The Dynamic Interactions of Actin Stress Fibers and Microtubules

By

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ABSTRACT

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The regulation of actin stress fibers (SF) are linked to the stability of microtubule (MT) structures. The exact method of interaction is not understood. To study the interaction, live cells containing fluorescent α-actinin were observed and treated with MT perturbing drugs as well as ATP-depletion. These treatments affect the stability of the microtubules and stress fibers respectively. A plasmid coding for fluorescent tubulin was also inserted into these cells to aid in observing both the MTs and SFs simultaneously. The observation relies on fluorescent microscopy and digital imaging. By observing the changes in the MTs and SFs in live cells, it is hoped that the mechanism of their interaction will be shown.

Introduction

The movement and contractility of cells is mediated through a wide range of cytoskeletal elements. Two of these cytoskeletal structures are the actin stress fibers and microtubules. Actin stress fibers are a contractile bundle formed from several proteins that aid in maintaining internal cell tension. Microtubules are dynamic structures that have been shown to play a role in providing a locomoting cell with directionality or polarity (Vasilev et al., 1970; Gail & Boone, 1971). Microtubules aid in cell movement, internal vesicle transport and swimming when found in cilia and flagella (Lodish et al., 1995). Microtubules have also been shown to affect contractility as well as stress fiber organization and stability (Danowski, 1989). In this way, microtubules seem to regulate the stress fiber strength and distribution. An example of the interaction is seen under conditions where the microtubules are caused to depolymerize. When the microtubules are depolymerized, stress fibers increase in number and become more prominent as do the adhesion plaques (Danowski, 1989).

Actin Stress Fibers

Actin is found in a variety of structures within cells (Lodish et al., 1995). Muscle cells use actin and myosin organized into sarcomeres for contraction. Actin filaments consist of filaments of globular actin monomers that have been polymerized into long strands. The monomers have a molecular weight of 42,000 (Zubay, 1998). The filaments form two main structures within a cell: bundles and a network of filaments. The bundles are parallel, closely packed filaments, while the network consists of filaments that crisscross and are loosely packed. The role of actin in these

structures is to provide a framework to support the plasma membrane of the cell and therefore to determine the shape of the cell (Lodish et al., 1995).

Actin is also present in another structure called a contractile bundle or stress fiber. These stress fibers differ from the actin used for cell support in that they are associated with myosin II. Myosin II is found at many locations along the actin filaments and provides the actin with contractility. Stress fibers have been shown to be contractile by experiments in which they were isolated and ATP was added. Upon addition of the ATP, the fibers contract (Lodish et al., 1995). Studies of the stress fibers reveal that they contain myosin and α-actinin in an alternating pattern along the fibers. This is similar to the alternating thick filaments and Z lines observed in muscle sarcomeres. Both muscle sarcomeres and stress fibers are capable of contraction. This contraction is used for different purposes in these structures: stress fibers use their contractile ability to aid in cell adhesion while muscle sarcomeres use the contractility for movement and generating tension.

Stress fibers are found along the ventral surfaces of cells. The ends of the stress fibers attach to structures called adhesion plaques that in turn attach the cell to the underlying substratum. By being attached to adhesion plaques, the stress fibers do not shorten extensively but instead generate isometric tension that helps to maintain the cell shape and adhesion to the substratum (Clement, 1998).

The adhesion plaques have a variety of components associated with them. Integrins are a key component of adhesion plaques; they are integral membrane proteins that function by binding to extracellular matrix components (Lodish et al., 1995). Stress fibers are attached to the adhesion plaques through adapter proteins.

Alpha-actinin and vinculin are examples of peripheral membrane proteins that associate with integrins.

The stress fibers in a cell help to maintain the cell shape, aid in the adhesion to the substratum, and provide the cell with contractility. Stress fibers also play a role in cell locomotion. The polymerization and depolymerization of actin in stress fibers as well as microtubule structures both affect cell motility and structure. Coordination of both structures is required to control their overall effects on cell structure and locomotion.

Microtubules

Microtubules also play an important role in cells. They are composed of subunits which polymerize to form a cylindrical tube (Lodish et al., 1995). The subunits are heterodimers of α-tubulin and β-tubulin, two globular protein monomers. The heterodimers bind two molecules of GTP. The GTP bound to α-tubulin is not released or hydrolyzed while the GTP on β-tubulin can be hydrolyzed to GDP. Once hydrolyzed, β-tubulin can exchange the GDP for another GTP. The hydrolysis of the GTP is linked to the addition of other subunits to the ends of the microtubule.

To form the microtubule, the subunits are linked into linear protofilaments. Thirteen of these protofilaments then aggregate to form an intact microtubule (Zubay, 1998). The protofilaments are theorized to align in one of two possible ways. One way involves having the protofilament being slightly staggered so that α-tubulin monomers of different filaments are in contact. In the second possible alignment, the protofilaments are staggered by one half subunit and therefore form an alternating

pattern of α-tubulin and β-tubulin. Once aggregated, thirteen of the protofilaments curve around to form a tube.

Microtubules have a distinct polarity that is linked to the composition of their subunits since they contain both α-tubulin and β-tubulin. Because of the way that each protofilament is formed, it retains an α-tubulin monomer on one of its ends and a β-tubulin monomer on the other. The protofilaments then arrange together in a way that provides them with the same terminal monomers on each end of the microtubule. Thus one end of the microtubule contains a terminal ring of α-tubulin, while the other end has a terminal β-tubulin ring.

The microtubules are in a state of dynamic instability, where they undergo periods of elongation, rapid shortening, and pause (Mitchison and Kirschner, 1987). Subunits are constantly being added and removed from these tubules. Studies of the polarity of the microtubule indicate that the (+) end of a microtubule (the end containing the α-tubulin ring), is the preferred site for addition and removal of subunits. The (-) end contains the β-tubulin ring and is constantly losing subunits. Thus the (+) end can be regulated to add subunits faster than what is lost at the (-) end and therefore cause microtubule elongation. The (+) end can also lose microtubules at a faster rate than the (-) and therefore cause microtubule shortening.

