Association of Cell Cycle Protein Kinases with the Cytoskeleton of Cultured Myoblasts

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Abstract

These studies investigated the role of the protein kinase cdk1 (cdc2) with regard to the cytoskeleton of cultured C2C12 skeletal muscle myoblasts. The cyclin-dependent protein kinases (cdks) are involved in regulating entry into and progression through various stages of the cell cycle. The cell cycle consists of four precise phases that dividing cells pass through: M, G1, S, and G2 phase. Cdc2, a member of the cdk family, is known for its role in facilitating the transition state from G2 to M phase. In order to examine the possibility that cdc2 is involved in cytokinesis two factors must be addressed. First, synchronization of C2C12 cells is critical to ensure that all cells are in the same phase of M phase before extraction of the cytoskeleton can occur. Establishing the presence of cdc2 during mitosis leads to the examination of its location and with what it is specifically linked to. Secondly, the removal of actin, a cytoskeletal protein, as well as nucleic acids is essential to determine the connection between cdc2 and actin or cdc2 and chromatin. This is important because actin and myosin are key components of the contractile ring that aids in the process of cytokinesis. If cdc2 is associated with actin, then cdc2 can possibly play a role in cytokinesis. After synchronization of C2C12 cells at mitosis, the cytoskeleton was isolated using detergent extraction protocols. Protein composition was analyzed by Western Blot to investigate the presence of cdc2 in the cytoskeleton fractions with and without actin and chromatin. Ultimately, the aim of this study was to show that cdc2 plays a part in cytokinesis by proving that cdc2 is linked to the cytoskeletal protein actin.

Introduction

Cells are composed of a network of filamentous structure in the cytoplasm collectively termed the cytoskeleton. The highly organized cytoskeleton houses over 10,000 different proteins including 3 families of structural proteins, microtubules, microfilaments, and intermediate filament protein that function in the movement of the cell. The cytoskeleton is responsible for cell locomotion, muscle contraction, transportation of organelles, cell division, and the ability to adapt to a changing environment. The vast array of proteins associated with the cytoskeleton all have precise and defined functions that are not all known. Furthermore, the cell cycle regulatory protein kinases also a part of the cytoskeleton adds yet another dimension to the role of the cytoskeleton. Previous studies using sea urchin blastomere showed that cdc2 is associated with the actin cytoskeleton by examining the relationship between protein phosphorylation and cytokinesis (Walker et al., 1997). By utilizing C2C12 rat skeletal muscle cells, it is the attempt of this study to establish a correlation between the structural proteins and the protein kinase cdk1 (cdc2) in the regulation of cytokinesis.

Microtubules, the largest of the structural proteins, are known to organize the cytoplasm and are involved in cell movement such as the transportation of membrane vesicles and the beating of cilia and flagella. Microtubules are also involved in the formation of spindle fibers that is associated with cell division and for the structural integrity of the cell. The highly conserved, large actin filaments

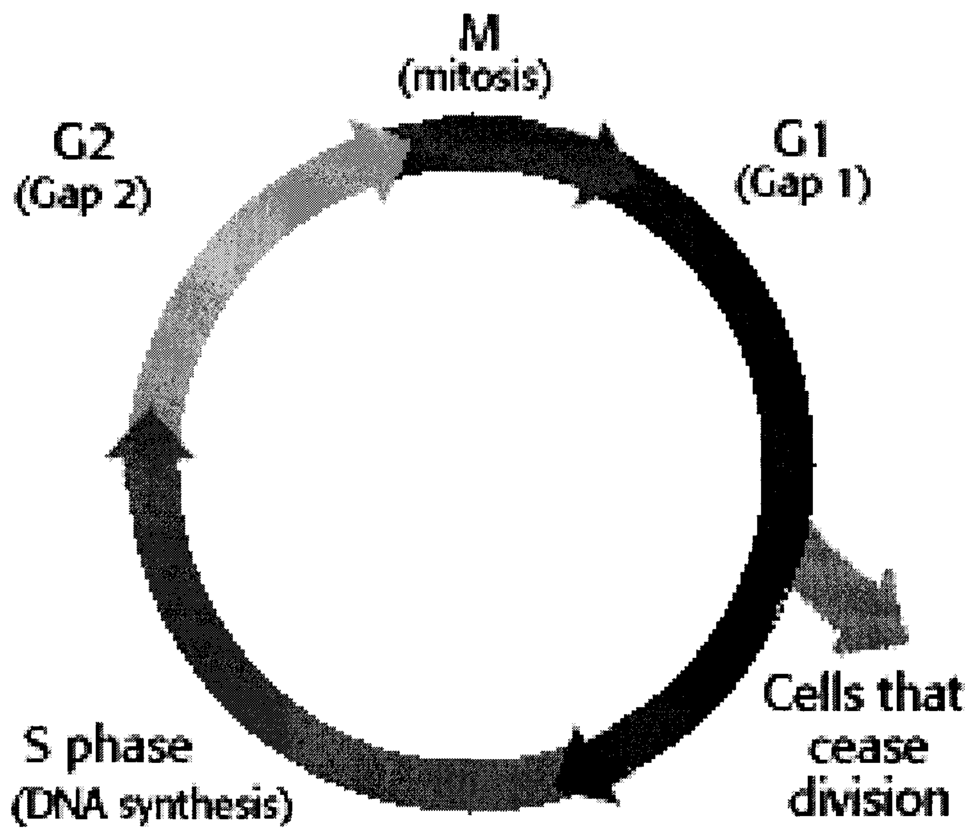
are also responsible for the integrity of cells. Actin, the most abundant intracellular protein, exists in two forms. A globular monomer called G-actin and a filamentous polymer termed F-actin. The polymerization of G-actins results in the formation of the filamentous actin and this linked to other proteins is known as microfilaments. The third structural element, intermediate filaments, is the most stable and thus provides the support of the cytoskeletal framework (Becker, W.M., *et al.*, 2000).

Dividing cells pass through a timed sequence of phases called the cell cycle. The timing of the cycles is precise and achieved in 4 phases; M, G1, S, and the G2 phase (Figure 1). M phase consists of mitosis and cytokinesis. The G1, S and G2 phases make up interphase which precedes mitosis. The cell spends the majority of its time in interphase. Here, the cell duplicates much of its contents needed in order for mitosis to occur. During interphase, the cell obtains nutrients, utilizes them to manufacture new living material, and maintains routine "housekeeping" functions (Shier, D., Butler, J., and Lewis, R., 2000). Interphase begins with G1, the first gap junction, which separates S phase from the preceding M phase. The synthesis of DNA occurs in S phase and that's followed by the second gap called G2 phase. Afterwards, the M phase begins with the division of the nucleus termed mitosis. Prophase, prometaphase, metaphase, anaphase, and telophase are the stages that constitute mitosis. Mitosis entails the duplication of chromosomes. The condensation of chromatins, the formation of the metaphase plate followed by the separation of the chromatids and then movement of the

chromosomes to the poles fulfills the first portion of M phase. However, prior to the completion of mitosis the formation of the mitotic spindle must occur. The mitotic spindle is critical for the capture, alignment, and separation of the chromosomes (Lodish *et al.,* 1995). This ultimately leads to the formation of two daughter cells. The mitotic spindle, consisting of microtubules, begins to grow in the late prophase where an increase in microtubules becomes evident. The connection occurring between microtubules and kinetochore from opposites ends of the cell, make up the mitotic spindle (Becker, W.M., *et al.*, 2000).

