cycle. A question that remains however, is the association of protein kinases with these processes. Such areas include the effects of protein kinase C on the actin cytoskeleton and the characterization of the association between protein kinases and the cytoskeleton.

The importance of the PKC family in cell regulation is usually assessed by activation of PKC with phorbol esters or by inhibition of PKC activity with non-selective pharmacological inhibitors. Through recent studies, it is believed that members of the PKC family play a critical role in the regulation of cell proliferation and differentiation. Staurosporine is an inhibitor of a number of protein kinases, including acting as a potent

Figure 2: A diagram of cdc2 and its regulation on cell division. Figure 2 courtesy of Dr. Gary Walker.
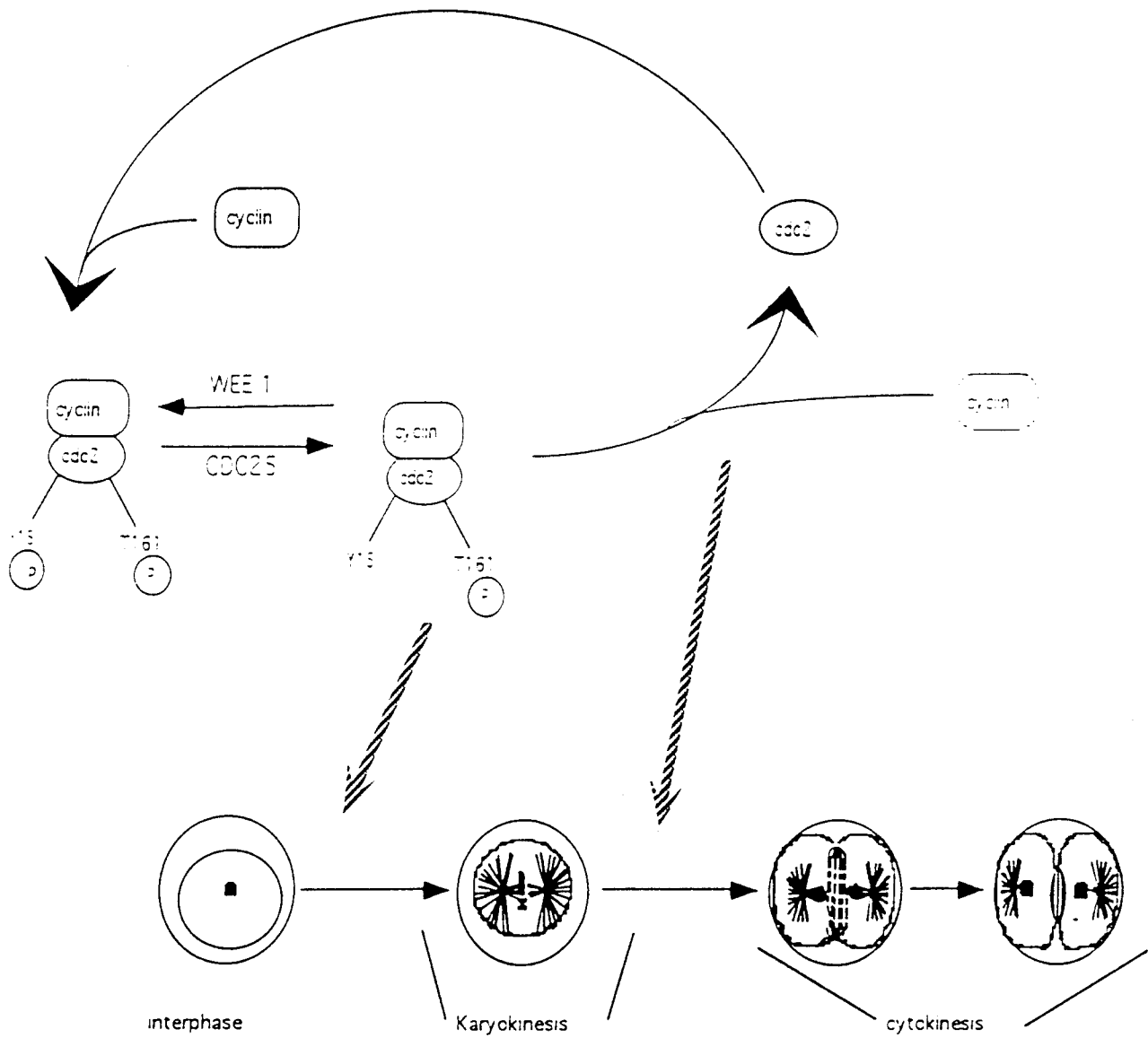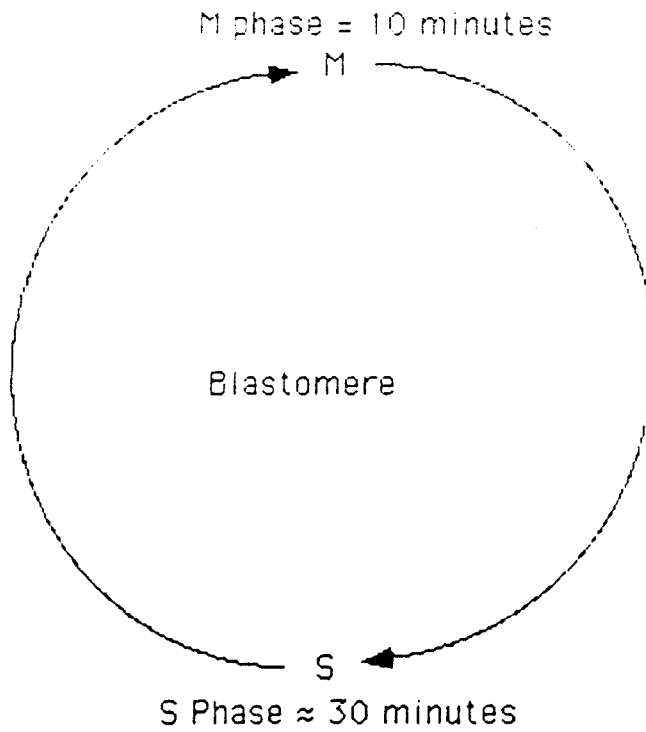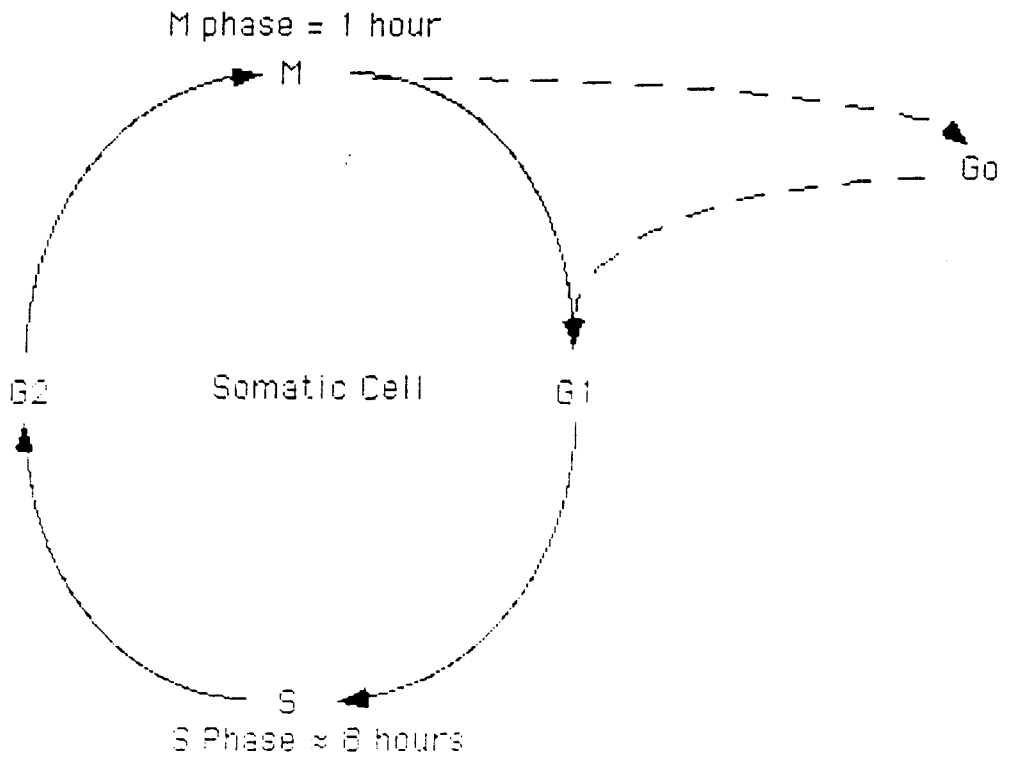
Cyclin B/ cdc2 complex regulates cell division.