Understanding the polarity of the microtubules aids in the study of their dynamics. Microtubules come in two main forms: a fairly stable form and a more short-lived form. The stable microtubule forms are commonly found in places where they provide some structural role as in a cilium. The short-lived microtubules are more commonly found during mitosis in the production of the spindle apparatus.

They are also used in maintaining some cell support, shape, and are constantly being lengthened and shortened as required by the cell.

Cell Observation
Fixed Cell Studies

Observation of cell structures is usually accomplished through the use of fluorescence microscopy, the most common technique being immunofluorescence. Immunofluorescence works by fluorescently labeling specific structures in the cell, but it is limited to fixed cell studies. Another technique relies on the use of fluorescent probes that can be inserted into live cells to observe cell structures in vivo. Both of these techniques will be discussed below.

Fluorescence is a chemical property that is dependent on light. For something to fluoresce it must first absorb light of a characteristic wavelength which would excite its electrons into a higher energy state. As the electrons return to a lower energy state they emit energy. This energy can be released in a variety of forms but in fluorescence it is released as light of a longer wavelength (less energy). The two main fluorescent dyes used in microscopy are rhodamine (which emits red light) and fluorescein (which emits green light). Other dyes exist, but these are the most commonly used.

In a fluorescence microscope, the sample being viewed is excited by a characteristic wavelength of light. The fluorescent light given off from the sample is then allowed to travel to the observer while filters absorb light of the excitatory wavelength. These filters allow the observer to only see the fluorescent light and therefore allow for clear viewing of the sample.

Immunofluorescence involves the use of antibodies. Antibodies exhibit specific binding and can therefore be used to bind either to tubulin subunits in the microtubules or actin in the stress fibers. Once the antibodies are bound to the desired structure, a secondary antibody containing a fluorescent protein can then be used to bind to the first antibody. In this way the desired structure can be tagged with a primary antibody, and then the tag can be used as a marker for the secondary antibody to bind and provide a fluorescent label. This method allows the location and distribution of sub-cellular structures to be determined. The disadvantage of using this labeling system is that it cannot be used in living cells. The cells must be fixed and their membranes permeabilized to allow the antibodies to enter and bind. By binding, the antibodies cause the structures to loose their functional abilities and therefore, cells must be fixed before this labeling method can be used. An advantage of using this system is that it provides clear labeling of the desired structures and the procedure is fairly easy to perform.

Live Cell Studies

The most effective method for observing sub-cellular structures in living cells is through the use of fluorescently-labeled proteins. Either α or β tubulin or g-actin can be linked to a fluorescent protein which does not hinder their functioning in the cell. These labeled proteins can then be inserted into the cells, thus allowing either the microtubules or stress fibers to be labeled in live cells and experiments to be performed. There are two ways of introducing the fluorescently-labeled proteins into the cell. One way is to microinject fluorescently-labeled proteins into living cells and allow them to incorporate into the native structures. Once incorporated, any

movement or shape changes of the fluorescent structures can be observed and recorded over time. This is only a temporary method of making the desired structure fluoresce.

A second method of fluorescently-labeling cell structures used in my experiments was to incorporate a fluorescent protein tag onto the actin or tubulin subunits in a way that does not affect the functioning of the subunits and therefore is conducive to live cell studies. The most commonly used fluorescent-protein tag is called GFP or Green Fluorescent Protein. It is a protein structure that was initially discovered in jellyfish, allowing them to glow green when stimulated by light. The GFP tag is incorporated into the actin and tubulin subunits by adding the DNA code for the GFP to the code for the actin or tubulin. In this way, the cell will produce the native protein with an added GFP tag as it reads in the DNA sequence. The DNA sequence is provided to the cell in the form of a manufactured plasmid or circular piece of DNA. GFP can be added to a wide range of subunits for various cell components and therefore is an important tool in aiding the fluorescent visualization of cell structures. The two main GFP-tagged structures used in the studies that were conducted are GFP-α-actinin and GFP-tubulin. This method can be used transiently for experiments or under rare occurrences can produce a cell line that permanently contains the labeled subunits. The cell line is produced when the plasmid is permanently expressed because it becomes incorporated into a chromosome of the cell. Permanent expression of the fluorescent subunits provides an easier way of working with the structures and also allows for performing experiments in live cells.

When the results obtained from fixed and living cells are combined, a better understanding of the interactions and dynamics of the structures can be obtained.

Two drug treatments used in my studies were sodium azide/2-deoxyglucose and nocodazole. It is known that stress fibers are disrupted by treatment with sodium azide and 2-deoxyglucose, which depletes cellular ATP levels (Sanger and Sanger, 1983). It is also known that nocodazole causes the depolymerization of microtubules. If the cells are first depleted of ATP and then treated with nocodazole, the microtubules do not depolymerize, therefore indicating that ATP is needed for the depolymerization (Bershadsky and Gelfand, 1981). Previous work done by Jessica M. Clement under the supervision of Prof. Barbara Danowski has shown that the microtubules somehow mediate the stability of the actin stress fibers. The work done by Jessica Clement indicates that when cells are first treated with nocodazole and then depleted of ATP, a significant number of stress fibers remain. This implies that the absence of the microtubules somehow enhances the stability of the stress fibers. These experiments were done using conventional immunofluorescence, which involves fixing the cells. I want to extend these studies using the fusion proteins GFP-α-actinin and GFP-tubulin to observe the interactions in living cells.

To gain a better understanding of the interactions observed by Jessica Clement, similar experiments were conducted using nocodazole and ATP depleting drugs in living cells expressing either GFP-α-actinin or GFP-tubulin. This allowed for the observation of drug effects and the dynamic nature of the structures. Cells that were permanently transfected with GFP-tagged α-actinin were used in the experiments that were conducted on stress fibers. The fluorescent α-actinin is seen

brightly in a dotted pattern along the fibers in living cells, and its disappearance when stress fibers are disrupted provides a clear indication on the state of the stress fibers. The presence of the GFP tag allowed for both the fixed and live cell studies that were conducted. To observe the microtubules, GFP-tubulin expressing cells were used. The GFP-tag allowed for the fluorescent visualization of the entire microtubule since each of its subunits are tagged. By using GFP-tubulin, both fixed and live cell microtubule studies could be conducted.