Figure 1: The illustration above represents the cell cycle which consists of interphase and mitosis. The diagram was obtained from the following website:
http://www.biology.arizona.edu/cell_bio/tutorials/cell_cycle/main.html

Typical Cell Cycle



Cytokinesis, or the division of the cytoplasm marks the end of cell division. Constriction of the cell membrane begins during anaphase. The contractile ring or the "ring of filaments" lies at right angles to the microtubules that pull the chromosomes to opposite ends of the cell. It appears that cytokinesis depends on the formation of the cortical actin-and myosin based ('actomyosin') contractile ring (Hales *et al.,* 1999). The ring pinches inward and as a result of completion of M phase, the formation of two daughter cells from a single parent cell occurs. An on going investigation in the regulation and mechanisms associated with cytokinesis reveals that targeted vesicle fusion at the cleavage plane plays an important role in animal cell cytokinesis. This along with contraction of the actinmyosin ring forms the foundation of cytokinesis (Hales *et al.,* 1999).

In order to understand the regulation of cell proliferation and the cell cycle, one must realize that the cell cycle depends on the regulation of proteins that are critical in accomplishing the steps needed for cell division. Mitogen-activated protein (MAP) kinases are a group of long-lasting serine/threonine protein kinases that are the major enzymes in this regulatory pathway. There are three known MAPK families in mammalian cells, ERKs, JNKs, and p38MAPKs. The activation of MAPK occurs as a result of the activation of MEK (or MAPKK). When MEK becomes activated, it phosphorylates a threonine and a tyrosine residue, one amino acid apart, on the MAPK thus activating it. MAPK then moves to the nucleus and phosphorylates the transcription factor Elk-1, which then turns on the fos gene. The activation of MAPK by MEK affects cell morphology,

induces the expression of fos and the entry into the S phase (Gotoh *et al.*, 1999). This suggests that MAPK activity affects many processes in the cytoplasm, the nucleus, the cytoskeleton, and the membrane. For example, Klemke and colleagues showed how ERK1 and ERK2 regulate cell migration by directly impacting the migratory machinery. MAPKs can also be dephosphorylated by phosphatases. When this occurs, a decrease in function of myosin light chain kinase (MLCK) and MLC phosphorylation is observed. This results in a decrease of cell migration on extracellular matrix proteins (Klemke *et al.*, 1997).

Extracellular kinase 1 (ERK 1), a member of the MAPK family, becomes activated as a result of mitogenic stimulation. The activation of ERK/MAPK is believed to be connected with cell migration in rat aortic smooth muscle (RASM) cells. These results indicate that cell migration can occur due to many signaling pathways. In RASM cells, the ERK1 pathway becomes activated in response to activin A, transforming growth factor-β (TGF-β), and angiotensin II (ATII). Furthermore, TGF-β1 and TGF-β2 have been shown to activate both ERK1 and Ras. Ras are a group of proteins that send signals from receptor tyrosine kinases to the nucleus. For ERK1 to become activated, the activation of Ras must occur first. In addition to the activation of ERK/MAPK pathway, activin A, ATII, and TGF-β1 are also responsible for changes in the cytoskeleton (Riedy, M.C. *et al.*, 1999).

The second MAPK family is the c-Jun NH2 –terminal (JNK) kinases, also known as SPK1. They are activated by stress-induced events as well as by inflammatory responses. According to Yujiri *et al.* (1999), the addition of microtubule toxins to cells resulted in the loss of JNK pathway and thus cell death. In response to microtubule toxins, only when MEKK1 is present do we get activation of the JNK pathway. In other words, microtubule toxins activate MEKK1. However, when Cytochalsin D is added to cells, activation of MEKK1 does not occur. Cytochalsin D disrupts actin filaments, but unlike microtubule toxins, activates the JNK pathway without first activating MEKK1 (Yujiri *et al.*, 1999).

The third family of MAPKs is the p38MAPKs. P38MAPKs, also known as CSBP2, are induced by stress and inflammatory responses. The activation of p38MAPKs may result from many factors including lipopolysaccharide (LPS) and hyperosmolarity due to sorbitol addition. There are two distinct regions among the p38MAPs; one region is specifically for substrate binding while the other region engages in substrate phosphorylation (Gum, R.J. and Young, P.R., 1999).

Another family of protein kinases that is known to regulate entry into and progression through various stages of the cell cycle are the cyclin-dependent kinases (cdks) (Holmes and Solomon, 1996). Cdc2, a member of the cdk family, is known for its role in facilitating the transition state from G2 to M phase. Cdc2 binds to cyclin and becomes an inactive MPF (maturation promoting factor), which is dephosphorylated by cdc25 (figure 2). Cdc25 is a dual-specificity protein

phosphatase that removes the inhibitory phosphate on the cdc2 of the complex leaving behind the activating kinase (Peng *et al.*, 2000). The Cdc2-cyclin complex (MPF) becomes activated and mitosis occurs. Thus, the timing of mitosis in embryonic cells is coordinated by MPF, a complex comprised of $p34^{cdc2}$ kinase and cyclin B that drives the G2 to M transition (Pines, 1995).

Furthermore, there are several checkpoints that must be met before progressing to the next phase of the cell cycle. These checkpoints determine the cell's fate. The first checkpoint is known as the G1 checkpoint or the restriction point and it is acted upon by growth factors. The second checkpoint, known as the G2 checkpoint, is acted upon by the p34cdc2-cyclin B complex to facilitate mitosis. The third checkpoint is the spindle assembly checkpoint. This checkpoint ensures that the chromosomes are properly attached to the mitotic spindle in order to assure that each daughter cell will receive a complete set of chromosomes. According to Pines (1995), the Cdk4-cyclin D is necessary for passage through G1, $p33^{cdk2}$-cyclin E is necessary for the transition from G1 to S phase, $p33^{cdk2}$-cyclin A is necessary for progression through S, and $p34^{cdc2}$-cyclin B is necessary for the transition from G2 to M phase. Moreover, the cdc2-cyclin B complex is involved in re-organizing the architecture of the cell at mitosis. It also causes dramatic changes in the behavior of the microtubule network, the actin microfilaments and the nuclear lamina. The cdc2-cyclin B complex has been shown to phosphorylate nuclear lamins thus leading to their disassembly (Peter *et*

al., 1990). This is important because the destruction of the nuclear lamins means that the breakdown of the nuclear envelope will follow.