inhibitor of protein kinase C. Studies were done with staurosporine on human lymphocytic leukemia MOLT-4 cells to see the possible effects it had as an inhibitor (Tragonas *et al.*, 1994). From these studies, several conclusions in regards to the relationship of staurosporine and cell division were drawn. For one, it was evident that staurosporine caused some cells to undergo DNA replication without undergoing cytokinesis. Data also indicated that staurosporine at concentrations up to $0.1 \mu M$ had no effect on MOLT-4 cell progression through G_1, S, or G_2 phase, but did inhibit cytokinesis (Tragonas *et al.*, 1994). Another aspect examined by staurosporine studies confirmed chromosome condensation in staurosporine treated cultures, correlated to the phosphorylation state of p34cdc2 or its kinase activity. It was concluded that p34cdc2/cyclin B activation was unaffected by staurosporine. Overall, the only observable change in MOLT-4 cells with treatment of staurosporine was the failure of cells to undergo cytokinesis. However, when staurosporine was removed, cell division was able to restart. Thus, it was concluded that MOLT-4 cells contained a kinase that controlled cytokinesis and was staurosporine sensitive, whereas all other kinase-mediated activities were "refractory" to staurosporine (Tragonos *et al.*, 1994).

These findings indicate that protein phosphorylation is critical in the regulation of the cell cycle and cytokinesis. Thus, examination of the physical relationship between cell cycle dependent protein kinases and the cytoskeleton may lead to a greater understanding of the regulation of cytokinesis.

The sea urchin blastomere and a somatic cell have differing cell cycles. The blastomere has a modified cell cycle as compared to the somatic cell (Figure 3). Thus, the goal of this study is to examine kinase location in two different, yet similar systems involving the sea urchin blastomere and the rat muscle fibroblast. The specific aim is to determine whether erk (mapk) and cdc2 (cdc1) are associated with a somatic cell cytoskeleton, and to compare any association with that of the sea urchin blastomere. Thus, my hypothesis is that erk and cdc2 are associated with the detergent insoluble extracted cytoskeleton of the rat muscle fibroblast.

Figure 3: A diagram comparing the cell cycle of a somatic cell versus the cell cycle of a blastomere. The somatic cell cycle is typically 18hrs: G_1 is about four hours, G_2 is about five hours, S phase is about eight hours, and M phase is about one hour. In a blastomere, G_1 and G_2 are virtually non-existent or very minimal and S is about 30 minutes and M phase about 10 minutes. Thus, the blastomere cell cycle is approximately 1hour. Figure 3 courtesy of Dr. Gary Walker.

M phase = 1 hour

M

G2   Somatic Cell   G1

Go

S

S Phase ≈ 8 hours

M phase = 10 minutes

M

Blastomere

S

S Phase ≈ 30 minutes

III. Materials and Methods

A. Experimental Design:

Cultured fibroblasts were grown in vitro from rat muscle obtained from sacrificed rats. After an adequate amount of cells were attained, isolation of the detergent insoluble cytoskeleton extract was performed. This isolation was performed at various time points analogous to times in the cell cycle. The cells were synchronized by utilizing an agent (nocodozole), which arrested all cells at the same point of M phase, thus ensuring that when released from cell cycle inhibition, all cells would start at the same point in the cell cycle. After isolation of the cytoskeleton, SDS-PAGE and Western blot analysis was used to determine if erk and cdc2 were associated with the detergent insoluble cytoskeleton extract.

B. Cell Isolation and Growth of Fibroblasts:

Muscle fibroblasts were isolated by the method of Askanas et. al (1997). Male rats were sacrificed by CO_2, and then transferred to a sterile surgical compartment where sections of the rat pectoral muscle were cut and placed into sterile saline. The muscle was cut into fine pieces and small sections were placed into each well of a 24 well culture plate. To each well, approximately 1ml of complete media was added.

All cells were maintained and grown in complete media, containing DMEM (Delbecco's Modified Eagle Medium), 0.2 mM pyruvate, 0.5 mM glucose, 10% FBS (fetal bovine serum), 1/1000 units penicillin, and 1/1000 units streptomyocin. The penicillin and streptomyocin were added to prevent bacterial growth. This media was filter sterilized to prevent bacterial growth in culture. After adding complete media to each well (which was all performed in the Bioflow chamber from the Germfree laboratories, Inc.), plates incubated at 37°C, in an atmosphere of 5% CO_2 and 95% air. The media was changed daily and the cells were monitored closely to assure that there was no contamination. After approximately one week, the first round of fibroblast cells migrated out of the muscle. At approximately three weeks in culture a confluent layer of fibroblasts formed in each well. At about the fourth week, the muscle sections were removed and placed into another 24 well tray for the production of a second fibroblast culture. Once the cells achieved confluency and the muscle tissue was removed, extraction procedures were begun.

Fibroblast cells were treated with nocodozole to synchronize all of the cells at the same point in the cell cycle. This agent arrests cells in M phase by disrupting microtubule assembly. The media in each culture plate well was replaced by approximately 1 ml of media containing nocodozole (10

µl). The cultures were incubated for 24 hours to allow ample time for all cells to be arrested at M phase. Then four time points were selected for analysis at various points in the cell cycle. Cells from six culture wells were pooled for each time point.   These were:

T=0 @ 0 hours

T=1 @ 5 hours

T=2 @ 18 hours

T=3 @ 27 hours

After incubation in nocodozole for 24 hours, the media was removed and replaced for two minutes with incomplete media to all the wells (that was strictly DMEM with no other components). This was done to wash away any remnants of nocodozole. The incomplete media was then once again replaced with complete media. The cells were then able to resume their cell cycle.

C. Fibroblast Cytoskeleton Preparation

Once the cells had undergone M phase arrest by treatment with nocodozole, detergent extraction procedures were begun at the selected four time points. Six wells were used at each extraction time point. For the first time point of T=0, which was immediately after the 24 hour treatment with nocodozole, the media was removed from six wells and 1 ml of isolation

buffer was added to each of the six wells. The isolation buffer contained .99 mM glycerol, 19.9 mM PIPES, 4.8 mM EGTA, 4.9 mM $MgCl_2$ $6H_2O$ and titrated to pH 6.9 with 10N NaOH. After sitting in isolation buffer for approximately 2 minutes, the isolation buffer was removed and 500µl of extraction buffer was added to each of the six wells. The extraction buffer contained a 1:1 dilution of isolation buffer and Walker buffer. The Walker buffer was composed of 12 ml 10% Nonidet Octylphenoxypolyethoxyethanol (NP-40) and 88 ml isolation buffer. The cells were exposed to extraction buffer for ten minutes. This was then removed and 1 ml of isolation buffer was added to each well for two minutes. The isolation buffer was removed and 250 µl of SDS sample buffer was added to each well for ten minutes. The samples from each well were then collected and placed into a microcentrifuge tube. This tube was boiled for one minute, labeled T=0, and frozen at -30°C until needed for analysis.

