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Identification and Analysis of Prohibitin in B16 Mouse Melanoma Cells

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IDENTIFICATION AND ANALYSIS OF PROHIBITIN IN B16 MOUSE MELANOMA CELLS

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by

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Abstract

Prohibitin, a highly conserved protein found in eukaryotic cells, has been found in recent years to possess a wide variety of functions in the cell. Whereas the mechanisms by which the protein functions are still largely unknown, it is clear that prohibitin possesses very strong anti-proliferative properties in the cell, as well as potentially both nuclear and mitochondrial functions based upon its movement and localization upon apoptotic signaling. Upon treatment with retinoic acid, transformed B16 mouse melanoma cells also indicate a differential expression of prohibitin in which production of the protein is increased. The purpose of this work was to both validate the initial findings reported using this cell line and 2D gel electrophoresis, as well as attempt to identify, isolate, and analyze prohibitin using mass spectrometry in order to determine if in its function within the cell, prohibitin was in any way being modified. Although several proteins such as carbamoyl phosphate synthase I, heat shock protein 70, and annexin A5 were identified as part of this experiment, prohibitin was never isolated by immunoprecipitation and was only observed using western blotting.

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Introduction

Prohibitin (PHB), also known as B-cell associated receptor protein 32 (BAP32) is a highly conserved protein found across multiple organisms ranging from cyanobacteria to yeast, plants, and *Drosophila* as well as mice and humans.[1,2,3,4,5] Prohibitin has been proposed to have both nuclear as well as mitochondrial roles based upon localization experiments, however although the protein appears to be present mainly in the inner mitochondrial membrane, it has also been observed inside the nucleus itself. Prohibitin is a 32kDa protein that was initially named due to its proposed role in arresting cell growth and development and preventing the cell from proceeding along the cell cycle under certain conditions. Initial studies of the protein showed that upon microinjection of the prohibitin mRNA into fibroblasts, synthesis of new DNA was halted[6], however it was later found that this was only an effect of the 3' untranslated region of the mRNA as opposed to a function of the protein itself.[7] Prohibitin seems to be highly vital to the survival of an organism due not only to the degree by which its sequence is conserved, but also the effects of removing it at an early stage of development, since cells in which prohibitin has been removed fail to develop. In mammals such as *Mus Musculus* or *Rattus Norvegicus*, prohibitin shows a homology greater than 96%, indicating a high degree of conservation of structure and likely function as well.[8]

In experiments involving fluorescent probes attached to prohibitin injected into hamster kidney cells, immunoelectron microscopy has also revealed that the protein appeared to be localized in the outer mitochondrial membrane under some circumstances, although localization to the inner membrane has been proposed to be the final target.[9] However, recent research involving the treatment of cells with chemotherapeutic agents

such as camptothecin have also indicated that prohibitin may be initially localized in the nucleus, and contain a tag allowing it to export other proteins such as E2F before being inserted into the mitochondrial membrane.[10] Supporting this possibility is evidence that prohibitin also contains a mitochondrial import tag on its amino terminus, perhaps facilitating movement to this compartment of the cell. Studies involving attaching a cMyc epitope tag to both the amino and carboxy termini indicated that it was the N-terminus that facilitated the movement of prohibitin into the mitochondria through the use of a cleavable sequence at the beginning of the protein.[9] Although it does appear as if the prohibitin is being localized to the mitochondria, the possibility still exists that it could be removed to function elsewhere in the cell, as well. As much of the function of a protein may be elucidated based upon its localization within the cell, the fact that a consensus cannot be reached upon its initial location within the cell raises several concerns and is of the utmost importance.

Prohibitin has been proposed to have nearly as many functions within the cell as points of localization. Early studies of the protein have proposed that it functioned in the regulation of cell proliferation pathways.[11] Mutations in the prohibitin gene were reported to lead to such effects as the generation of sporadic breast cancers.[12, 15] Secondly, some studies also indicated that prohibitin had a role similar to the retinoblastoma (Rb) protein in inhibiting the function of E2F1, adding support to the claim that prohibitin functions in promoting cell proliferation.[16] However, conflicting studies also reported that prohibitin was a regulator involved in determining whether cells should undergo an apoptotic pathway, instead. In co localization experiments, prohibitin was observed as being associated in the nucleus with Rb, E2F, and p53, as well as associated with some of these

proteins after likely facilitating their movement outside of the nucleus, which supported the possible apoptotic role for the protein.[17]