Cytokinesis depends partly on the interaction of two proteins, actin and myosin. As I referred to earlier, actin protein is the most abundant protein in the eukaryotic cell. It can exist in cells in two forms, as a monomer and as a filament. The myosin proteins (myosin I and myosin II) are a group of motor proteins. Myosin Is are associated with vesicular transportation. It has been proven that vesicles derived from the Golgi complex travel to the apical surface of epithelial cells via myosin I. Myosin II, muscle myosin, is an important protein in muscle contraction. The formation of the thin contractile rings associated with cell division occurs as a result of the accumulation of myosin II and actin. Contractile rings form in early anaphase. When contracted, it ensures that each daughter cell receives half of its parent's chromosomes and half of the organelles. The activity of $p34^{cdc2}$, a negative regulator of myosin II, remains high until anaphase when the destruction of cyclin B occurs resulting in the reduction of $p34^{cdc2}$ activity (Walker *et al.*, 1997).

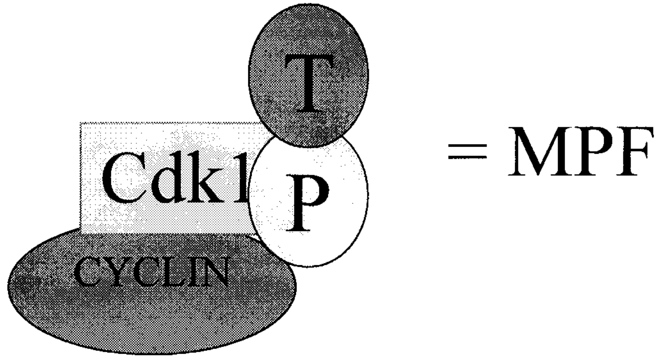
The cell cycle, like other cellular processes, occurs as a result of a series of protein phosphorylations/dephosphorylations. Protein kinases are the essential elements that begin the cascade for the many cellular processes. For example, a key element in the regulation of cortical contraction during cytokinesis is the timing of protein kinase activities that is in turn regulated by mitotic apparatus (Walker *et al.*, 1997). Therefore, it is important to determine the role(s) of protein

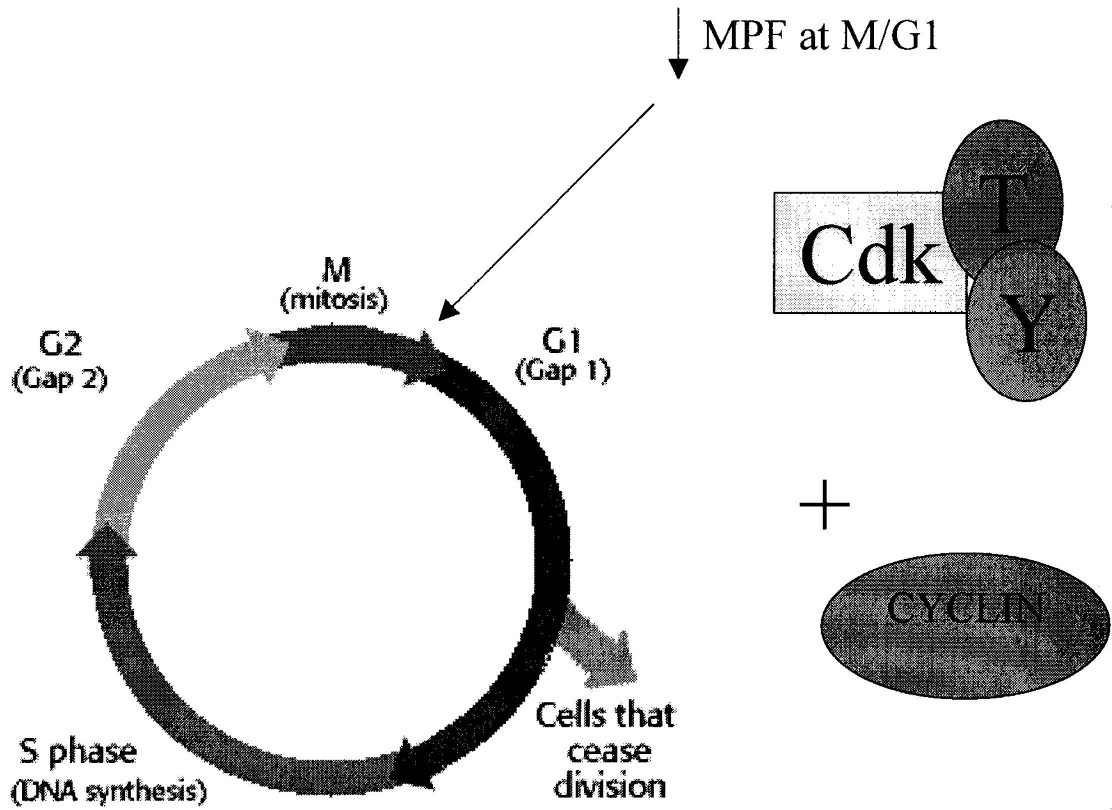
kinases, the cyclins they associate with, their substrates, and the affect this kinase-cyclin complex has on their targets. In doing so, we can better understand the pathway that occurs not only to initiate mitosis but also for its completion.

Statement of Specific Aims

This study investigated the nature of the physical association between the actin cytoskeleton and the cell cycle relevant protein kinase, cdk1 (cdc2)

Figure 2: Cyclin B/cdk1 complex regulates cell division. Cdk1 (cdc2) binds to cyclin B and becomes an inactive Maturation Promoting Factor (MPF). Only when Cdc25 dephosphorylates the inhibitory phosphate (Y) on cdc2 does it become an active kinase. Modified from
http://www.biology.arizona.edu/cell_bio/tutorials/cell_cycle/main.html


T
Cdk1
P
CYCLIN
= MPF


MPF at M/G1
M
(mitosis)
G2
(Gap 2)
G1
(Gap 1)
S phase
(DNA synthesis)
Cells that
cease
division
Cdk
T
Y
+
CYCLIN

Materials and Methods

C2C12 Rat Skeletal Muscle myoblasts were cultured in vitro until enough cells are present to extract the detergent insoluble actin cytoskeleton at various times of the cell cycle.