Additional cell samples consisting of fibroblasts from six culture wells were also obtained for the T=1 (5 hr.), T=2 (18 hr.), and T=3 (22 hr.) time points. To assure adequate sample sizes, two additional rounds of time points were similarly collected and prepared for analysis. The cells undergoing extraction were remarkably different in appearance (Figure 4),

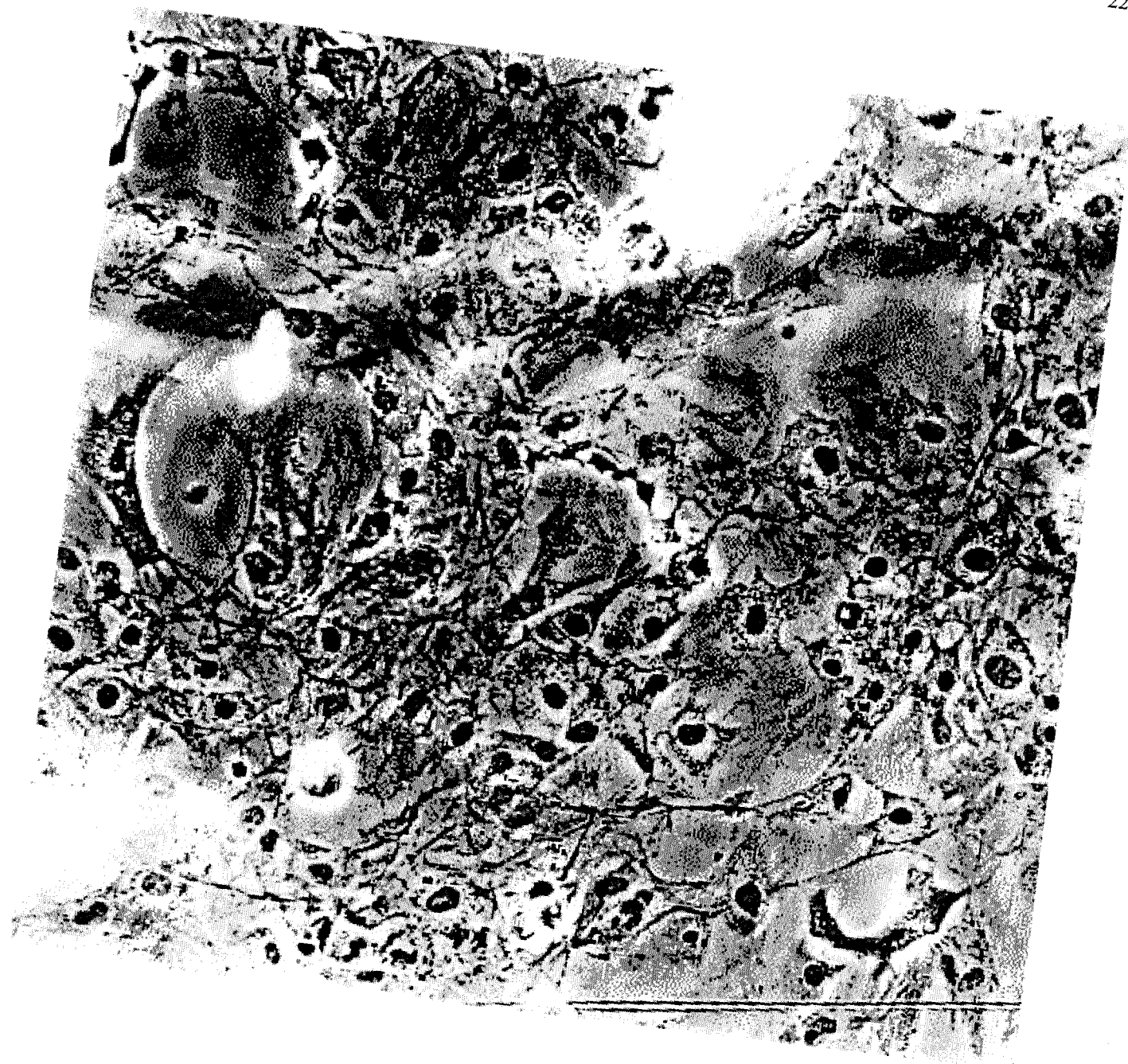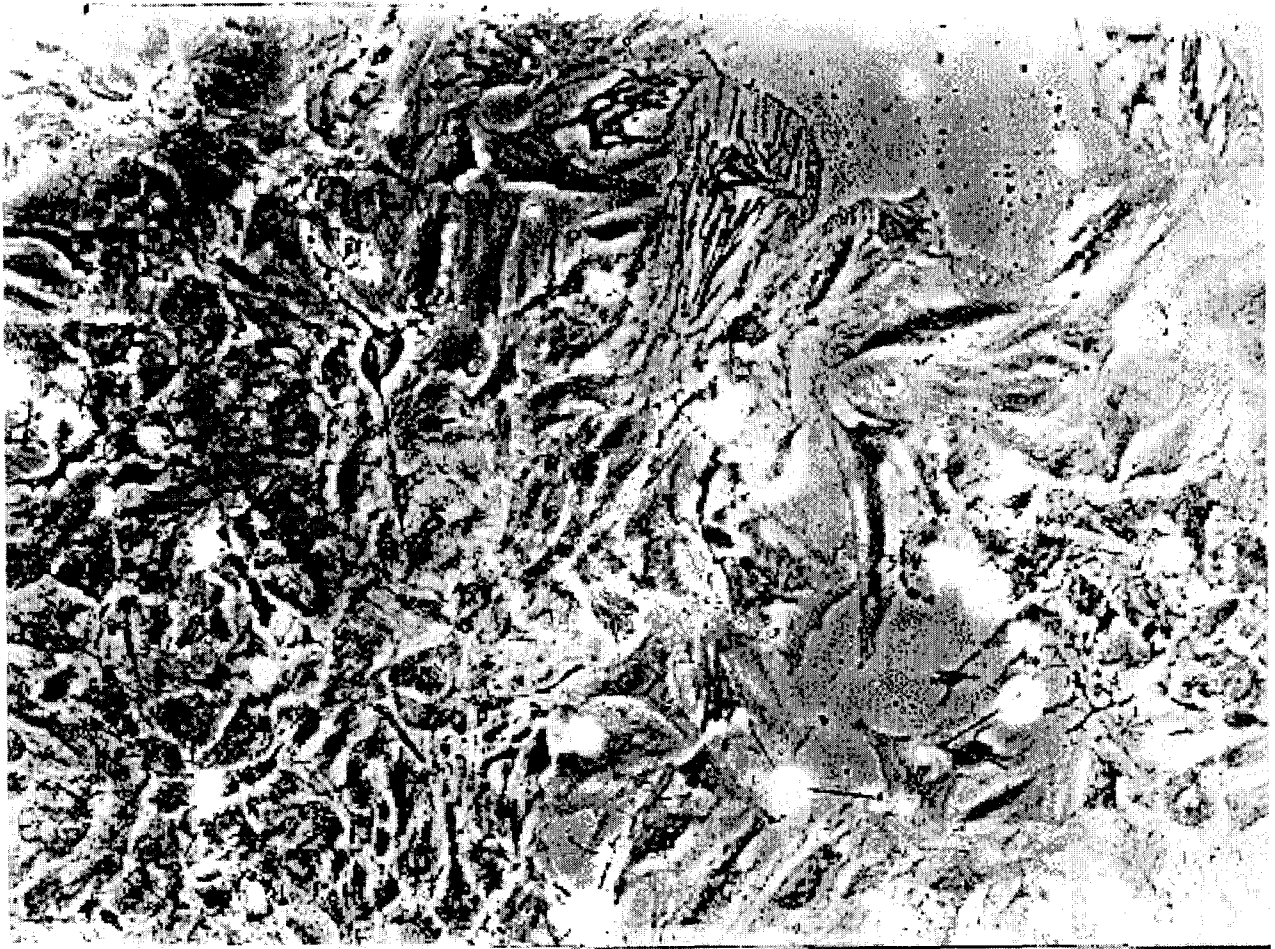Figure 4: Rat muscle fibroblasts while in extraction buffer.