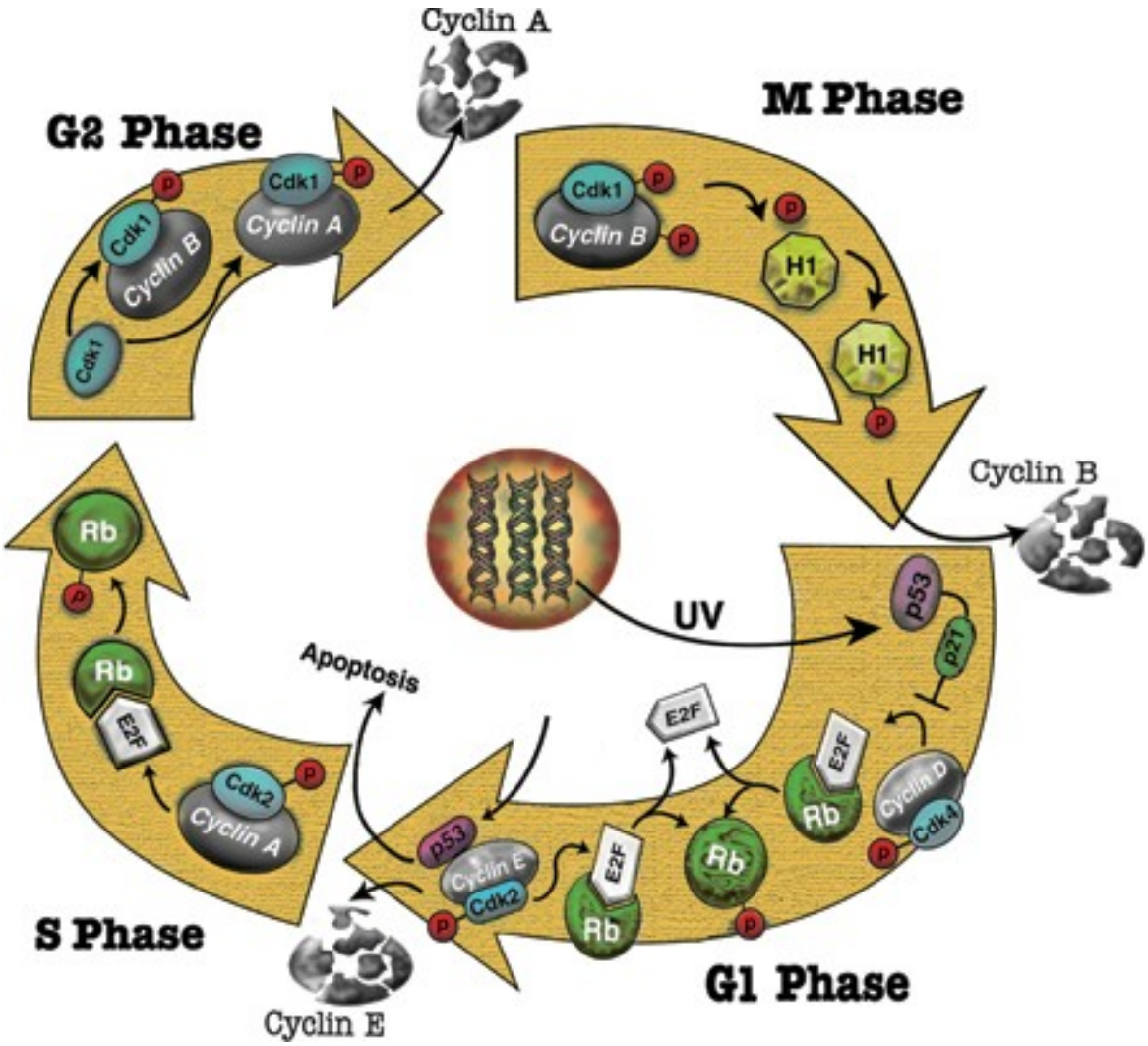


Figure 1: Generic Diagram of Cell Cycle. Several proteins known to be affected by prohibitin such as Rb and E2F are indicated within the G1 and S phases

Furthermore, studies into the sequence of prohibitin have noted its high degree of homology with Cc, a protein found in *Drosophila melanogaster.*[3,6] Cc has been shown to be present at times in the mitochondrial envelope of cells, as well as have reported roles in DNA replication and the regulation of progression through the cell cycle. In some studies where the Cc gene has been deleted , embryogenesis has prevented the fly from

developing past the larval stage, thus supporting the claim that Cc is vital the development and survival of the animal. Prohibitin shows many of the same characteristics as Cc, as well as homology with the protein, indicating that it too must serve a very vital role for the cell.