Cell Cultural Procedures

Initially, the cells were frozen so to obtain good, quality cells the vials of C2C12 cells were thawed quickly. The addition of 1ml of 10% fetal bovine serum (FBS) was added to the pellet to resuspend the cells. This was followed by a 10 minute waiting period. Next, 1ml of 10%FBS was again added (1:1 dilution) followed by a 10 minute waiting period. Subsequently 2ml of 10% FBS was added the final volume equaled 8mls. The suspension of the C2C12 cells in 8mls of 10% FBS was then separated into flasks and about 6mls of 10% FBS was added to the flasks. These flasks were then placed in an incubator set at 37˚C, 5% CO_2, and 95% air. The medium was changed at least twice a week. The 10% FBS medium contains a 1:9 dilution of FBS stock and DMEM (Dulbecco's Modified Eagle Medium), 1/1000 units penicillin, and 1/1000 units streptomycin. When enough cells were harvested, to about 80% confluence, cells are incubated with nocodazole for 24 hours.

Synchronization Procedure

Nocodazole is an antimitotic drug that binds to tubulin molecules and thus leads to the destruction of the mitotic spindle. The addition of nocodazole for 24 hours guarantees that all cells will be synchronized in M phase. Moreover, removal of this drug ensures that all the cells enter the cell cycle at the same point.

Not only is synchronization of cells important, but also the elimination of chromatin must be attained. This provides accurate results that determines the association of the protein kinase cdc2 with changes in the cytoskeleton and with the cell cycle. There are a number of methods to eliminate chromatin. The method used entails extracting the CSK at varying time points then using DNase as well as RNase to degrade the DNA and RNA respectively.

Experimental Procedures

Part 1

After growing and maintaining enough cells, nocodazole was added to ensure that all cells are synchronized in the same phase of M phase. A 1: 15000 dilution ratio was used (6.7μl of nocodazole/100ml of 10 % FBS). Next, four time points are chosen corresponding to the phases of the cell cycle to perform the cytoskeleton extraction procedure. The four time points are:

T=0: right after treatment of nocodazole

T=1: 3 hours later

T=2: 9 hours later

T=3: 18 hours later

Subsequent to the incubation period, nocodazole was washed and replaced with incomplete medium (DMEM) for about five minutes. DMEM was removed and 10% FBS (complete medium) was added to the flasks for the duration of the time points mentioned above. This was done to allow the cells to continue with the cell cycle all at the same time.

For each of the 4 chosen time points of the cell cycle, the extraction of the detergent insoluble actin cytoskeleton was performed. At each time point 10 mls of Isolation Buffer replaced the 10% FBS for approximately two minutes. After the two minutes, the Isolation Buffer was removed and replaced with 10 mls of Extraction Buffer for 10 minutes. If after the 10 minutes the cytoskeleton appeared to be floating, it was necessary to centrifuge this mixture to avoid losing the extracted cytoskeleton. Next, wash with Isolation Buffer then remove and replace with 1x SDS Sample Buffer. The samples were boiled for about 3 minutes and stored at -30˚C until needed for analysis. Analyzing the samples included using SDS-PAGE, which provides data based on the size of the proteins rather than their charge. Then electrophoretic transfer of proteins, Western Blot, and finally Immunoblotting were performed to determine the presence of cdc2 and actin at each phase of the cell cycle.

The Isolation Buffer consisted of .99mMGycerol, 19.9mM PIPES, 4.8mM EGTA, 4.9mM $MgCl_2$ $6H_2O$ and titrated to pH 6.9 with 10N NaOH. The

Extraction Buffer contained a 1:1 dilution of Isolation Buffer and Walker Buffer that contained 1ml of Nonidet NP-40 octlyphenoxypolyethoxyethanol and 99ml Isolation Buffer.

Additionally, cell samples at the same time points were collected according to the protocol above with the exception of the addition of certain cell treatments. DNase and RNase were added with the Isolation Buffer to eliminate nucleic acids and Cytochalasin D was added to remove actin from the detergent extracted cytoskeleton.

Part 2

C2C12 cells were cultured in 3: 12-well plates until confluent to 80%. The treatment of cells with Nocodazole for 24 hours was done to synchronize the cells in M phase followed by the timely collection of samples. Three samples from each time point were collected; t=0h, t=3h, t=14h, t-15h, t-16h, t=17h, t=18h, t=19h, and t=20h. As before, SDS-PAGE and Western Blot analysis were performed on the samples collected followed by Immunoblotting with anti-phosphoHistone H3 mitosis marker. Anti-phosphoHistone H3 mitosis marker phosphorylates histones.

Part 3

Using the data obtained from part 2, the next step was to determine if cdc2 was associated with actin or chromatin. To do this, actin was removed from the

cytoskeleton by using Cytochalasin D. Cytochalasin D is a drug that prevents cell division by blocking formation of microfilaments. C2C12 cells were cultured on cover-slips until the surface of the cover-slips were 80% confluent. Afterwards nocodazole was added for 24 hours to synchronize the cells. One hour before the collection of each time point, the cells were incubated in Cytochalasin D. After which the detergent insoluble cytoskeleton fractions were collected at time points t=0h, t=3h, t=18h, and t=19h followed by SDS-PAGE analysis, Western Blotting, and Immunoblotting with cdc2. Similarly, after incubation with DNase and RNase for 30 minutes, the detergent extracted cytoskeleton at t=0h, t=3h, t=18h, and t=19h was collected after release from nocodazole.

SDS-PAGE

SDS-PAGE is an analytical electrophoretic method for analyzing proteins based on differences in molecular size. This technique uses SDS (sodium dodecyl sulfate), which is an ionic detergent that denatures proteins and gives a negative charge to all the proteins. Thus proteins are separated based on size only. The SDS-PAGE gels contained the samples collected at the various time points as well as a molecular weight standard. This helped us determine what proteins were present in our sample.

Preparation of Resolving and Stacking Gels

The 10% Resolving Gel contained 4.1ml ddH_20, 3.3ml 30% acrylamide, 2.6 resolving gel buffer (Sigma™), 50µl 10% APS (ammonium persulfate), and 5µl TEMED (Sigma™). Mix the components of the resolving gel in a beaker and quickly pour in between two glass plates to about 75% of the way up. Then follow with butanol. Let stand for about 1 hour or until the gel has polymerized.

After the resolving gel polymerized, the butanol was poured off and the 5% acrylamide stacking gel was made which contained 6.3ml ddH_2O, 1.7ml 30% acrylamide, 2.0ml continuous buffer, 50µl APS, and 10µl TEMED. The stacking gel was quickly poured in between the plates and the 10 well comb was placed between the plates to form 10 wells. Polymerization of the stacking gel took about 20 minutes, after which the comb was removed. The gel was then loaded in the chamber and SDS-PAGE Electrode Buffer (43.26gr glycine, 9.075gr TRIS base, 3gr SDS to 3000ml ddH_2O) was poured in the chamber. Then 4µl of Prestained SDS-PAGE Standards, Low Range or High Range as well as 20µl of the samples collected at the time points mentioned above were loaded in the wells. Additional electrode buffer was added to the wells and in the chamber to allow them to be conductive. The chamber was hooked up to a power supply and allowed to run for about one hour at a constant current of 0.025 amps. After which the glass plates were pried open and the gels were analyzed by either coomassie staining or western blot.