Figure 5: Rat muscle fibroblasts before extraction.

as compared to those not extracted (Figure 5). The extraction was assessed visually using an Olympus IMT-2 microscope where photographs of the cells could be taken while undergoing extraction.

D. SDS-PAGE:

Once samples for the four time points were collected, the first step of analysis involving SDS-PAGE (sodium dodecyl sulfate- polyacrylamide gel electrophoresis) was begun (Matsudiara *et al.*, 1978). This technique separates proteins on the basis of size only. By using the ionic detergent SDS, proteins are given the same charge to mass ratio and so they move at the same rate in an electric field. β-mercaptoethanol is added to break disulfide bonds. The proteins are then electrophoresed in a gel, small proteins moving faster than large. A series of molecular weight standards are also run with the experimental samples to serve as controls for data analysis and interpretation of the results.

1. Preparation of minislab gels:

Resolving gels were prepared in a gel-pouring chamber at a concentration of 10% acrylamide. These resolving gels were prepared in batches of 12. The 10% gels were made by the addition of 6.4 gr glycerol, 23 ml resolving gel buffer (Sigma™), 24 ml acrylamide, 240 μl APS (ammonium persulfate) and 96 μl TEMED (Sigma™) to 41 ml ddH$_2$0.

The acrylamide solution was poured between glass plates separated by 0.5 mm spacers. This solution was overlaid with resolving gel buffer diluted 4:1 with ddH$_2$0. These gels were allowed to polymerize overnight at room temperature. Gels were removed from the pouring chamber, the overlay solution was discarded, and the gels were stored in an airtight container at 4°C.

A 10% gel was clamped into place in the gel chamber. The top part of the chamber was sealed with agarose to prevent any leaking and then overlaid with stacking gel. This stacking gel was made by the addition of 1.1 ml stacking gel buffer (prepared from stock consisting of 6.05gr Tris, pH 6.8-7.0, + 0.4 gr SDS + dH$_2$0 to 100mls) + 2.6 ml H$_2$0 + 0.75 ml acrylamide stock + 30µl of 10% ammonium persulfate and 11µl of TEMED. Two pasteur pipet full of stacking gel solution were quickly added in between the plates and a ten well comb was inserted evenly between the glass plates to allow space for the samples. The gel was allowed to polymerize for 30 minutes, after which the comb was gently removed, the lanes straightened, and bubbles popped in the wells. One set of samples were thawed (T=0, T=1, T=2, and T=3), and loaded in the gel. The first lane was loaded with 5 µl of pre-stained standard. Additional lanes were individually loaded with 20 µl of T=0, T=1, T=2, and T=3 samples. Each well was topped off with

electrode buffer [consisting of 6.05 gr Tris + 28.84 gr glycine + 2.00 gr of SDS and distilled water to 2000 ml]. Both the top and bottom chambers were filled with electrode buffer to allow them to be conductive. The gel was then connected to a power supply with the negative electrode attached to the upper half of the chamber and the positive electrode attached to the bottom half of the chamber. This arrangement resulted in negatively charged proteins running to the bottom of the gel. The acrylamide gels were run for one hour at a constant current of 0.025 amp. After this time, the glass plates were pried apart and the gels were removed for further analysis by either coomassie staining or western blotting.

In some gels, whole cell samples (the cell with all of its components in tact) were used as a standard. The whole cell sample was prepared by using only isolation buffer and SDS-sample buffer, extraction buffer was not added. Lanes were individually loaded with 20μl of T=0, T=1, T=2, T=3, and whole cell samples and run following the same protocol described above.

2. Coomassie and GelCode Staining of minislab gels:

For gels to be stained by coomassie or GelCode, the first lane was loaded with 5 μl of a molecular weight standard, 15μl of whole cell sample was loaded in the next lane, and additional lanes were individually loaded

with 20 µl of T=0, T=1, T=2, and T=3 samples. This gel was run slower at a constant current of 0.015 amp for approximately two hours. Two different staining methods were utilized, coomassie and GelCode staining.

In coomassie staining, the minislab gels were placed in a solution of 0.2% Coomassie Brilliant Blue R-250, 45% methanol, and 10% acetic acid. The gels were stained for a period of about 30 minutes. The gels were destained in a solution of 45% methanol and 10% acetic acid for a period of one hour with frequent changes of the destaining solution. The stained gels were dried between two sheets of dialysis membrane and analyzed for total protein distribution by densimetric analysis.

For GelCode staining, the acrylamide gels were transferred to a clean tray after electrophoresis and rinsed 3 times, 5 minutes each, with 100-200 ml of deionized water, and then placed into 20 ml of Gel Code Blue Stain Reagent. The tray was placed on a shaker and stained for approximately 1-2 hours. After this, the staining reagent was replaced with deionized water. Water equilibration enhances staining sensitivity as weak protein bands further develop in deionized water. The stained gels were then dried between two sheets of dialysis membrane and analyzed for total protein distribution.

E. Electrophoretic transfer of proteins:

For gels to be used for Western blotting, it was necessary to transfer the proteins that had previously been separated by SDS-PAGE onto a support membrane that binds proteins for immunoblot analysis. Minislab gels prepared as previously described were transferred onto sheets of PVDF membrane by the use of a semiwet blotting apparatus from Idea Scientific "Genie".

The tray was assembled from the bottom up. First the negative electrode was placed in the transfer chamber, followed by a bubble screen. The next layer in the transfer chamber consists of two Scotch Brite pads. The chamber was then filled to the level of the Scotch Brite pads with transfer buffer. This transfer buffer consisted of 57.6 gr glycine, 12.1 gr TRIS, 800 ml methanol and 3200 ml ddH_2O. A piece of filter paper cut to the size of the Scotch Brite pads was placed in the chamber and the bubbles were squeezed out to ensure a clean transfer of proteins. The gel was gently placed on top of the filter paper, ensuring that there were no bubbles between the layers. The PVDF membrane was prepared for transfer by first wetting it in methanol, then rinsing in distilled water. This sheet was carefully placed on top of the gel. A second piece of filter paper was placed over the PVDF. Two more Scotch Brite pads were placed in the chamber.

A second bubble screen was placed in the chamber, followed by the positive electrode. The chamber was closed with a Plexiglas sheet and placed into the running chamber. The whole apparatus was tilted into running position, and the positive and negative electrodes were connected to the power supply. Proteins were transferred at a constant 25 volts for one hour.

F. Western Blotting:

Western blotting is a process of using protein specific antibodies to selectively stain a membrane that contains proteins of interest.

1. Blocking of PVDF:

PVDF sheets were placed in a blocking solution for one hour in order to prevent non-specific binding of antibodies to the PVDF membrane. This solution consisted of 5% powdered non-fat dry milk and TBS-T (Tris buffered saline containing 0.5 M NaCl + 20 mM TRIS (hydroxymethylaminomethane) + 2% Tween 20 detergent and adjusted to a pH of 7.3).