The purpose of my study was to observe the loss of stress fibers in response to treatment with sodium azide and 2-deoxyglucose as well as to determine the time course for disruption. A second goal was to observe the protection of stress fibers from complete disruption by sodium azide and 2-deoxyglucose when the microtubules were previously disrupted using nocodazole. These real-time studies hope to reveal some evidence as to the nature of the interactions between microtubules and stress fibers.

Materials and Methods

Cell Culturing

The cells used in the live cell studies of actin stress fibers and microtubules are Swiss 3T3 mouse fibroblasts. Fibroblasts are cells that commonly secrete proteins that are found in the fibrous connective tissue of animals. The cultured fibroblasts used in the experiments are similar to tissue fibroblasts but they retain the ability to differentiate into other mesodermal cell types. They adhere to the glass or plastic by secreting matrix proteins such as fibronectin, laminin, and collagen and then adhering to the secretions. Fibroblasts will not usually grow if they have not adhered to some substratum. They are fairly easy to maintain and provide a convenient cell type for experimentation.

Swiss 3T3 cells and Swiss 3T3 cells with GFP α-actinin (provided by Carol Otey, UNC-Chapel Hill) were both maintained in Dulbecco's modified minimum essential medium (DMEM, Gibco-BRL) with the following components added: 10% fetal calf serum, glutamine, sodium pyruvate, penicillin/streptomycin, and G418 at 200 ug/mL (a selection antibiotic used only on the GFP α-actinin or GFP tubulin expressing cells for selection of cells containing resistance). The cells were maintained in an incubator under 37°C and 5% CO_2. Constant care was taken to prevent the cells from reaching confluence due to its negative effects on this cell line. Cells were transferred and subcultured when sufficient quantities of cells were present. They were transferred by removing the growth media and adding trypsin to detach the cells from the culture flasks used. After approximately 10 minutes, 2 mL

of normal growth media was added to the cells. The cells were then transferred to new flasks containing media at dilutions of 1:8 or 1:10.

To provide conditions more favorable to cell growth, sodium pyruvate was added to cell culture media. The media was also changed from low to high glucose content. The cells were maintained under these new conditions which provided adequate growth for the experiments conducted.

Immunofluorescence

To observe the effects of several drug treatments on Swiss 3T3 cells, these cells were plated overnight onto square coverslips. The cells were then treated with either nocodazole for 30 minutes, sodium azide with 2-deoxyglucose for 90 minutes, or a combination of nocodazole followed by sodium azide treatment. The effects were observed by fixing the cells. This was accomplished by removing the growth media and adding approximately 500 µl of formaldehyde for 10 minutes to each coverslip. The coverslips were then rinsed 2 to 3 times with calcium and magnesium free phosphate buffered saline (CMF-PBS). Next Triton-X solution was added to the coverslips for 3 minutes. The coverslips were then rinsed 2 times with CMF-PBS and transferred to a moist chamber. Fifty microliters of the desired antibodies were then added to each coverslip. To visualize the stress fibers, rhodamin-phalloidin was diluted at 1:75 and added to the coverslips for 45-60 minutes. The coverslips were then mounted onto slides with Mowiol and observed on a Nikon Diaphot microscope using a 100X, 1.4 n.a. objective.

To visualize the microtubules, the cells required a methanol fixation since formaldehyde fixation fails to work on microtubules. The coverslips were submerged

in ice cold methanol for approximately 6 minutes. The coverslips were then rinsed 2-3 times with CMF-PBS and the primary antibody was added. For microtubule staining an anti-tubulin antibody was used at 1:100 dilution and 50 µl was added per coverslip for 1 hour. Rinsing with CMF-PBS was then required 2-3 times prior to the addition of the secondary antibody. Fifty microliters of the secondary antibody (1:100) was then added to each coverslip for 45 minutes. The coverslip was then rinsed with CMF-PBS and mounted onto a slide as per the previous procedure.

Optimization of Immunofluorescence

Fluorescence experiments required the use of primary and secondary antibodies to label the microtubules and stress fibers. Microtubules were labeled using tubulin at a dilution of 1:100 from stock while the secondary antibody of FITC goat anti mouse was also used at 1:100. These conditions provided sufficient labeling to visualize the microtubules under the fluorescent microscope. To label the stress fibers an antibody to actin was used. Rhodamine-Phalloidin was used at a dilution from stock of 1:50. This produced very bright actin staining that seemed to interfere with observation of microtubules during double staining with FITC antibodies. Rhodamine-Phalloidin was then tested at different dilutions to find a level where it was effective yet did not interfere with viewing FITC antibody labeled structures. The optimum dilution was found to be 1:75 which provided bright staining but decreased the interference with viewing other structures.

Plasmid Purification

To aid in the visualization of the microtubules and stress fibers, a plasmid coding for fluorescent α-actinin and a second plasmid coding for fluorescent tubulin were utilized. Before a plasmid can be inserted into a cell, there must be a sufficient quantity of it in a usable form. When purchased, there was very little plasmid provided. The procedure for inserting the plasmid into the cells requires a fairly high concentration and therefore more plasmid must be produced. This was accomplished by transforming bacteria with the plasmid and allowing them to produce large quantities of the plasmid. Once cultured, the plasmid was then removed from the bacteria through a series of purification steps. This method of producing the plasmid provides large quantities that can later be used to transfect eukaryotic cells. The original plasmid, pEGFP-Tub, was purchased from Clontech Laboratories (CA). This plasmid codes for the GFP-tubulin used in microtubule experiments.