Coomassie Staining

Gels used for coomassie stain were placed in a 0.2% Coomassie Brilliant Blue R-250, 45% methanol, and 10% acetic acid solution for about 30 minutes. This was followed by destaining the gel in a solution of 45% methanol and 10% acetic acid for about one hour with frequent changes of the destaining solution. The gels were then dried between two sheets of dialysis membrane and analyzed for total protein distribution.

Western Blotting

Western blotting relies on the use of specific antibodies to identify proteins of interest. This process of transferring the proteins to a membrane uses the Genie Blotting Apparatus. The proteins were electrophoretically transferred from the gels to the blotting membrane (PVDF). After the transfer of the proteins to the PVDF membranes, the membranes were blocked for immuno-detection. The PVDF sheets were then placed in blocking solution (5% powdered non-fat dry milk and TBS-T) for one hour.

Primary and Secondary Antibody Application

After the membranes were blocked, the PVDF sheets were incubated in primary antibody for one hour. The primary antibody used was either mouse anti-cdc2 or mouse anti-actin. The membrane was then rinsed with TBS-T three times

for five minutes each and then incubated in secondary antibodies that are linked to Horseradish Peroxidase (HRP). The secondary antibody goat anti-mouse was used to label anti-cdc2 and anti-actin. The blot was rinsed with TBS-T again three times followed by one washing with TBS. The TBS-T washing frees any unbound secondary antibodies and washing with TBS removed tween 20. Once this was finished, the gels were taken to the dark room for development.

Chemiluminescent Detection

Chemiluminescent Detection relied on the substrate that was purchased from PIERCE Super Signal ULTRA. It is composed of a Luminol/ Enhancer Solution and Stable Peroxide Buffer. In the dark room, the blots were incubated with the chemiluminescent substrate. The substrate reacts with the HRP linked to the secondary antibody. Luminol was reduced by HRP giving off light that was detected on autoradiographs.

Results

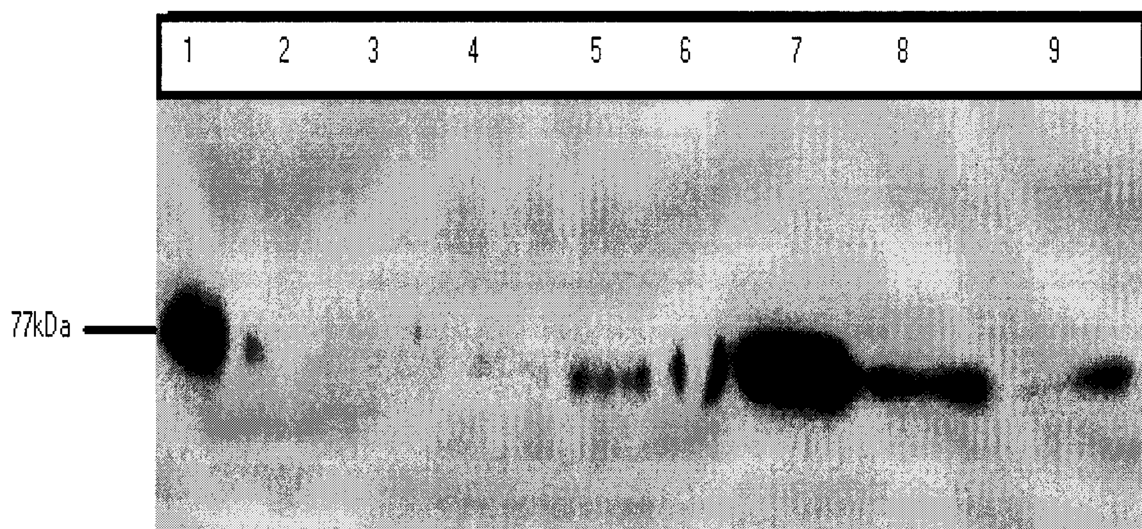
The insoluble detergent extracted cytoskeleton from C2C12 Rat Skeletal Muscle cells is a highly protein rich fraction. Actin is an abundant protein both in the detergent extracted cytoskeleton and in whole cell samples. The contractile ring, composed of actin and myosin II, functions in the constriction of the cell membrane. Thus actin and myosin II participate in cytokinesis. However, the reorganization of the actin cytoskeleton to form the contractile ring must be stimulated. The purpose of this study is to determine if the protein kinase cdk1 (cdc2) is involved in cytokinesis by proving whether a correlation exists between actin and cdc2.

In order to determine that a physical correlation exists between cdc2 and actin and if this complex plays a role in cytokinesis, C2C12 cells must be synchronized. Synchronization of cells ensures that all the cells are in the same phase of mitosis. This must be achieved before performing any experiments because cdc2 is a cyclical protein kinase so it is present throughout the cell cycle. In this study, the M phase/cytokinesis junction was analyzed to determine the action of cdc2. To do this, nocodazole was added to the C2C12 cell line for 24 hours to synchronize the cells in M phase followed by extraction of the detergent insoluble cytoskeleton. Nocodazole destroys the mitotic spindle by binding to tubulin. The cells continue through the cell cycle until they enter mitosis at which

point they become arrested in this phase. Following release from nocodazole, the detergent insoluble cytoskeleton was extracted at time points t=0h, t=3h, t=14h, t=15h, t=16h, t=17h, t=18h, t=19h, and t=20h. To determine precisely the time point corresponding to M phase, anti-phoshoHistone H3 mitosis marker was used. After running SDS-PAGE on the above samples, the gel was placed between 2 dialysis membranes and dried. The gel was scanned and quantitation of each lane was analyzed (figure 3). Histone is present throughout the cell cycle; however, only in M phase does it become phosphorylated. The increase in intensity observed at time points t=0h, t=18h, t=19h, and t=20h hours after treatment with nocodazole showed that those time points corresponded to the phosphorylation of histone. However, at time point t=18h (lane 7), there was a marked increase in intensity compared to t=0h, t=19h, and t=20h (figure 3). Therefore, the results indicated that t=18h represents M phase. This data confirmed that the cells were in M phase, and were synchronized.

Figure 3: After treating C2C12 rat skeletal muscle cells with nocodazole for 24 hours, the detergent insoluble extracted cytoskeleton was collected. The collection of the samples began with t=0h (lane 1-after release from nocodazole), t=3h (lane 2), t=14h (lane 3), t=15h(lane 4), t=16h (lane 5), t=17h (lane 6), t=18h (lane 7), t=19h (lane 8), and t=20h (lane 9). Western analysis of histone detected that phosphorylation of histone occurred at t=0 t=18h, t=19h, and t=20h, but we found that the higher intensity observed at t=18h (lane 7) corresponds to M phase.