2. Primary Antibody Application:

After blocking, the PVDF sheets were placed in primary antibody (either rabbit anti-Cdc2 or mouse anti-Erk). A total volume of 20 ml was used (that is 16 ml of TBS-T + 4 ml blocking buffer + 10μl of primary

antibody). After one hour incubation with primary antibody, the PVDF sheet was rinsed 3 times, 5 minutes each, in TBS-T.

3. Application of Secondary Antibody:

After the three washes, they were placed in secondary antibody for one hour with a total volume of 30 ml (22.5 ml TBS-T + 7.5 ml blocking buffer + 10μl of secondary antibody). The secondary antibodies were obtained from SIGMA Chemical Co., and were either goat anti-rabbit to label anti-cdc2 or goat anti-mouse to label anti-erk. Any unbound secondary antibody was removed by rinsing the PVDF sheets 3x with TBS.

4. Chemiluminescent Detection:

Secondary antibodies, with attached HRP labels (Sigma Chem. Co.™), consisted of either goat-antirabbit (for visualization of anti-cdc2) or goat-antimouse (for visualization of anti-erk). The detection of bound secondary antibodies was performed by the addition of a chemiluminescent substrate that would react with the HRP labeled secondary antibodies. This substrate was PIERCE Super Signal™ ULTRA. Substrate (2 ml) was applied to each membrane and allowed to react for a period of 2 minutes. The PVDF membrane was then placed between two sheets of clear plastic. Care was taken to avoid the occurrence of bubbles between the membrane and the sheets. In the darkroom, light sensitive paper was layered over the PVDF

membrane. The initial exposure was for 30 seconds, with subsequent exposures being either longer or shorter depending on the amount of chemiluminescent signal.

After developing, the photos were dried for latter scanning and analysis. Due to different variables that were beyond our control, it was critical to duplicate this process several times to eliminate any variables and to confirm that the results attained were consistent.

G. Gel Scanning and Analysis:

Images of gels and blots were digitally scanned and computer stored for analysis. The developed autoradiograms were scanned using a StudioScanIIsi flatbed scanner, a Macintosh Quadra 800 computer, and Adobe Photoshop 3.0 software. The blots were scanned using the following perimeters: a gray-scale mode, a reflective original, scaled to 100%, high sharpness, general preferences, and optimum quality. Scanned images were analyzed using the NIH Imager software program. This program allows specific lanes on a gel or blot to be isolated and calibrated for densimetric calculations. Base to peak quantitations can be calculated and compared to known control values of proteins such as actin.

IV. Results

Extraction procedures were utilized to isolate a detergent insoluble cytoskeletal fraction to investigate cytoskeleton regulation during cell division. Characteristic proteins that define the cytoskeleton are found in the detergent insoluble extracted sample. Changes in the optical properties of cells are also noticeable during the extraction process, (Figure 4 & Figure 5) when distinct filamentous stress fiber-like structures within the extracted cells are observed. Stress fibers are prominent components of the cytoskeleton of fibroblast cells. After extraction it is also noticeable that the membranes have dissolved, changing the appearance of the cell's edges. Thus, the optical properties of the cell have changed. Also during extraction, the nuclei are still visible which suggests the continued presence of chromatin associated with the cytoskeleton (Figure 4). An abundance of actin was found in the detergent insoluble fraction, indicating that actin is an insoluble protein.

The fibroblast's cytoskeleton was found to have a characteristic protein composition. Figure 6 shows acrylamide gel separations of fibroblast proteins, comparing whole cell proteins to the insoluble protein fraction. The whole cell sample shows a more complex protein composition. The detergent insoluble fraction (cytoskeleton) has a higher concentration of

cytoskeleton proteins, such as actin, since all of the soluble non-cytoskeletal proteins are removed by the extraction. Actin is present in both the whole cell sample and cytoskeleton sample. Actin is found in a higher concentration in the cytoskeleton sample, primarily because the cytoskeleton fraction is enriched in cytoskeletal proteins.

There is also an association of growth-related protein kinases with the extracted cytoskeleton. Specifically, mapk's (erk) are found in the detergent insoluble fraction (Figure 7). Map kinase has a molecular weight of around 42-44 kD. This association also indicates that some portion of the soluble kinase remains attached to the cytoskeleton after detergent extraction, suggesting that erk is not strictly a soluble protein, but can be associated with other areas of the cell. The presence of two bands in both the whole cell and cytoskeleton sample indicates the presence of different isoforms of erk.

The protein cdc2, another cell growth related protein kinase, was also found to be associated with the cytoskeleton after extraction. Cdc2 has a molecular weight of 32 kD. Figure 8 shows a western blot of whole cell (labeled W) and cytoskeleton (labeled C) protein blotted with anti-cdc2 antibody. Protein bands are noticeable in both the whole cell and cytoskeleton lanes. Cdc2 appears as multiple bands in both blots. The

presence of multiple bands may be due to different phosphorlylation states of the protein, which causes changes in protein conformation, or may indicate the possibility of different isoforms. This phenomenon can also be seen with the erk blots (Figure 7). Alternatively, discrepancies could arise from the use of the antibody. Antigen specificity of the primary antibody may also effect the banding pattern. For these reasons, the possibility of non-specific binding by the primary antibody can not be ruled out.

The amount of actin in detergent extracted cytoskeletal samples is not reflective of the total actin amount present in the cell. Actin exists as both filamentous actin and soluble actin. The soluble form is removed by detergent extraction, leaving the filamentous form as part of the insoluble cytoskeleton protein fraction. Actin is one of the most abundant proteins in the cell and is the best protein to use in normalization for quantitative analysis. The degree of actin enrichment in the cytoskeletal fraction was determined by comparing the ratio of actin to total protein in whole cell versus cytoskeleton samples (Figure 9). Actin quantitation was carried out by densitometric analysis of coomassie stained gels. The identity of the peaks were determined based on known molecular weights and by comparing the plot with the actual coomassie gel. The top profile shows whole cell proteins, with cytoskeleton proteins shown in the lower profile.

Approximately 2.97% of the total protein content is actin in the whole cell sample, while approximately 14.8% of total protein is actin in the insoluble fraction. This is a 5-fold increase in the proportion of actin in the cytoskeleton compared to whole cell. Actin levels in whole cell and extracted samples were also determined from densitometric plots using different approaches. The method that was most appropriate involved selecting an arbitrary baseline and then calculating the relative density (Figure 9).

The relative amounts of immunoreactive bands in western blots were determined by normalizing the optical density of the western blot band to actin optical density in the lane profiles of whole cell protein and cytoskeleton protein (Figure 10). The optical density ratio of erk to actin was calculated for whole cell protein samples and cytoskeleton samples. The erk/mapk ratio was 4.8 in whole cell compared with 6.8 for cytoskeleton. The fact that the concentration of erk/mapk rose in cytoskeleton suggests that a certain portion of erk/mapk is specifically associated with the cytoskeleton.

Figure 6: Gel Code stained 10% gel showing total protein distribution in a

sample prepared by SDS-PAGE. Lane 1 Bio-Rad prestained molecular

weight standard, lane 2 shows total of whole cell protein compsotion, and

lane 3 shows cytoskeletal protein compositon obtained from the insoluble

fraction.