Transformation

To transform bacteria so that the plasmid can be mass-produced, DH5α bacterial cells were used. Three microliters of the plasmid was added to 50 μl of DH5α cells. The mixture was then incubated on ice for 30 minutes and the cells were heat shocked at 42°C for 2 minutes. After the heat shock the cells were returned to ice for 3 minutes. Thirty-five microliters of the heat shocked cell/DNA mixture was then added to 500 μl of liquid SOC medium. The mixture was incubated at 37°C for one hour while the tubes were inverted every 15-20 minutes. Next, 200 μl of the cells were plated onto agar plates with antibiotics and then the plates were incubated at 37°C for 16 hours.

Mini-Prep

The mini-prep was performed to verify the presence and intact nature of the plasmid in the colony picked from the transformation culture plates. The mini-prep consists of inoculating 5 mL of LB (containing 30 μg/mL kanamysin) with one colony from the transformation plate. The inoculated culture was then allowed to shake overnight at 30°C.

To perform the mini-prep, the cells from the overnight culture were pelleted, resuspended, and then lysed to allow the cell contents to be released. Next the solution was neutralized with a special solution and centrifuged. The supernatant was then decanted and combined with a purification resin. The resin/DNA mixture was then run through a purification column and the DNA was later washed and eluted. The exact procedure can be found in the Wizard Mini-Prep instructions (Qiagen, California).

Test for the Plasmid

To test for the presence of the plasmid, the DNA was then digested into fragments that were of a predicted molecular weight depending on the enzymes used. The digestive enzymes chosen were Xho1 and PVUII because their sites of cleavage are known from the plasmid structure. Three digestion samples were produced by combining 13 μl of the mini-prep DNA with a total of 1 μl of enzyme and 1.5 μl of a buffer. Three samples consisting of Xho1, PVUII, and a combination of both were digested for 1 hour at 37°C. Two microliters of the digests were then combined with 2 ul of loading dye and 6 μl of water. The samples were then run on an agarose gel

along with molecular weight markers. The digest produced the expected fragment sizes, indicating that the plasmid was intact.

Maxi-Prep

Once the presence and size of the plasmid was verified, a maxi-prep was performed to produce a large quantity of plasmid. The day prior to the maxi-prep, the single colony chosen from the transformation plates was used to inoculate 2 mL of LB medium with selective antibody. The cells were incubated for approximately 8 hours at 37°C with vigorous shaking. After the incubation, the starter culture was diluted 1:1000 into selective LB medium and grown at 37°C for 12-16 hours with vigorous shaking. The second culture was then used in the maxi-prep, which is very similar to the mini-prep. The only difference between the procedures was the larger quantities of material used and produced. The exact maxi-prep procedure can be obtained from Gibco (Grand Island, NY).

Once the maxi-prep had been performed, the purified plasmid DNA was then used to transfect eukaryotic cells. The plasmid was also verified by performing another restriction digest of the plasmid and running the fragments on an agarose gel.

Transfection

Insertion of the purified plasmid into eukaryotic cells requires a combination of scientific techniques and many trials. Inserting a plasmid into eukaryotic cells is a process called transfection. One of the most common methods of transfecting cells is lipofection. This method works by shielding the plasmid in a sphere of lipids or micelle type structure. Once in this form, these encased plasmids are exposed to the target cells. The lipid spheres interact with the cell membranes of the cells and

commonly fuse to allow the plasmid to enter a cell. After the cell has taken in the plasmid, it begins to read it and produce the target proteins encoded by the plasmid. The cells begin to express the desired protein and fluorescence experiments can then be conducted.

Prior to the transfection, Swiss 3T3 cells (both those expressing GFP α-actinin and the non-expressing cells) were plated onto 8 coverslips which were sterilely inserted into 30 mm petri dishes. Plating occurred the night before the transfection and it required that the cells be at approximately 80% confluence so that many cells could be transfected.

Lipofectamine PLUS reagent (Gibco, NY), was mixed with the plasmid DNA and allowed to incubated for 15-30 minutes. During this time the growth medium of the cells was replaced by serum-free medium. The lipofectamine/DNA solution was then added to the cells and allowed to incubate for approximately 8 hours. Serum was then added to the cells at the normal level used in growth media (10%). The cultures were allowed to incubate for approximately 24 hours. The exact procedure for the Lipofectamine PLUS transfection was obtained from Sigma Chemical Corporation (MO). After 24 hours, the cultures were observed for expression of the GFP-tubulin by the use of low-light level fluorescence microscopy. This was accomplished by placing Vaseline on the corners of the culture coverslip and inverting it (cell side down) onto a glass slide containing a drop of media. The slide was then viewed on the microscope.

The transfected cells were observed for expression levels at approximately every 8 hours after the initial 24-hour incubation. The goal was to determine the time

in which the highest number of cells expressed the plasmid after transfection, thus allowing for experiments to be conducted on these transiently transfected cells.

Live Cell Viewing

The method used in these experiments involves plating the desired cells in glass bottom dishes that could be directly placed on the microscope for observation. The cells were grown in normal growth serum, but for live cell observation this serum was replaced by CO_2 free medium since the outside atmosphere has a lower percentage of CO_2 when compared to the incubators used. To keep the cells at a consistent temperature (approximately 37°C), an air curtain was also prepared. This curtain regulated the temperature of the cell media by using a probe and an air heater to maintain the temperature.

By using the glass bottom dish, the cells were directly observed without moving them or having to sacrifice them on a coverslip. They were observed through standard fluorescence microscopy techniques and images were taken by using the attached 35mm camera or the CCD video camera. The video camera allowed for live viewing of the cells on a larger screen as well as the manipulation of the image using computer software (the software used is IMAGE 1 by Universal Imaging). Using these techniques, experiments were performed where the cells were observed after several drug treatments (nocodazole, and sodium azide with 2-deoxyglucose).

Nocodazole Drug Treatment

The studies of microtubule depolymerization were conducted using the drug nocodazole. Nocodazole causes the microtubules to favor disassembly and therefore

leads to the breakdown of the microtubule structures. Nocodazole was used at a concentration of 1.65 mM for a duration of 30 minutes. Images of the cells during the treatment were taken at intervals of 5 minutes during the 30-minute drug treatment. The images were taken using a CCD camera and then compiled to form a time-lapse film loop showing the depolymerization of the microtubules during the treatment.