To add even more complexity to the many roles that prohibitin is proposed to take within the cell, research has also suggested that prohibitin may be one of the few proteins that may result in the loss of the senescent phenotype.[18, 19] Cellular senescence is the process by which cells, instead of undergoing apoptosis, lose the ability to divide and replicate. Under normal circumstances this may occur naturally along with such processes as the shortening of the telomeres, however prohibitin is thought to have a different mechanism by which it functions. Instead of stopping mitosis and leading to cell death over time, prohibitin seems to lead the affected cells into becoming immortalized in some cases. Finally, it has been proposed by researchers looking at lower organisms that the function of prohibitin may be more concerned with protein folding than a direct interaction with the regulation of the cell cycle.[2,20,21] Prohibitin is known to interact with a closely related 37kDa protein known as prohibitone.[22] The two proteins often form ring structures within the mitochondrial membranes, and it has been suggested that they may serve as chaperone proteins by stabilizing newly synthesized gene products within the mitochondria, including many involved in the respiratory cycle.[23] Work done with the establishment of a new rat ovarian granulosa cell line (RGA-1) found a drastic up regulation of prohibitin contained in the cells, even just prior to the induction of apoptosis.[24] In that particular cell line, localization of prohibitin to the mitochondria was not only confirmed, but determined to be present in a larger amount

than was expected, indicating that in these cell lines, prohibitin may aid in proliferation either by performing its role in a chaperone complex, or that perhaps with destabilization of mitochondrial function, prohibitin may migrate and assist in some way with the process of cellular respiration. In this sort of example, prohibitin then would be acting in a fashion to protect against apoptosis in cells during times of metabolic stress. [9]

Furthermore, as stated above, prohibitin not only affects other proteins within the cells, but also seems to have an effect on gene regulation within it. Co-localization of prohibitin with proteins such as Rb, p53, and E2F only begin to touch the surface of the extent of its action within the nucleus, as research has also shown it to have interactions with other nuclear proteins. Prohibitin has been observed interacting with several proteins including HDAC1, BRG1/BRM, N-CoR, and in one heavily studied example, estrogen receptors.[25] In the interaction with estrogen receptors, a correlation has been found between the removal of prohibitin and the decrease in function of growth inhibition by anti-estrogens in human breast cancer cell lines.[26, 27, 28]

If the information presented above is true, then the possibility exists that the function of prohibitin is in fact somewhere between the proliferatory and apoptotic pathways, and instead acts to somehow regulate the cell between those pathways. This possibility has been suggested; however, the exact mechanism by which prohibitin functions in order to do this is still unknown. At a glance, many of the studies of which prohibitin has been the target appear to have conflicting results, as they describe a vast array of proposed functions for the protein. However this may only further support the possibility that instead of having a specific, defined function, it may instead serve a vital regulatory role within the cell. It is entirely plausible to suggest that perhaps the role of

prohibitin may change depending on not only the type of cell that it is found in but also the state of that cell. Even if the role of the protein may change, this would not change the fact that it is obviously vital to the cell in order for survival. For the protein to be as crucial to cell development as it clearly as, while at the same time have mechanisms of action that are not clearly understood, it is evident that a better understanding of this protein, its targets, and the changes that it undergoes would be of great value.

In addition to this, research specifically targeting prohibitin in recent years has noticed some interesting results when using the compound known as 4-ethyl-4-hydroxy-1*H*-pyrano[3',4':6,7]indolizino[1,2-b]quinoline-3,14-(4*H*,12*H*)-dione, also known as camptothecin.[29] Camptothecin is a quinoline-based alkalioid whose derivatives are frequently used as chemotherapeutic agents. The common name for camptothecin was given after the initial discovery of the compound was made in samples taken from the bark of the Chinese camptotheca tree, and to date it is one of only five naturally occurring known inhibitors of topoisomerase I. Topoisomerase I is a protein that functions by creating single strand breaks in chromosomal DNA, and then loosening the coiling before ligating the strands back together. Camptothecin functions by binding to topoisomerase I, and allowing it to break the DNA strand, but preventing re-ligation from taking place.[30, 31] Due to the increased amount of DNA replication that takes place in cancer cells, they tend to be more prone to being affected than normal cell types. It is for this reason that camptothecin was originally researched to be used as a chemotherapeutic agent.[32]