116kD ——

97kD ——

66kD ——

45kD ——

29.5kD ——

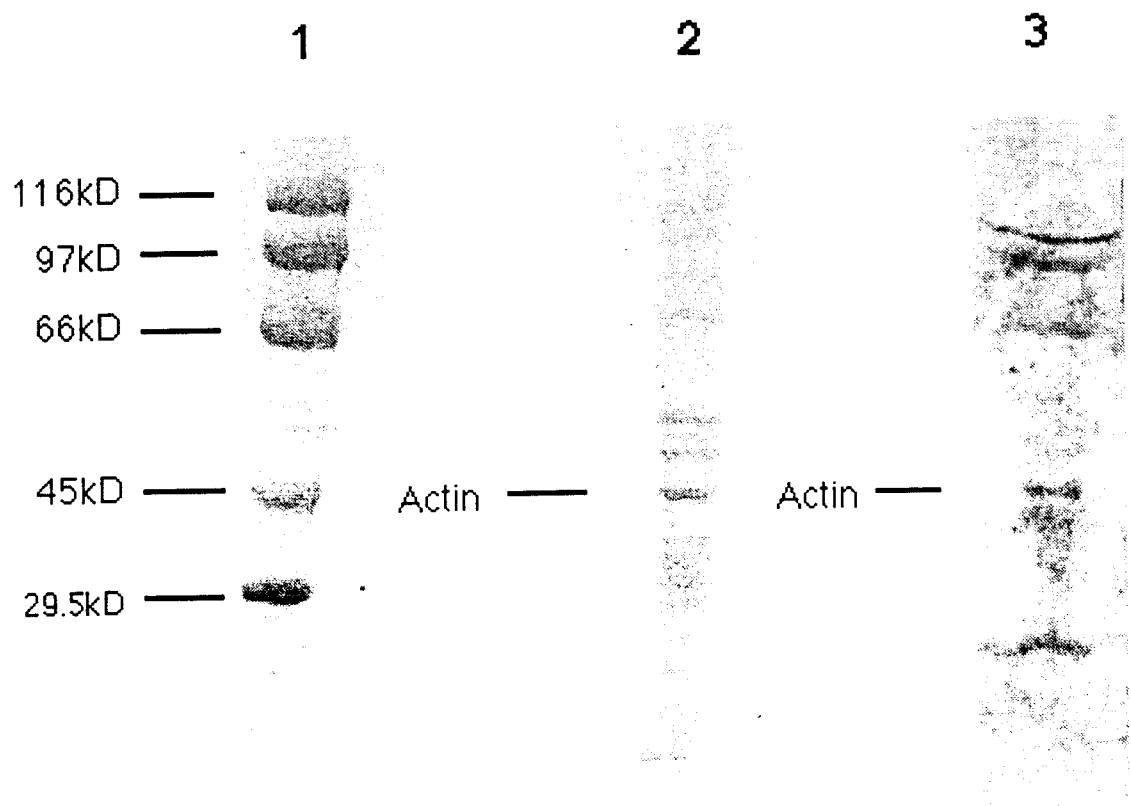1   2   3

Actin ——

Actin ——

Figure 7: Immuno detection of mapk in this detergent insoluble fraction

reveals an association of growth kinases with the cytoskeleton. Western

blots of 10% acrylamide gel using anti-erk, lane "W" is a whole cell sample

labeled with anti-erk primary antibody at a 1:3000 dilution, lane "C" is a

cytoskeleton sample labeled with anti-erk primary antibody at a 1:3000
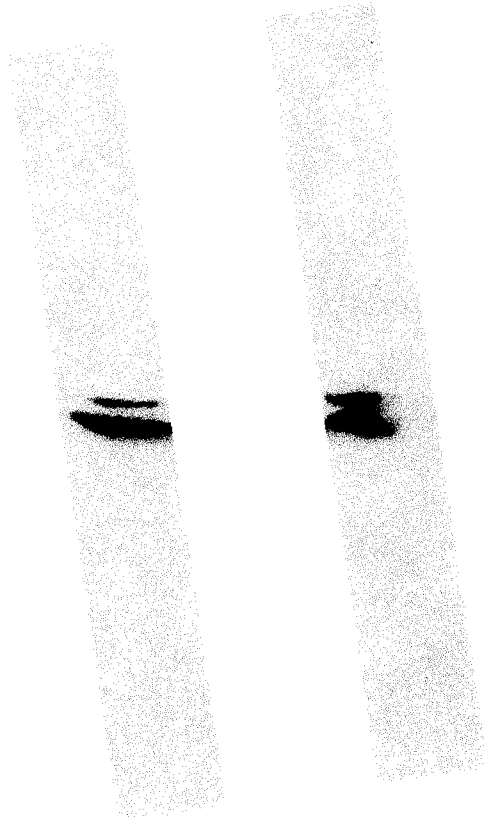
dilution.

Figure 8: Immunodetection of cdc2 in this detergent insoluble fraction reveals an association of growth kinases with the cytoskeleton. Western blots of 10% acrylamide gel using anti-cdc2, lane "W" is a whole cell sample labeled with anti-cdc2 primary antibody at a 1:3000 dilution, lane "C" is a cytoskeleton sample labeled with anti-cdc2 primary antibody at a 1:3000 dilution.

W   C

Figure 9: Quantitation was carried out by gel scanning of coomassie stained

gels. Optical density lane profiles of whole cell protein and the CSK fraction.

The top profile is whole cell and the bottom profile is CSK. The actin band

is indicated.