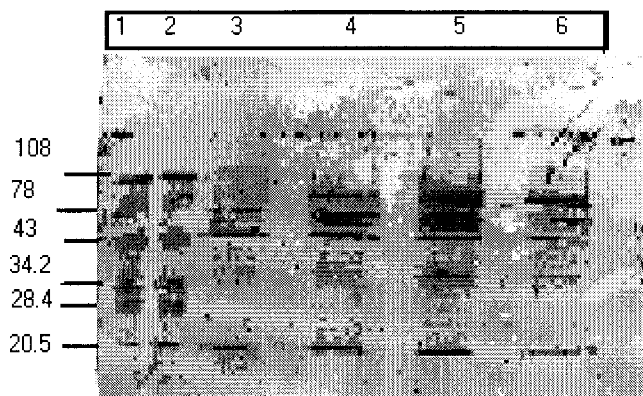
Western Blot Analysis of Histone



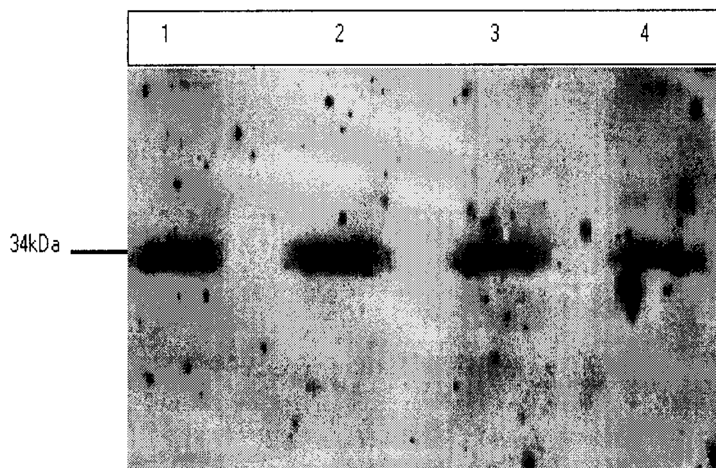
The next step was to analyze the detergent extracted cytoskeleton for cdc2 and actin. Four time points corresponding to the cell cycle were selected: t=0 (after release from nocodazole), t=3 (3 hr later), t=9 (9 hr later), and t=18 (18 hr later). After analyzing the intensity of the bands at each time point compared to the total protein concentration in each lane, an increase in intensity was observed at t=18h indicating that a higher level of cdc2 was found in M phase (figure 4). First, this proves that cdc2 is associated with the cytoskeleton and secondly, it proves that there is a cyclic accumulation of cdc2. Before proving that a correlation exists between the protein cytoskeleton and the protein kinase cdc2, it must be determined that is also a cytoskeletal protein. The detergent extracted cytoskeleton samples corresponding to time points t=0h, t=3h, t=9h, t=18h and whole cell sample were probed with anti-actin (figure 5). This data shows that actin is indeed present in the detergent extracted cytoskeleton as well as in the whole cell sample. Up to this point, it was found that both cdc2 and actin are associated with the detergent extracted cytoskeleton. However, it doesn't reveal that there is a correlation between cdc2 and actin. Chromatin was also retained in these fractions and so there is a possibility that cdc2 is linked to DNA. Degrading chromatin and actin and testing for the presence of cdc2 determined whether or not an association exists between cdc2 and chromatin or cdc2 and actin and if this complex functions in cytokinesis.

Therefore, degrading DNA must first occur to confirm that cdc2 is actually linked to the protein cytoskeleton rather than to DNA. DNase was utilized to degrade chromatin followed by western blot analysis of cdc2 (figure 6). The absence of a band at 34kD suggested that cdc2 is associated with chromatin and not with the detergent extracted cytoskeleton. However, there is a possibility that chromatin was not completely degraded from the cytoskeletal fractions that were collected. To verify that chromatin is degraded, cover-slips treated with DNase showed that when DNase permeates through the cell membrane, the nucleus becomes fragmented (figure 7). Therefore, the data suggests that chromatin is completely removed from the cytoskeleton since the cytoskeletal fractions were first extracted before DNase was added. This makes it easier for DNase to degrade DNA because there is no cell membrane blocking DNase from eliminating DNA.

Figure 4: C2C12 Skeletal Muscle Cells were treated with nocodazole for 24-h. A) Coomassie Stain of blot probed with anti-cdc2. Lanes 1 & 2 represents prestained SDS-PAGE standards. Lane 3, 4, 5,and 6 represents samples t=0h, t=3h, t=9h, and t=18h respectively. B) Autoradiography illustrated that cdc2 (34.2kDa) was present in the samples t=0h, t=3h, t=9h, and t=18h.



Coomassie



Autoradiography

Figure 5: Western analysis of actin in C2C12 Skeletal Muscle cells. After release from nocodazole, different time points corresponding to the cell cycle were collected. Lanes1 and 2: molecular standard, lane 3: t=0h, lane 4: t=3h, lane 5: t=9h, and lane 6: t=18h. At 48kDa, actin was detected at t=0h, t=3h, and t=9h. At t=18h, actin was present in coomassie stain, but was undetected in the autoradiography.

Western Blot Analysis of Actin

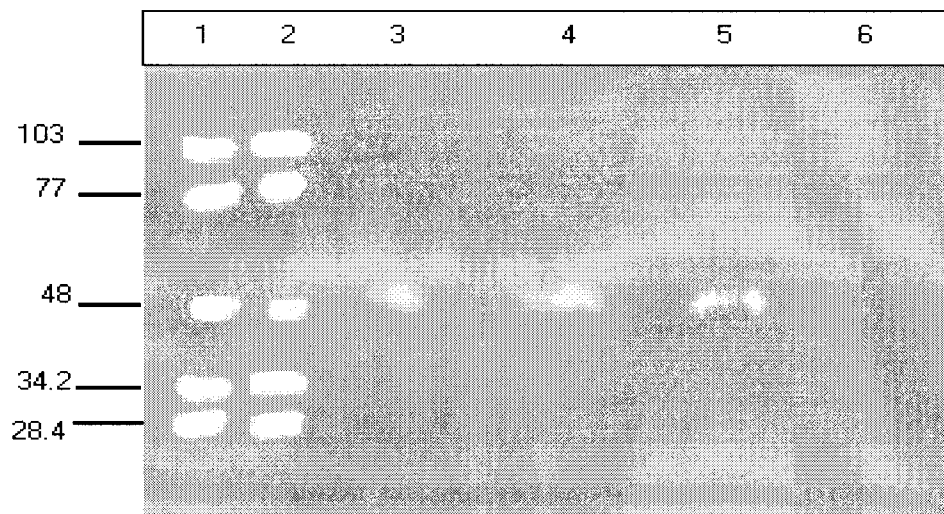


Figure 6: DNase treated C2C12 cells at time points t=0h, t=3h, t=9h, and t=18h. Lane 1: molecular standard, lane 2: whole cell sample, lanes 3 and 7: t=0h, lanes 4 and 8: t=3h, lanes 4 and 9: t=18h, and lanes 5 and 10: t=19h. Western analysis of cdc2 showed that cdc2 (34.2kDa) was present only in whole cell sample.