Sodium Azide/2-Deoxyglucose

Studies of the structure and stability of actin stress fibers were conducted using GFP α-actinin expressing cells. Sodium azide with 2-deoxyglucose affects the cell by causing a depletion of cellular ATP (adenosine triphosphate) levels. This depletion causes a number of changes in the cell, one being the disruption of the stress fibers. Stress fibers disappear when cells are treated with sodium azide/2deoxyglucose due to the decrease in ATP. A mixture of 20 mM sodium azide and 10 mM 2-deoxyglucose was used to study the stress fibers. GFP α-actinin cells were treated with sodium azide and 2-deoxyglucose for 90 to 120 minutes. Images of the cells were obtained at 10-minute intervals for a total of 120 minutes using a CCD camera. These pictures were then compiled into a time-lapse film loop showing the stress fiber disruption during drug treatment over time.

Double Drug Treatment: Nocodazole followed by Sodium Azide/2-Deoxyglucose

To study the interactions of the microtubules and stress fibers, a double drug treatment of nocodazole for 30 minutes followed by sodium azide and 2-deoxyglucose for 120 minutes was conducted. GFP α-actinin cells were used to

observe the effects of the drugs on the stress fibers. Images were taken and time-lapse films showing the effects on stress fiber structure were obtained using the techniques previously described.

Results

Verification of Previous Research: Nocodazole Treatment, Sodium Azide/2-Deoxyglucose Treatment and a Combination of Drug Treatments In Fixed Cells

To observe the stress fibers and microtubules, cells were grown on coverslips and then treated using nocodazole, sodium azide and 2-deoxyglucose, or a combination of the two. The cells were then fixed and immunofluorescence microscopy was conducted using a tubulin antibody for the microtubule and rhodamin-phalloidin for imaging the actin in the stress fibers. Cells treated with nocodazole lost all their microtubules while those treated with sodium azide and 2-deoxyglucose showed disruption of the stress fibers. Treatment with both drugs (nocodazole for 90 minutes followed by sodium azide/2-deoxyglucose for 120 minutes) showed a disruption of stress fibers; however, some small number of stress fibers remained intact. This indicates that the depolymerization of microtubules somehow stabilized certain stress fibers. The stabilized stress fibers appeared randomly throughout the cell in no predictable pattern. These results are consistent with those observed by Jessica Clement.

Interestingly, cells treated with sodium azide and 2-deoxyglucose developed scattered triangular actin-containing structures throughout the cell. These structures appear to be formed from several small actin filaments as shown in Figure 1. The triangular structures always formed after stress fiber disruption and therefore may be aggregates of stress fiber proteins that appear as the stress fibers are disrupted since they are not seen prior to disruption.

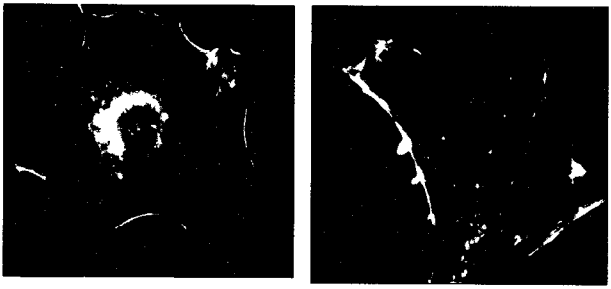

Figure 1. Swiss 3T3 cells marked with actin antibodies after treatment with sodium azide and 2-deoxyglucose. These pictures show the triangular structures observed after stress fiber disruption.

Optimization of Drug Treatments, Antibody Concentrations and Cell Culture Conditions

The nocodazole was tested at a concentration of 1.65 mM and was found to be sufficient to disrupt the microtubules in the Swiss 3T3 cells. However the sodium azide (20mM) and 2-deoxyglucose (10mM) did not appear to totally disrupt all stress fibers. To find an optimal treatment time, cells were treated with three different concentrations of sodium azide and 2-deoxyglucose for 90 minutes using 20mM sodium azide/10mM 2-deoxyglucose, 40mM sodium azide/20mM 2-deoxyglucose, or 60mM sodium azide/30mM 2-deoxyglucose. In addition, 90 minute and 120 minute drug treatment times were investigated. I found that 40mM sodium azide and 20mM 2-deoxyglucose concentrations were sufficient to totally disrupt the stress fibers in the cell line used. The 60mM sodium azide and 30mM 2-deoxyglucose concentrations were also successful but the lower concentration was chosen to minimize any adverse effects the drug may have on the cell at such high levels.

Transformation of Swiss 3T3 cells with GFP-Tubulin

To visualize microtubule structures in living cells, a plasmid containing the gene for GFP-tubulin was obtained from Clontech Laboratories and transfected into two cell lines, either Swiss 3T3 or Swiss 3T3 cells expressing GFP-α-actinin. The optimal transfection conditions were those initially followed in the protocol listed in the methods. Transfection rates for both Swiss 3T3 cells and GFP-α-actinin expressing Swiss 3T3 cells during two attempts can be seen in Table 1.

Table 1: Transfection Rates for GFP-Tubulin Expression in Swiss 3T3 Cells During Two Attempts

Transfection Attempt	Cells Line	Time after transfection (Hours)	Percent expressing some level of non-specific fluorescence	Percent expressing fluorescently labeled microtubules
1st	Swiss 3T3	72	67%	9.7%
2nd	Swiss 3T3 GFP α-actinin	24	----	19%
2nd	Swiss 3T3 GFP α-actinin	34	----	26.4%
2nd	Swiss 3T3 GFP α-actinin	44	----	27%
2nd	Swiss 3T3 GFP α-actinin	55	----	26%
2nd	Swiss 3T3	24	60%	13%
2nd	Swiss 3T3	44	71%	6%
2nd	Swiss 3T3	55	75%	12.5%

The repeated transfection attempts coupled with growth selection media containing G418 produced a permanently expressing cell line of GFP-tubulin. Transfection of the GFP α-actinin expressing cell line with the GFP-tubulin plasmid did not result in a cell line. Many of the cells in the new GFP-tubulin expressing cell line contained a large amount of background or non-incorporated fluorescent protein as seen in Figure 2. This background made it difficult to visualize microtubules in

live cells. The overall fluorescence brightness level of these cells is also fairly low, thus complicating imaging of the microtubules.