Actin

Whole Cell
Fraction

Bottom of
Gel

Top
of
Gel

Cytoskeleton
Fraction

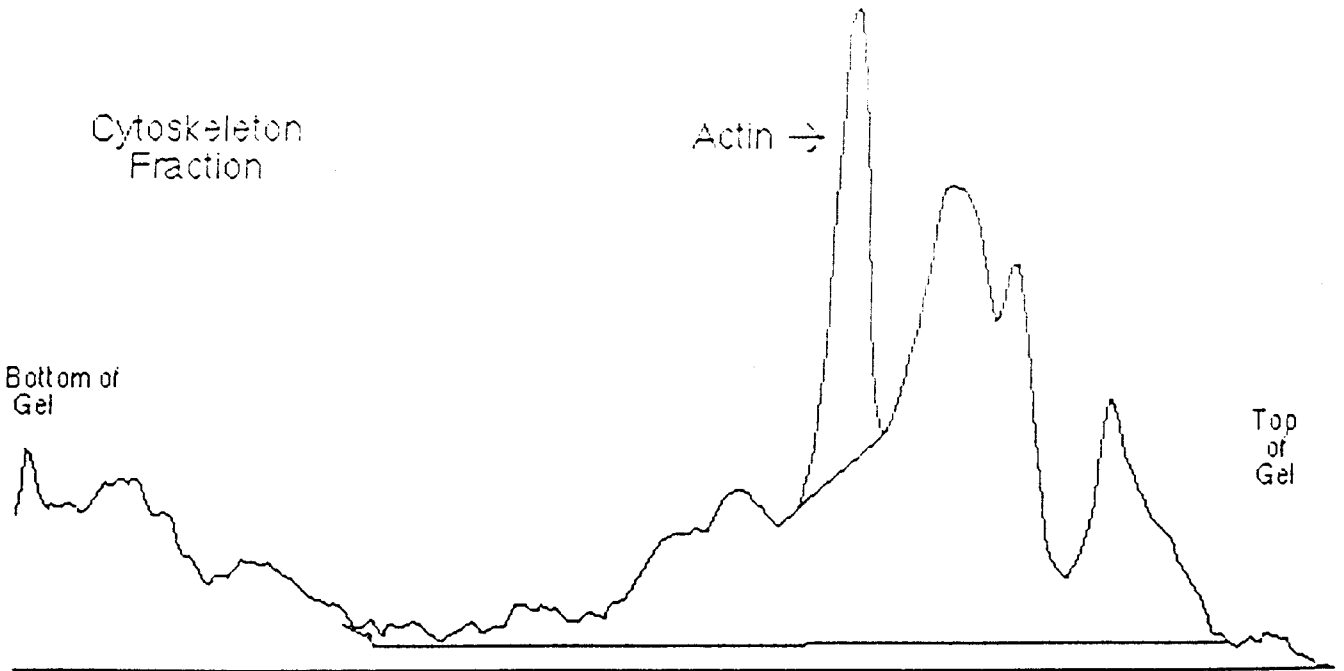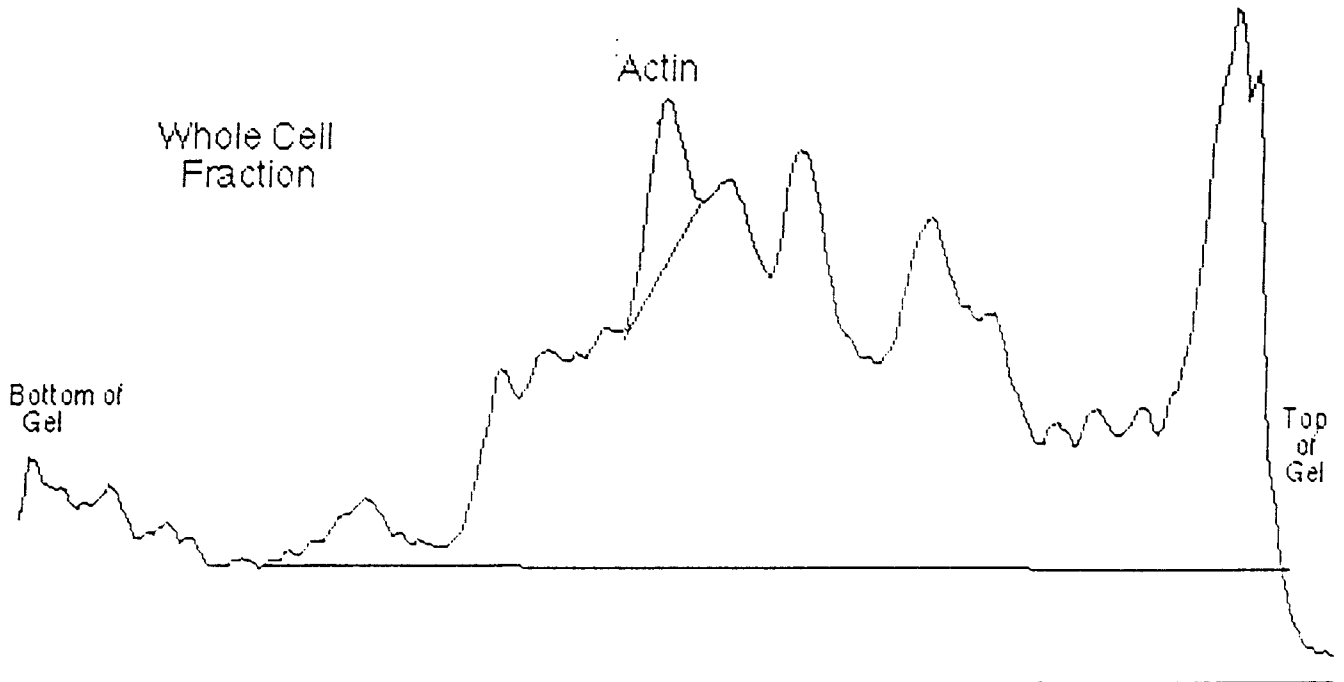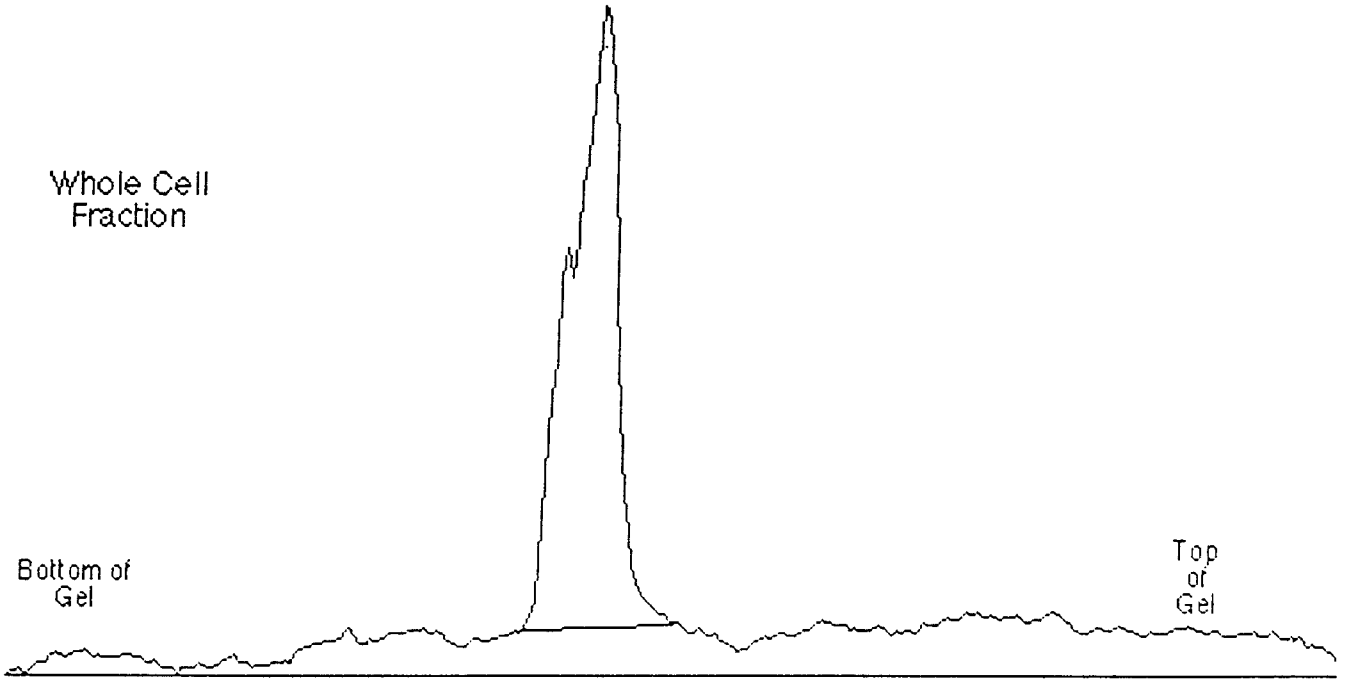Actin →

Bottom of
Gel

Top
of
Gel

Figure 10: Optical density of the western blot autoradiographs were determined and the relative amount of erk was determined for the whole cell and cytoskeleton protein samples. The top profile is a whole cell protein sample and the bottom profile is a extracted protein sample.
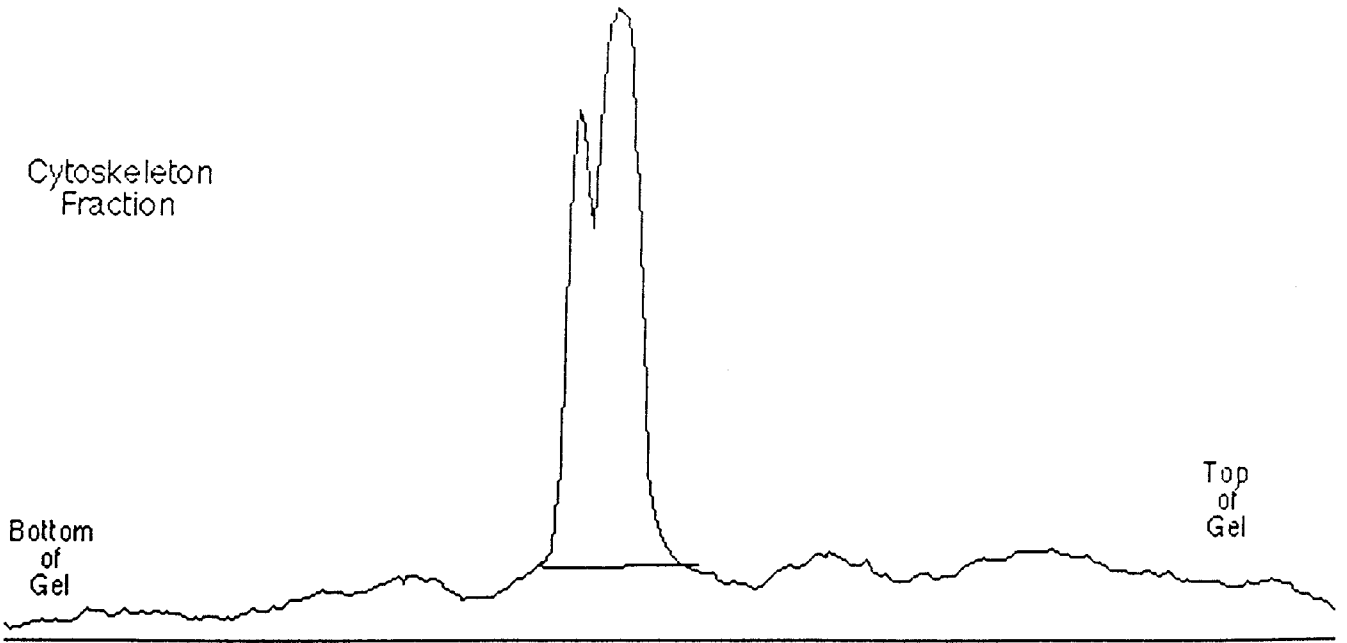
Whole Cell
Fraction