Western Blot Analysis of Cdc2

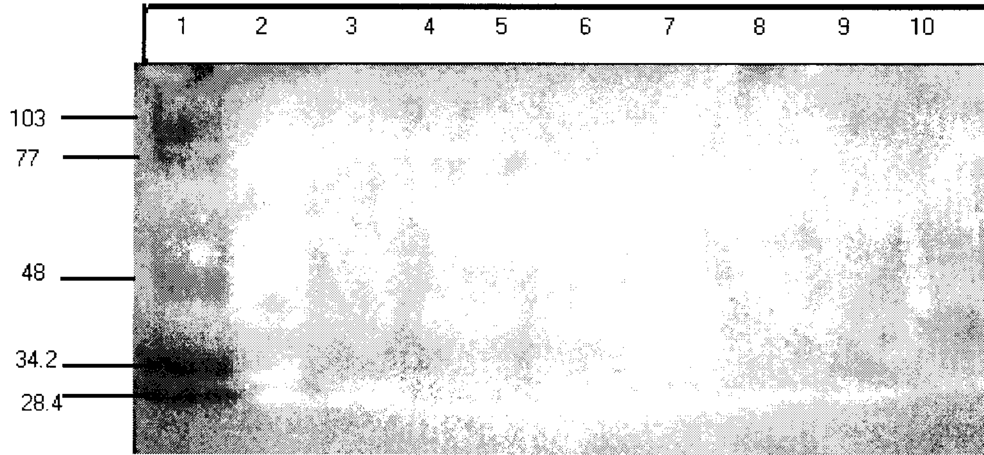
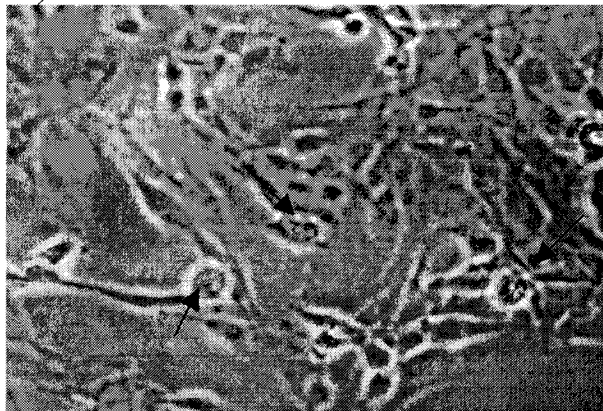


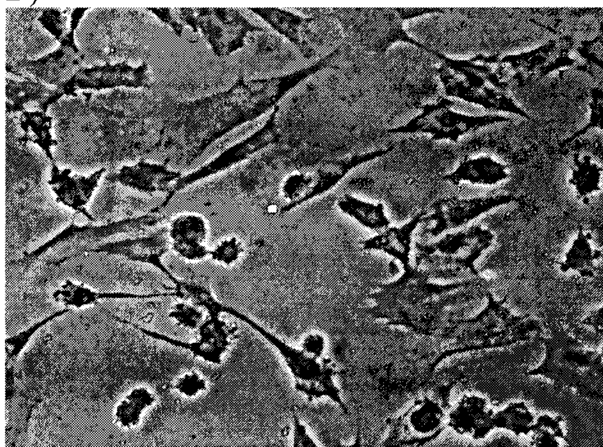
Figure 7: A) This micrograph of the C2C12 cell line, shown in phase contrast, illustrates the fragmentation of the nucleus (arrows) as a result of incubation with DNase for 30 minutes. B) C2C12 cell line shown in phase contrast with Giemsa staining.

A)



DNase Treated C2C12 Cells

B)



Giemsa Stain

Based on the data, it was determined that cdc2 and chromatin are linked; however, at this point it hasn't been proven that cdc2 is also associated with actin. To test whether a complex between cdc2 and actin occurs and as a result plays a role(s) in the regulation of cytokinesis, Cytochalasin D was added to C2C12 Rat Skeletal Muscle Cells. Cytochalasin D disrupts the formation of microfilaments which is important in determining a possible pathway that dividing cells partake in. After release from nocodazole, the detergent insoluble cytoskeleton fractions were collected at t=0h, t=3h, t=18h, and t=19h. Probing with anti-actin and anti-cdc2 resulted in the absence of actin in the Cytochalasin D treated cells, but not cdc2 (figure 8 and 9, respectively). This data points to the conclusion that the formation of an association between cdc2 and actin does not occur and therefore, contrary to what was believed, cdc2 associates with chromatin. Knowing how critical the proper alignment and separation of chromosomes to the division of cells may mean that cdc2 plays a role in cytokinesis through its connection with chromatin and not with actin.

Figure 8: Western analysis of actin in Cytochalasin D treated C2C12 Rat Skeletal Muscle cells. Lane 1 represents molecular standard, lane 2: whole cell sample, lane 3: t=0h, lane 4: t=3h, lane 5: t=18h, lane 6: t=19h, lane 7: t=0h, lane 8: t=3h, lane 9: 18h, and lane 10: 19h. Actin (48kDa) was detected only in the whole cell sample and not in the Cytochalasin D treated fractions. Bands present at about 70kDa represent albumin protein.

Western Blot Analysis of Actin

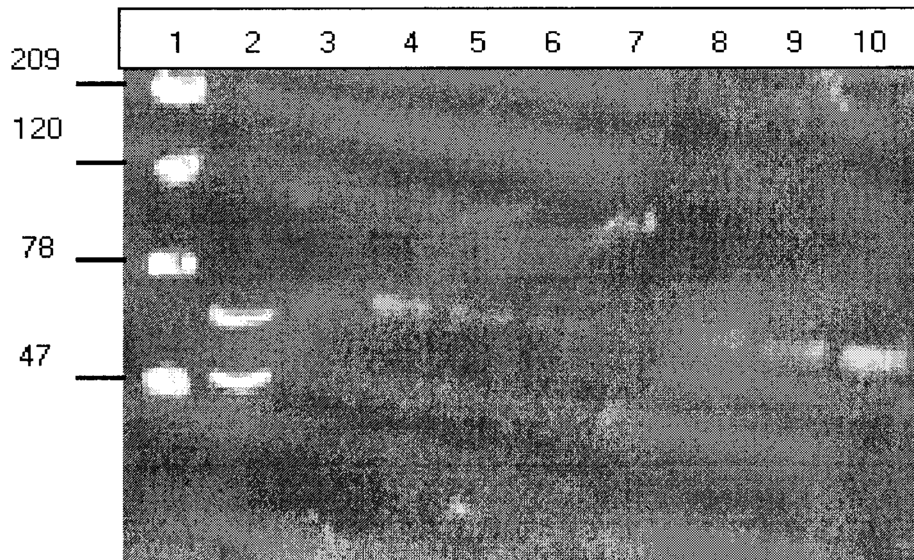
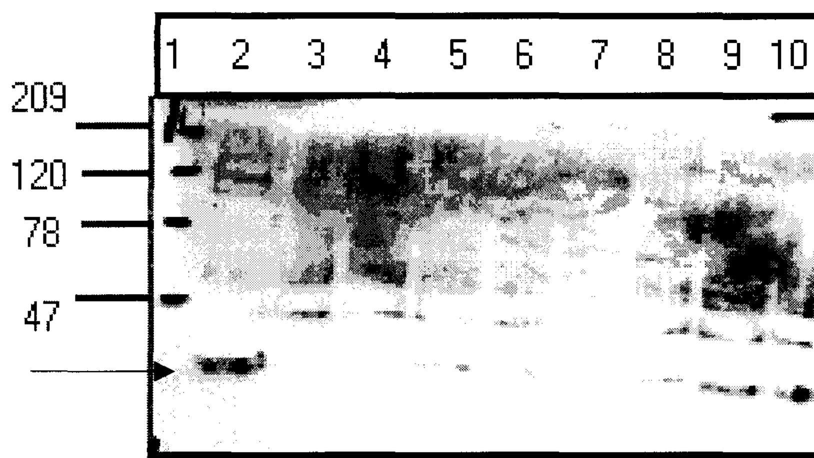