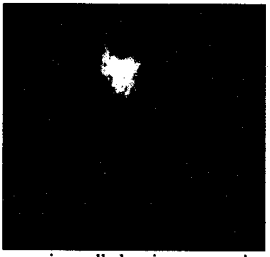

Figure 2. A GFP-tubulin expressing cell showing some microtubule structures with a large amount of background fluorescence.

The microtubules seen during transient transfection of GFP-α-actinin expressing cells are also difficult to visualize in live cells. The difficulty arises due to the background fluorescent levels of the GFP-α-actinin combined with background GFP-tubulin. Even though microtubule structures can be seen in these cells, they are difficult to follow since both the microtubules and stress fibers are marked with the same GFP probe. However, these limitations are offset by the increased experimental capabilities for live cell studies by the use of these fluorescently labeled proteins that are produced in living cells.

Live Cell Experiments: Nocodazole Treatment Initiates Microtubule Disruption

Nocodazole was added to the cell culture media and its effects on the microtubule structure in the cells were followed in the newly established GFP-tubulin cell line. During the 20 minutes that the cell was followed, the microtubules readily

disappeared within the first 4-8 minutes and background cell fluorescence levels increased (Figure 3). The microtubules disappeared quickly from the cell periphery and after 4-8 minutes no individual microtubules could be seen. A characteristic of this cell line is that the cells do not become well spread, thus making it difficult to image the thin microtubules because many of them overlapped in these rounded cells. The overlapping made it difficult to track individual microtubules even though some could be seen along the periphery or flattest parts of the cells prior to nocodazole treatment.

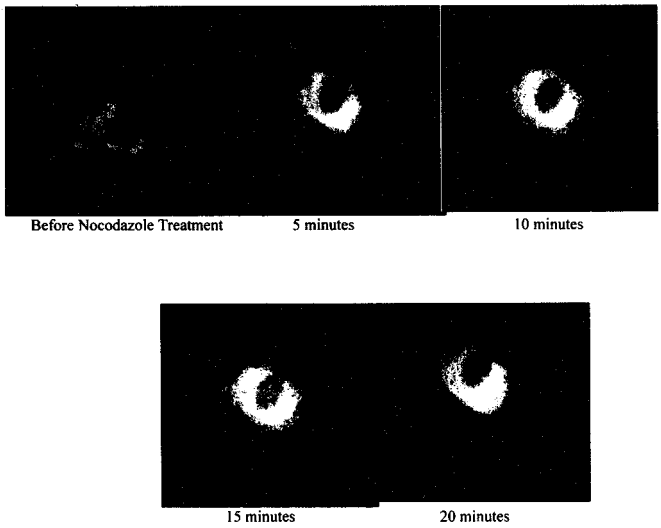

Figure 3. GFP-tubulin expressing cells were treated with nocodazole to disrupt the microtubule structures. These pictures show a cell prior to treatment and during nocodazole treatment at intervals of 5 minutes.

Live Cell Experiments: Nocodazole Treatment Causes Stress Fiber Strengthening

GFP-α-actinin expressing cells were observed after nocodazole was added to the cell media. The drug is known to disrupt microtubules and therefore the effects of microtubule disruption on stress fiber stability were observed in these cells. After drug treatment, the dotted pattern of α-actinin along the stress fibers did not change its positioning but some dots found along a small percentage of stress fibers seemed to become brighter and more prominent. No large increase in stress fibers or the presence of α-actinin in focal adhesions was observed. The only effect was a slight increase in the brightness of α-actinin found along the stress fibers and therefore a slight increase in stress fiber thickness.

Live Cell Experiments: Sodium Azide/2-deoxyglucose Treatment Causes Stress Fiber Disruption

Cells containing either GFP-α-actinin or both GFP-α-actinin and GFP-tubulin were treated with sodium azide and 2-deoxyglucose to observe the effects of the drug on stress fiber stability. As was observed in fixed cell studies, live cells responded to the drug by disruption of the stress fibers. The drug treatment was conducted for 120 minutes. During the treatment, the cells began to slowly round up and the adhesion plaques were seen to move from the cell periphery toward the main body of the cell. As the cells began to pull in on themselves they appeared to lose the stress fibers from the area around the nucleus first, followed by those along the periphery. The stress fibers along the cell periphery appeared to be intact during the first 80 minutes of drug treatment and then they slowly disappeared. As the cell began to shrink, the peripheral stress fibers also appeared to shorten in length but did not seem as

disrupted as those found toward the interior of the cells. After approximately 120 minutes, the stress fibers throughout the cell seemed to have been disrupted. This experiment was also repeated with double the concentration of sodium azide and 2-deoxyglucose but the results were the same. The results can be seen in Figure 4.

Cells expressing both GFP-α-actinin and GFP-tubulin responded similarly to the drug treatment. The microtubules are difficult to visualize in the double-expressing cells and therefore visualization of their structures is further complicated during stress fiber disruption due to its effect of increasing background fluorescence. Thus the simultaneous visualization of microtubules and stress fibers during this drug treatment provided no additional insight into the interactions between the microtubules and stress fibers.

Live Cell Experiments: Nocodazole Treatment Followed by Sodium Azide/2-deoxyglucose Treatment Stabilizes Stress Fiber Structures from Disruption

To further study the interactions between stress fibers and microtubules, a double drug treatment was conducted. A 30-minute treatment of nocodazole was performed on GFP-α-actinin expressing cells to disrupt the microtubules. This treatment was followed by the addition of sodium azide and 2-deoxyglucose. The cells were then observed over 120 minutes.