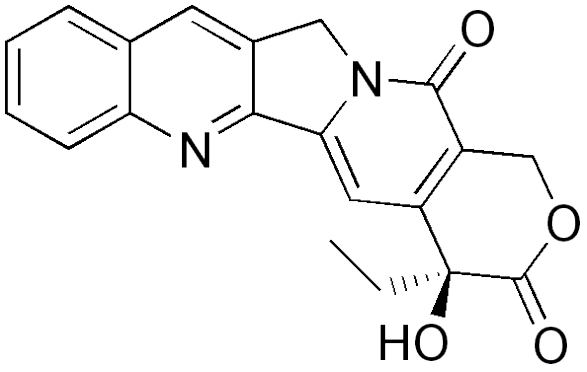


Figure 2: Structure of Camptothecin molecule

In recent studies involving the treatment of human breast cancer cells with camptothecin, it has been found that the compound exhibits properties that lead to the movement of prohibitin from the nucleus of the cell toward the mitochondria. Since it is known that the compound has a very well defined effect on prohibitin, it represents one more mechanism that can be studied, as camptothecin clearly is able to affect the cell in some way that forces the protein to react to the changes affecting the cell.

Previous research performed by undergraduates at Marshall University indicated through both 2D gel electrophoresis and western blotting that prohibitin was being differentially expressed in B16 mouse melanoma cells treated with retinoic acid, and that perhaps some sort of modification had taken place, causing the protein to migrate slightly different between the two gels. These results were of particular interest, because retinoic acid does not influence cell proliferation or apoptosis in B16 cells, but rather acts by arresting cell growth in the G1 phase.

Melanoma is a highly aggressive form of skin cancer that causes the majority of skin cancer related deaths in the US each year. Despite advances in medical knowledge, the primary form of treatment for melanoma remains surgical treatment, as there is no

effective chemotherapeutical treatment once the tissue begins to metastasize. The B16 cell line is a rapidly spreading form of melanoma found in mice. Retinoic acid is the biologically active form of retinol, and has been shown to induce terminal differentiation in B16 cells through interaction with retinoic acid response elements (RARE) within the nucleus of the cells.[33,34,35] The reported results involving prohibitin in these cells are interesting, as they indicate a previously unreported effect of retinoic acid on these cells.

If prohibitin does in fact regulate so many processes within the cell, it is of the utmost importance that the protein be isolated and more thoroughly analyzed for any structural characteristics that may assist in elucidating its mechanism more precisely. Although the concentration of the protein between treated and untreated cell lines has been noted to be drastically different, it may be possible that the interaction of the protein with the cell may be different as well. Although in previous studies it has been noted that prohibitin contained within the nucleus contains a nuclear export tag, as well as a mitochondrial import tag, the possibility still remains that in its interaction with the cell, a peptide could be cleaved after localization granting it further mobility between compartments of the cell.[9, 36] Another likely possibility exists that a post-translational modification such as phosphorlyation or methylation could take place, thus modifying characteristics such as charge or conformation, and also allowing the protein to show a different interaction with the B16 cells. It has been observed in some studies that prohibitin may undergo phosphorlyation or ubiquitination, thus making this a viable possibility for how it may interact with the cell.[37] One simple way to directly analyze the protein and search for modifications that may be present is through the use of mass spectroscopy.

By digesting the prohibitin protein into smaller peptide fragments, it becomes possible to directly analyze the protein and see the physical weight differences between potentially modified portions of the protein.[38] The challenge in performing this technique is that the protein sample being analyzed must be purified before it can be digested and analyzed. One potential method for isolating the protein in a cellular lysate is immunoprecipitation.

With immunoprecipitation, an antibody generated to be specific only to the protein of interest is generated, and allowed to react with epitopes on the protein so that it specifically binds.[39, 40] The complex between the antibody and protein is then recovered and purified using a protein bead while the rest of the solution containing other proteins not of interest is removed. With this technique, it is possible to isolate prohibitin and ensure with a greater degree of accuracy that the protein digests contain only one protein for analysis.

In this experiment, attempts were made at purifying the protein by immunoprecipitation for analysis with mass spectrometry. However, after initial difficulties from the immunoprecipitation of the protein, it became necessary to analyze the initial identification of the protein and attempt to validate the previously reported results regarding this important regulator of both cell proliferation and apoptosis. By repeating the initial groundwork of the experiment, it could be ensured that the foundation of the experiment in fact held true before more attempts at immunoprecipitation were made.