Figure 9: Western analysis of cdc2 in Cytochalasin D treated C2C12 Rat Skeletal Muscle cells. After release from nocodazole, the detergent insoluble cytoskeleton fractions were collected. Lane 1: molecular standard, lane 2: whole cell sample, lanes 3 and 7: t=0h, lanes 4 and 8: t=3h, lanes 5 and 9: t=18h, and lanes 6 and 10 t=19h. Cdc2 (34.2kDa) can be detected in all the lanes; however, some bands appear at a higher intensity than others.

Western Blot Analysis of Cdc2



Discussion

The transition from interphase to M-phase in dividing cells is under strict control of the intracellular regulatory machinery. This machinery involves the interaction of several cell cycle regulatory proteins, most of which can be grouped into two families of proteins: the cyclins and the cyclin dependent kinases (cdk). The best characterized cell cycle complex is MPF (maturation promotion factor). MPF consists of cdc2, which is a catalytic subunit, coupled to the regulatory protein cyclin B that forms the M-phase promoting factor (MPF). MPF regulates the transition of the cell through M-phase.

During M-phase two divisional processes must occur in a precise and timely order to ensure that cells divide properly. Karyokinesis divides the genome while cytokinesis divides the cytoplasm into two cells each containing one of the nuclei produced during karyokinesis. Cytokinesis and karyokinesis are mutually exclusive. Premature cytokinesis is never observed in normally dividing cells. Cytokinesis however depends on completion of karyokinesis. Inhibition of karyokinesis results in the absence of cytokinesis. Evidence supporting the theory that the mitotic apparatus determines where and when the contractile ring forms and its function comes from experiments in dividing sea urchin zygotes. Re-positioning of the mitotic apparatus at anaphase resulted in a repositioning of the cleavage furrow (Rappaport, 1986). These studies suggested that some factor(s)

emanates(s) from the mitotic apparatus and influences the formation and subsequent activity of the contractile ring resulting in proper cytokinesis.

MPF is known to regulate myosin activity in an inhibitory way (Satterwhite and Pollard, 1992). Myosin is required for the contractile force needed to divide the cytoplasm during cytokinesis (Mabuchi and Okuno, 1977). This may insure that cytokinesis does not prematurely occur. However it does not answer the central question of how and what regulates the rearrangement of actin filaments at the onset of cytokinesis.

Past studies using the detergent extracted sea urchin blastomere cytoskeleton showed that the actin enriched cytoskeleton is still capable of some functional behavior reminiscent of behavior observed in dividing whole cells (Walker *et al.*, 1997). Addition of ATP reactivated the cleavage furrow and it's contractile behavior. Later studies indicated that p34cdc2 is specifically associated with this detergent insoluble actin filament enriched fraction and may be playing a regulatory role in determining function and structural activity of the contractile ring (Walker *et al.*, 1997).

The sea urchin cell cycle is dramatically different from the normal somatic cell. The cell cycle is shorter than the somatic cell cycle. The stages of the cell cycle are altered in the dividing blastomere, no G1 or G2 phase. For this reason studies like those of Walker et al. (1997) were initiated in cultured myoblast cells. It was reported that cdc2 was found to be associated with the detergent extracted cytoskeleton of the sea urchin blastomere cell cortex. Analysis of the percentage

of cdc2 revealed that 1.5% of cdc2 is present in an unfertilized egg, 2.4% in the interphase zygote, and 1.0% in the mitotic zygote.

First, the evidence from this study established which time point corresponded to M phase. Immunoblotting with anti-phosphoHistone H3 mitosis marker revealed that the time point t=18h has the greatest intensity and therefore corresponds to mitosis. Histone being present throughout the cell cycle becomes phosphorylated only during M phase. This is important because it confirmed that the C2C12 cells were synchronized and were in M phase. Secondly, this study confirmed the basic observations of the above studies that the detergent insoluble fractions of myoblasts contain a significant amount of associated cdc2. This also supported the same observations in asynchronously dividing myoblasts (Atway, 1999).

However, actin filaments are not the only cellular component found in these fractions, microtubules, intermediate filaments and chromatin are all present. It is known that cdc2 is localized to the centrosome region of the cell, the microtubule organizing center, and is tightly bound to the nuclear matrix-intermediate filament scaffold (Pockwinse *et al.*, 1997). Evidence from many studies suggested an association between cdks with chromosomes and microtubules. Many cdk substrates are known to be associated with chromosomes and microtubules. This study confirmed those results that cdc2 is associated with chromatin. When the detergent insoluble fraction was digested with nucleases cdc2 was no longer present within the insoluble fractions. Agarose gels confirmed that chromatin

was not present in the detergent insoluble fractions; however, there is a possibility that fragments of chromatin were still present within these fractions. Furthermore, the affects of the addition of nucleases in regards to other cytoskeletal proteins are not known. Therefore further analysis of the cytoskeletal fractions is needed to corroborate the results that suggest that cdc2 is associated with chromatin.

Moreover, cdc2 could be associated with actin as in the case of the sea urchin blastomeres. To determine if actin and cdc2 are associated with each other, Cytochalasin D was added to the detergent insoluble fractions. Cytochalasin D eliminates actin by blocking the polymerization of G-actin. Western blot analysis revealed that actin was not present, but cdc2 was depicted on the autoradiographs. These results contradicted those found in the sea urchin blastomere (Walker *et al.*, 1997). However, the two cell types have very different cell cycle mechanisms and thus might account for the discrepancies.

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