Bottom of
Gel

Top
of
Gel

Cytoskeleton
Fraction

Bottom
of
Gel

Top
of
Gel

V. Discussion

Cytokinesis represents the terminal event of mitosis in which the cytoplasm is partitioned into two daughter cells through the constriction of an actomyosin contractile ring. However, cytokinesis cannot occur until karyokinesis (division of the nucleus) is complete. One possibility is that the mitotic apparatus involved in karyokinesis contributes to the regulation of the cortical cytoskeleton contractile ring. This hypothesis is supported by experiments demonstrating that the position and induction of the cleavage furrow is influenced by the mitotic apparatus (Rappaport, 1986; 1996). Thus, karyokinesis and cytokinesis though separate in nature, may work together in a very dependent manner.

The cell cycle is a regular sequence of growth and division phases a cell must pass through before it can physically divide into daughter cells. These phases are carefully regulated by the cell as it passes through each cell cycle. Current evidence indicates that the master controllers of these events are protein kinases, which regulate the activities of multiple cell cycle proteins. Two key kinases involved in various regulation mechanisms are erk and cdc2. These two kinases play several roles that contribute to regulation of the cell cycle.

Weber et al. (1997) showed that sustained activation of erk1 was required for the continued expression of cyclin D1 during the G_1 phase. Inhibition of erk1 in hamster fibroblasts led to a loss of cyclin D1 mRNA and protein expression, and G_1 growth arrest (Weber *et al.*, 1997). This suggests that erk activity is required for the continued expression of cyclin D1, and thus for progression of the cell through G_1 phase of the cell cycle (Weber *et al.*, 1997).

Cdc2 protein kinase is a cell cycle regulating kinase that helps to organize cell cycle dependent changes in cell structure and function. It is a catalytic subunit of a larger protein kinase complex called the M-phase promoting factor. This factor induces entry into mitosis and is found in all eukaryotic cells (Draetta *et al.*, 1988). In most multicellular organisms, cdc2 acts as a major player in the initiation of mitosis. A study by Pockwinse et. al (1997) revealed that cdc2 is localized to the centrosome region of the cell and is tightly bound to the nuclear matrix-intermediate filament scaffold. This study illustrated that all cells, regardless of position within cell cycle, showed cdc2 labeling of the centrosomes, which implied an independence of the stage in the cell cycle. Therefore, it was suggested that cdc2/cdk1 interacts with the centrosome in a cell cycle independent manner.

The role of cell growth related kinases (cdc2 and erk in particular) were recently examined closely in the detergent insoluble, extracted cytoskeleton of the sea urchin blastomere cell cortex (Walker *et al.*, 1997). These isolated cortices were capable of cytokinesis-like activity. This study demonstrated the presence of numerous cortex-associated, phosphorylated proteins and that the appearance of these phosphorylated proteins was cyclical and synchronized with the cell cycle. Protein phosphorylation appeared to result from the action of endogenous cytoskeletal associated protein kinases. The percentage of proteins associated with the cortical cytoskeleton was determined in unfertilized eggs, and interphase or mitotic (dividing) zygotes. Map kinase was found to be associated with the sea urchin cell cortex: 1.0% in an unfertilized egg, 2.4% in the interphase zygote, and 1.0% in the mitotic zygote. Cdc2 was also determined to be associated with the cytoskeleton: 1.5% in an unfertilized egg, 2.4% in the interphase zygote, and 1.0% in the mitotic zygote. It was concluded that these kinases associated with the detergent insoluble, extracted cytoskeleton play a role in regulating the actin cortical cytoskeleton.

The sea urchin blastomere has a modified cell cycle of about one-hour, considerably longer than the 18 hour cycle of a typical somatic cell. For this reason, it was important to examine the relationship of these same

kinases in a somatic cell and to look for any similarities or differences in regard to the role of map kinases and cdc2. The present study addressed this question by examining the association of cdc2 and erk with the cytoskeleton in rat muscle fibroblasts. It was hypothesized that these kinases may be associated with the detergent insoluble extracted cytoskeleton, in a manner similar to that of the sea urchin blastomere, despite the longer cell cycle of fibroblasts.

After extraction of the rat muscle fibroblast was completed, the detergent insoluble cytoskeleton was examined by western blot. Cdc2 and erk are found present in the fibroblast cytoskeletal fraction (detergent insoluble). Similar findings were also observed in sea urchin blastomeres (Walker *et al.*, 1997). This association is consistent with the possibility that these kinases play a role in the regulation of the fibroblast cytoskeleton during cytokinesis.

The presence of cdc2 and erk in the cytoskeleton sample is not due to incomplete washing of the detergent insoluble fraction. Data was obtained concerning the relative quantities of proteins in the cytoskeleton and whole cell fractions. In whole cell samples, actin is approximately 2.9% of the total protein. In cytoskeleton sample, actin represents about 14% of total protein. This suggests that the detergent insoluble fractions were

enriched in proteins specifically associated with the cytoskeleton. Since actin is an insoluble protein and forms the major component of the cytoskeleton, it is found at a higher concentration in the detergent insoluble cytoskeleton fraction when compared to whole cell (unextracted) samples.

In the study by Walker *et al.* (1997), the amount of erk in the cortex of mitotic cells was found to be 1.0%. In this present study, the amount of erk in the cytoskeleton sample was 6.8%. One explanation for the discrepancy in percentages is the use of differing extraction procedures. In the present study, the extraction process retained chromatin in the detergent insoluble samples. The study by Walker et al. used homogenized samples in which chromatin was removed. In the presence of chromatin, more erk is present in the samples. In samples with very little chromatin present, a lower percentage of map kinase is found, suggesting the possibility of limited association between map kinase and the cytoskeleton. Thus, there is evidence of enrichment of map kinase in these samples, which in turn demonstrate an association of the kinase with the cytoskeleton.

One significant conclusion can be drawn from this study comparing the association of cell cycle regulating kinases with the cytoskeleton. The findings presented here suggest that erk and cdc2 are associated with the somatic cell cytoskeleton. Thus, the soluble proteins, erk and cdc2 were

found in the extracted, detergent insoluble cytoskeleton samples obtained from somatic fibroblast cells. This finding supports previously obtained results from sea urchin blastomere cells, suggesting an important similarity between embryonic and somatic cells. This link may serve as a basis for future studies concerning cytoskeleton regulation during the cell cycle.

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