Furthermore, with access to camptothecin, a secondary experiment was undertaken in order to attempt to determine if prohibitin could again be visualized using

2D SDS-PAGE so that it could be analyzed and determined if there were any modifications present to support its export from the nucleus as reported in the literature, as well as note if the regulation of the protein had changed at all under conditions that would highly favor the cells undergoing apoptosis.[41, 42, 43]

Materials and Methods

2D SDS-PAGE

Cell lysates were separated by a two dimensional gel electrophoresis by first diluting the samples with 125µL of a rehydration buffer placed in a rehydration/equilibration tray and then placing a 7cm IPG strip with a pH gradient of 3-10 (Biorad) into the solution. The IPG strips were allowed to absorb the rehydration buffer for one hour before being overlayed with mineral oil to prevent further evaporation. The strips were then allowed to rehydrate and load the cell lysate overnight. The mineral oil was removed from the IPG strips and they were placed into a focusing tray. Paper wicks were wet with 10mL of deionized water and used to create direct contact between the IPG strips and the electrodes in the tray. Another layer of mineral oil was placed over the strips to prevent evaporation, and the IPG strips were left overnight to focus with a starting voltage of 0V ending at 4,000V, 8-10,000 V-hr, and a rapid ramp in voltage at a constant temperature of 20°C, with a maximum current of 50µA per strip.

After the first dimension of charge separation was complete, the mineral oil was removed from the strips, and 2.5 mL of a SDS-PAGE equilibration buffer was added into the tray over each IPG strip. The strips were allowed to sit for 10 minutes after which

point the buffer was decanted and replaced with a second equilibration buffer. The strips were allowed to sit in the buffer for an additional 10 minutes before it was removed, and then the gels were washed using a solution of 10% SDS/Tris/Glycine running buffer to remove any excess equilibration buffer present.

IPG strips were laid onto a precast 12% polyacrylamide gel (Biorad) and run at 200V for 45 minutes until the prestained molecular weight markers appeared to be significantly separated. The gels were washed in deionized water before being stained overnight using Coomassie Blue (Biorad). Analysis of the gel was performed first by imaging on a Genomic Solutions gel imaging system to visualize the staining pattern, and then the spots were excised, digested, and processed for mass spectrometry as detailed below. All recipes listed for the 2D SDS-PAGE may be found in Appendix I.

Cell Culture

B16 mouse melanoma cells were cultured from stocks obtained from Dr. Richard Niles using RPMI growth medium supplemented with L-glutamine, 5% bovine calf serum and 1% Penicillin/Streptomycin. The media was mixed from a powder and supplemented with sodium bicarbonate. Media was removed from the stock flasks, and replaced with 10mL of 1X PBS to wash the cells. The PBS was removed and replaced with 2.5 mL of 1X trypsin, and the cells were allowed to sit in the flask for 5 minutes to allow them to lift off of the surface. The cells were then resuspended in a 50mL conical tube in 35mL of RPMI medium. A cell count was performed using a hemacytometer, and approximately 200,000 cells were added along with 10mL of media in a 10cm dish. Cells were allowed to incubate for 24h at 37°C. At this point, some of the plates were

labeled as control dishes and allowed a 48h incubation, while another fraction of the plates were treated with 10μM retinoic acid and also allowed to incubate for 48h. After 48h, the cells were examined and the media was removed. 2mL of cold 1X PBS was added to each plate to wash the cells, the PBS was removed, and another 2mL of PBS was added before the cells were scraped from the plates into a centrifuge tube. Cells were spun at 1,000 rpm for 5 minutes, and then the PBS was removed. 200μL of lysis buffer was added to each tube, and the samples were sonicated, centrifuged for 5 minutes at 1000 rpm, and stored at -80°C. The recipe for the lysis buffer may be found in Appendix I.