The dotted α-actinin distribution on the stress fibers was seen under the fluorescence microscope. During the drug treatment some of the stress fibers around the cell nucleus appeared to be disrupted (Figure 5). Those stress fibers found at the cell periphery did not appear to become disrupted during the treatment. These stress fibers neither looked strengthened nor more prominent than in control cells, but the

stress fibers failed tc disrupt completely. These fibers also failed to shorten in length to the same extent as that seen in the drug treatment using only sodium azide and 2-deoxyglucose. Even though many of the stress fibers remained after the treatment, parts of the cell still seemed to contract and become less spread on the substratum.

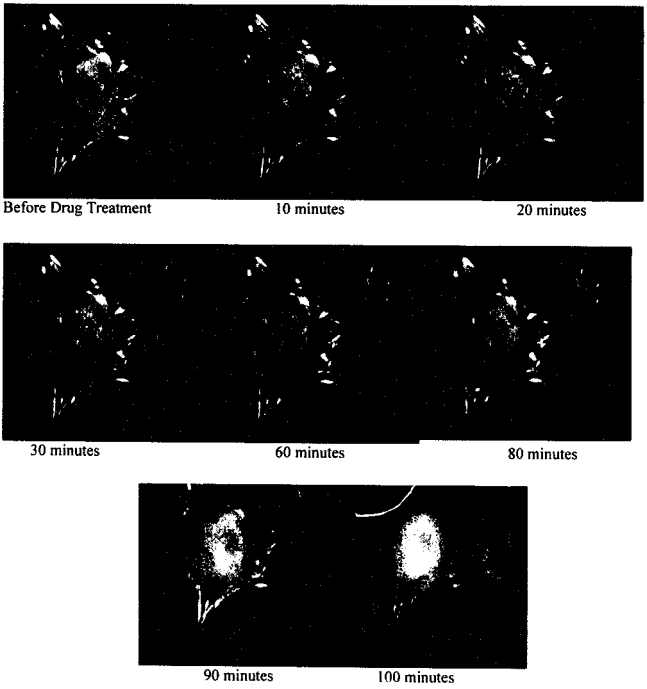

Figure 4. GFP α-actinin expressing cells were treated with sodium azide and 2-deoxyglucose for 100 minutes. Pictures of the cells were taken at 10-minute intervals. The disruption of the stress fibers can be seen over time as well as the rounding up of the cell.

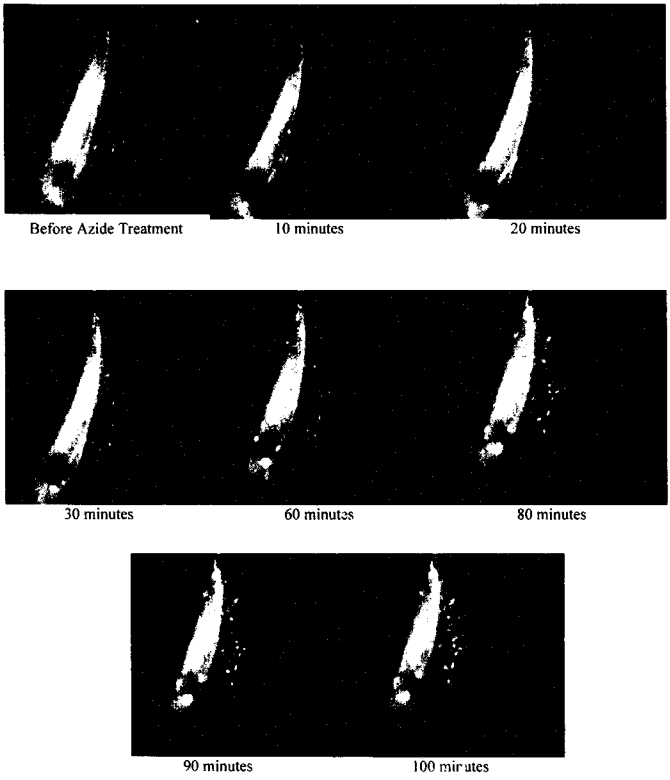

Figure 5. GFP-α-actinin expressing cells were treated with nocodazole to disrupt the microtubule structures, followed by treatment with sodium azide and 2-deoxyglucose to disrupt the stress fibers. These pictures show a cell prior to treatment and during sodium azide/2-deoxyglucose treatment.

Discussion

The mechanism of interaction between microtubules and actin stress fibers still remains unknown. Previous studies have relied on fixed cell experiments (Clement, 1998; Enomoto, 1996). These studies were repeated using Swiss 3T3 mouse fibroblasts to verify the observed interactions. As shown in previous studies the actin stress fibers were disrupted when the cells were depleted of ATP using sodium azide and 2-deoxyglucose (Glascott et al., 1987; Sanger et al., 1983). Also, microtubule disruption by nocodazole treatment prior to ATP depletion led to a net strengthening of stress fibers and increased contractility (Enomoto, 1996; Kajstura and Bereiter-Hahn, 1993). Observation using live cells allows for the dynamic viewing of the effects of the drug treatments. By observing the effects in live cells, it was thought that some component of the interaction between the microtubules and stress fibers could be deduced. The use of live cells also confirms the fixed cell studies since the interactions are being observed in vivo.

My studies concentrated at first on optimizing drug treatment conditions, followed by reproducing the fixed cell studies in living cells. The optimum treatment of nocodazole to disrupt microtubules was maintained as in previous studies during an exposure time of 30 minutes (Clement, 1998). To disrupt the stress fibers, 40 mM sodium azide and 20 mM 2-deoxyglucose concentrations for 120 minutes were found to be optimal. The optimal drug treatments had to be found to prevent drug overdosing and thus any toxic effects they may have. Optimization was also necessary to verify that the concentration and treatment times were sufficient to totally disrupt the structures.