Treatment with Camptothecin

B16 mouse melanoma cells were cultured from stocks obtained from Dr. Richard Niles using RPMI growth medium supplemented with L-glutamine, 5% bovine calf serum and 1% Penicillin/Streptomycin. The media was mixed from a powder and supplemented with sodium bicarbonate. Media was removed from the stock flasks, and replaced with 10mL of 1X PBS to wash the cells. The PBS was removed and replaced with 2.5 mL of 1X trypsin, and the cells were allowed to sit in the flask for 5 minutes to allow them to lift off of the surface. The cells were then resuspended in a 50mL conical tube in 35mL of RPMI medium. A cell count was performed using a hemacytometer, and approximately 200,000 cells were added along with 10mL of media in a 10cm dish. Cells were allowed to incubate for 24h at 37°C. At this point, some of the plates were labeled as control dishes and allowed a 48h incubation, while another fraction of the plates were treated with camptothecin over a range of 10μM, 20μM, 30μM, and 40μM.

and allowed to incubate for 48h. After 48h, the cells were examined and the media was removed. 2mL of cold 1X PBS was added to each plate to wash the cells, the PBS was removed, and another 2mL of PBS was added before the cells were scraped from the plates and into a centrifuge tube. Cells were spun at 1,000 rpm for 5 minutes, and then the PBS was removed. 200µL of lysis buffer was added to each tube, and the samples were sonicated, centrifuged for 5 minutes at 1000 rpm, and then stored at -80°C. The recipe for the lysis buffer may be found in Appendix I.

Differential Cell Lysis

In preliminary experiments, rat liver tissue was finely ground, and the cells were lysed using the CelLytic™ NuCLEAR™ extraction kit (Sigma). 500µL of a hypotonic lysis buffer was added to the cell tissue for the differential cell lysis. The cells were allowed to incubate and swell in the lysis buffer on ice for 15 minutes before adding 30µL of 10% IGEPAL CA-630 to the mixture. Cells were vortexed for 10 seconds and then centrifuged at 10,000X for 30 seconds. The supernatant was transferred to a fresh tube and labeled as the cytoplasmic extract. The nuclear pellet was resuspended in 70µL of an extraction buffer mixed for the differential lysis. The mixture was vortexed for 20 minutes and then centrifuged for 5 minutes at 20,000X before being transferred to a clean tube and frozen as the nuclear extract. Recipes for the lysis buffers used may be found in Appendix I.

Western Blotting

The cell lysates were thawed, and the amount of protein contained in them was quantitated using the Bradford Assay.[44] A standard curve was created using samples containing 1mg/mL bovine serum albumin (BSA) over a range of 2µg to 50µg by combining BSA with 900µL of Bradford reagent, and then bringing the total volume to 1mL. The samples were tested by combining 2mL with 98mL of distilled water and 900µL of Bradford reagent. Samples were plotted onto the best fit curve to determine protein concentrations, and the samples were then normalized against the sample containing the lowest concentration. 10µL of each sample was then combined with 10µL of a gel loading buffer containing beta mercaptoethanol, and the samples were boiled for 5 minutes to denature the proteins. Proteins were loaded onto a precast 2-20% polyacrylamide gel (Biorad), and run in a 1X Tris-glycine buffer containing SDS at 75V for three hours. Subsequent experiments were run at 150V for one hour. The gels were then transferred onto nitrocellulose membranes using an electroblotting apparatus for 45 minutes at 150mA. TBST was prepared to have a final concentration of 1X TBS and 0.5% Triton X-100, and used to cover the membranes as they rocked overnight at 4°C.

The solution was then removed from the membranes, and replaced with 15mL of fresh TBST, and hybridized with 0.5µL of a prohibitin specific polyclonal rabbit antibody that was added to it. After 12 hours, the TBST and antibody were removed from the membrane, and replaced with 15 mL of a blocking buffer containing 5µL of a horseradish peroxidase conjugated goat anti-rabbit secondary antibody at room temperature. After one hour, the membranes were then washed for five minutes three times in TBST.

Membranes were blotted to remove excess TBST and placed briefly into a luminol solution that had been diluted 1:40 to prevent over exposure of the film. The solution was again blotted to remove any excess, and the membranes were placed into clear plastic sheet protectors and pressed against a sheet of X-ray film in an autoradiography cassette for varying exposure times. X-ray film was run through a film processor in a dark room, and the resulting developed film was used to analyze the results.

In repeated experiments using the same membranes, a stripping buffer was used. The membranes were incubated in the stripping buffer at 50°C for 30 minutes and occasionally shaken to cover the membranes. The stripping buffer was removed by washing the membranes three times in TBST for 10 minutes, and then the blocking and reprobing steps were repeated so that the blots could be developed again. Recipes for all buffers used may be found in Appendix I.

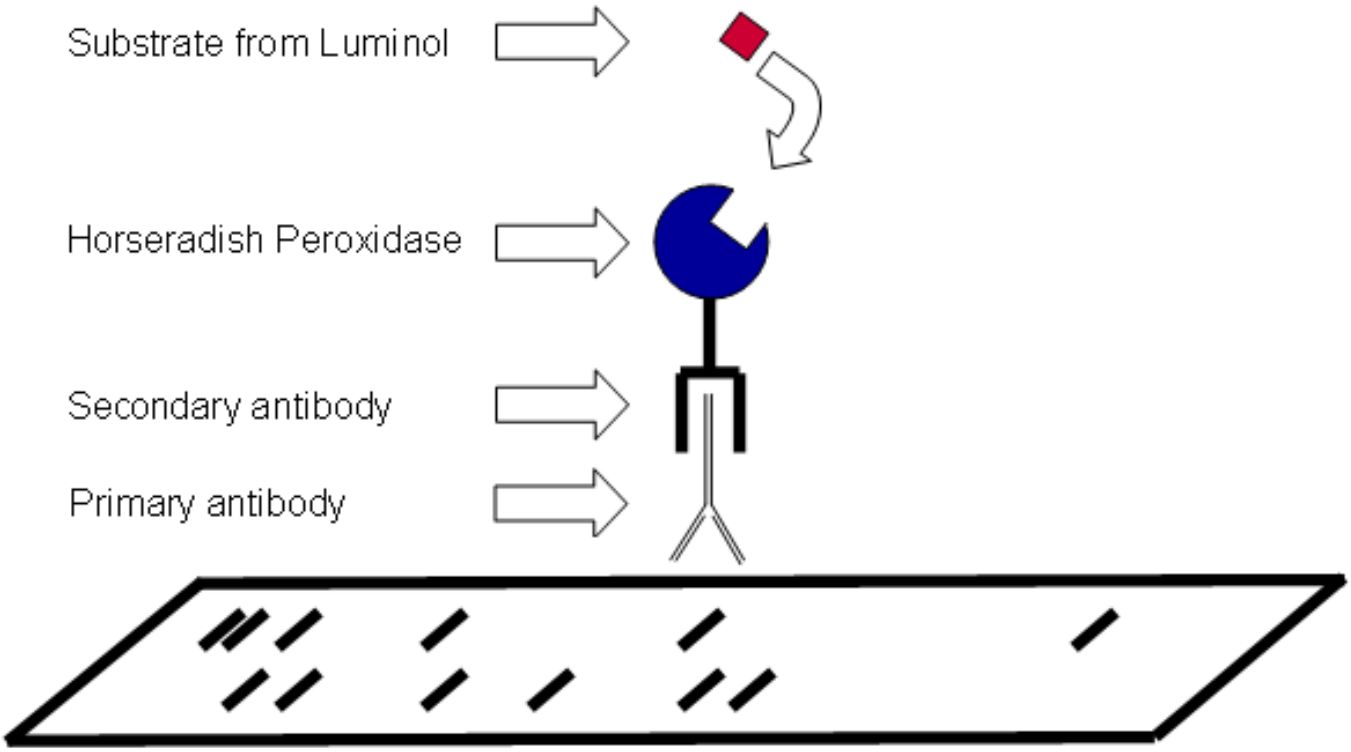


Figure 3: Schematic of Western Blotting procedure. The schematic shows the primary antibody attaching to the membrane, along with a complex of the secondary antibody and covalently attached enzyme leading to the chemiluminescent reaction with the luminol substrate.[45]

Preclearing of Cell Lysates

50µL of a slurry containing Protein A conjugated to sepharose beads was transferred into an eppendorf tube, and 450µL of a cold lysis buffer was added to the beads. The beads were spun at 10,000x for 30 seconds, and the lysis buffer was removed and replaced with 500µL of fresh solution. The beads were again spun, and the buffer was removed and replaced with 50µL of lysis buffer. 500µL of the cell lysate was then added to the washed beads, and this was allowed to incubate for one hour on a rocking platform in an ice bath. The samples were then spun for 10 minutes at 4°C, and the supernatant was transferred to a fresh eppendorf tube. The lysates were then prepared for

immunoprecipitation. The recipe for the cell lysis buffer used may be found in Appendix I.

Immunoprecipitation

Precleared cell lysates were immunoprecipitated using a mouse monoclonal IgG antibody (Abcam) following a procedure provided with the antibody. 10µl of antibody solution (0.10 mg/ml) was added to 200µl of each cell lysate before incubation in ice on a rocking platform for one hour. Following incubation, 100µl of protein A beads were added to each lysate with an additional incubation on ice for one hour. The beads were then precipitated by centrifugation at 10,000g for 15 seconds at 4ºC, and the supernatant was removed using a pipet. The beads were then washed three times using 1ml of lysis buffer, and the final wash solution was removed before the addition of 50ml of 1X SDS gel-loading buffer containing ditheithreitol (DTT). The proteins were denatured by boiling for five minutes, and 30ml of each sample was loaded onto a pre-cast gel (Biorad) for SDS-PAGE separation.