Study of the stress fibers and microtubules in living cells required the use of fluorescently labeled subunits. Microinjection of tubulin or α-actinin is a plausible method but the subunits are artificially tagged and therefore their function may be slightly altered when viewed in the cell. A method more conducive to in vivo studies relies on transfecting cells with a plasmid coding for a cytoskeletal subunit with a fluorescent tag (GFP) attached. GFP tagged to a subunit through the use of a plasmid minimizes any interference that artificial fluorescent labeling may cause in live cell studies.

GFP-tubulin expressing cells showed prominent, fairly bright microtubules that were slightly hidden by background fluorescence. Observation of microtubules in GFP-α-actinin expressing cells was also difficult due to background fluorescence. In this situation the background was greatly enhanced since both GFP-tubulin and GFP-α-actinin were present in the cells. Microtubules could be seen in some cells but not to any great extent. Also the transfection of GFP-α-actinin expressing cells with GFP-tubulin made selection for transfected cells virtually impossible since both plasmids used G418 as a selection antibiotic. Thus any cell expressing either plasmid would survive in the selection media. Some cells were initially observed to co-express the plasmids after transfection but the co-expression was lost during maintenance of these cells. These studies indicate that any experiment requiring co-expression of these plasmids must be performed immediately after transfection. A possible method to reduce the background in the co-expressing cells could be to induce full microtubule polymerization through the use of Taxol. This should clear some background fluorescence if it is caused by unincorporated tubulin.

Live cell experiments were performed after the successful transfection of GFP-tubulin, which formed a cell line. The first experiment observed the disruption of microtubules over a 30-minute treatment of nocodazole. During this treatment the microtubules were observed to disappear as background cell fluorescence increased. There remained no visible microtubules at the end of the treatment. This is consistent with previous studies using fixed cells (Enomoto, 1996; Danowski, 1989; Clement, 1998; Kajstura and Bereiter-Hahn, 1993). Increased background can be attributed to GFP-tubulin that was disrupted from the microtubules. The time course used may be too spread to allow for accurate visualization of the disruption since it occurs so quickly. Further studies using decreased time intervals may further help in understanding how the disruption occurs.

The next live cell experiment was conducted to observe stress fiber disruption during treatment with sodium azide and 2-deoxyglucose. These studies were mainly conducted on only GFP-α-actinin expressing cells due to the difficulties encountered with cells expressing both GFP-α-actinin and GFP tubulin. During the treatment in live cells the dotted pattern of α-actinin along the stress fibers was seen to disappear over time. The disruption occurred initially toward the inside of the cell followed by peripheral stress fibers. After treatment, the cells also seemed to become more rounded and lose some of there adhesion to the substratum even though adhesion plaques remained bright. These results are consistent with those observed in fixed cell studies (Glascott et al., 1987; Sanger et. al, 1983; Clement, 1998; Enomoto, 1996). The additional information provided by the live cell study pertains to where stress fiber disruption begins and how it propagates. This is difficult to observe in fixed

cells. The experiments performed show that disruption begins toward the center of the cell and then propagates to the periphery. These observations must be verified before a conclusive method of disruption can be obtained.

The final experiment conducted was aimed at observing the interactions between microtubules and stress fibers. Cells expressing GFP α-actinin were treated first with nocodazole to disrupt the microtubule structures followed by treatment with sodium azide and 2-deoxyglucose to disrupt the stress fibers. This live cell study also produced similar results to those found using fixed cells (Enomoto,1996; Clement, 1998; Danowski, 1989). Cells treated with both drugs were found to slightly resist total stress fiber disruption. Some stress fibers toward the interior of the cell did disrupt while others found along the periphery remained stable. The later disruption of the peripheral stress fibers may be due to their more important role in maintaining cell shape and tension. Therefore the less structurally important interior stress fibers are more easily disrupted while those along the periphery resist disruption. The cells were also observed to round up slightly but not to the same extent as those solely treated with sodium azide and 2-deoxyglucose. Rounding-up may be a side effect of the drug treatment in these cells. In the ideal case, the cells should not round up and should remain fairly spread. Stress fibers that were stabilized did seem to slightly shorten in length during the treatment but the α-actinin still remained prominent along their entire length. These results reinforce previous studies that found a link between microtubule stability and actin stress fiber stability.

There are several proposed mechanisms for the interactions between microtubules and the actin stress fibers. The first model reveals an inverse

relationship between microtubules and stress fibers. This model is referred to as tensegrity and portrays the microtubules as an incompressible meshwork to which the stress fibers can contract against and therefore generate cell tension (Ingber, 1993; Danowski, 1989). Therefore this model would indicate that the increased cell contractility and rounding up can be attributed to microtubule disruption thus removing the opposing force to stress fiber tension and allowing them to further contract.

Another explanation for the increased contractility and rounding up of the cells after microtubule disruption is the activation of the GTPase, rho. Studies of rho have shown that it stimulates cell contractility by activating a biochemical pathway that increases myosin light chain phosphorylation (Liu et. al, 1998). Increasing the phosphorylation of the myosin light chain should increase contractility in non-muscle cells (Liu et. al, 1998). Studies of rho have shown that microtubule depolymerization leads to rho activation and therefore could explain the observed increase in contractility (Liu et. al, 1998; Enomoto, 1996).

Microtubule associated proteins (MAPs) may also play a role in increasing the strength of stress fibers during microtubule disruption. It has been observed that MAPs help to organize bundles of microtubules (Griffith and Pollard, 1982). Therefore MAPs may be freed in the cell during microtubule disruption with nocodazole. MAPs have also been shown to cross-link actin filaments (Griffith and Pollard, 1982). The strengthening of some stress fibers even after attempted disruption may be due to the release of MAPs during microtubule disruption. This hypothesis is also consistent with the results of stress fiber disruption without

microtubule perturbation since none of the stress fibers appeared to have been strengthened.

Both MAPs and rho may play a role in the observed strengthening of stress fibers after microtubule disruption. Although my experiments may fail to promote one mechanism of the interaction, the results clearly indicate that some interaction exists. It is also clear that microtubules play a greater regulatory role than stress fibers in the interaction. The relationship that exists between microtubules and stress fibers is interesting and requires further studies that, over time may elucidate the exact mechanism of the interaction.

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