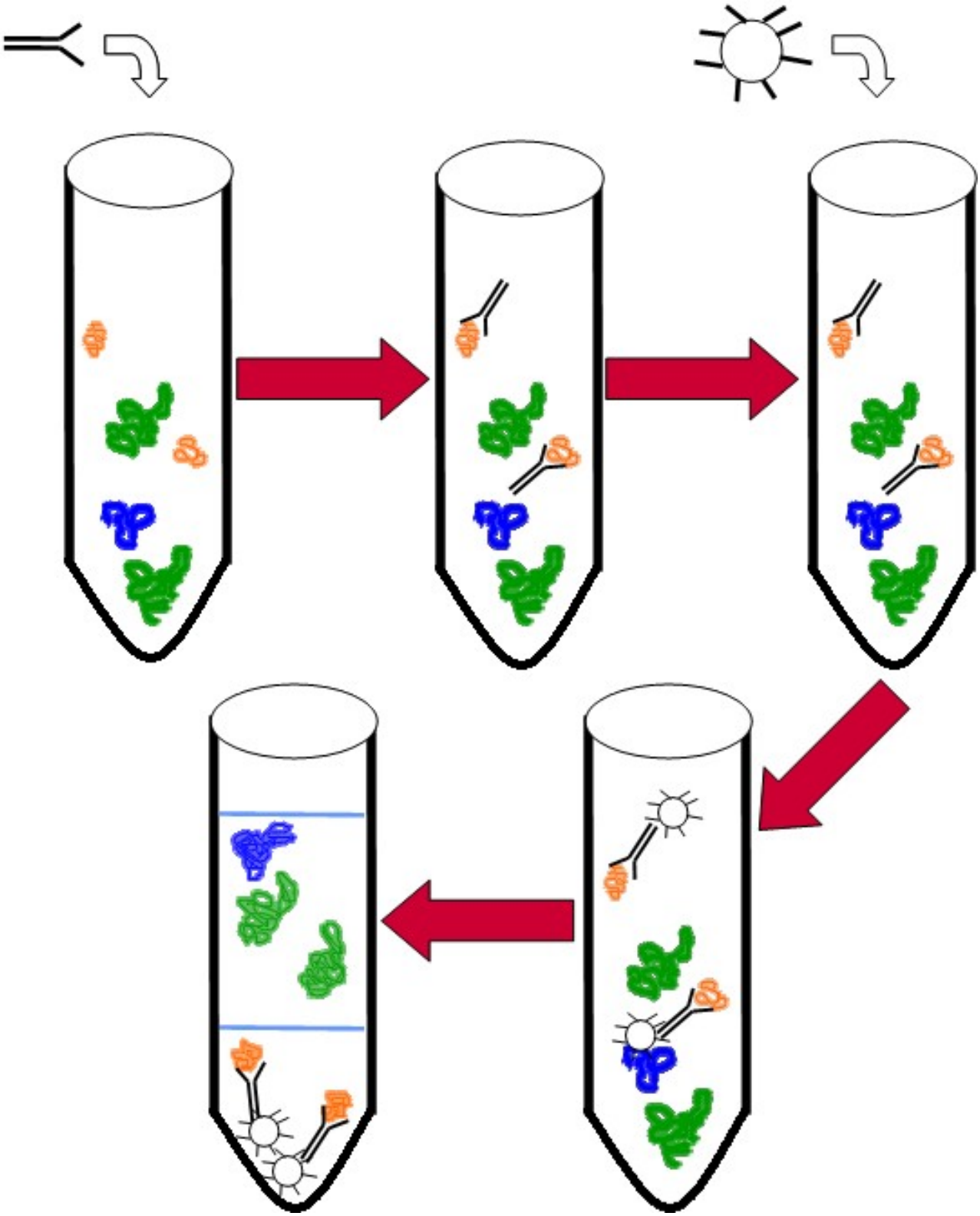


Figure 4: Schematic of Immunoprecipitation Protocol. Initially a sample containing a whole cell lysate is treated by the addition of an antibody designed to specifically target a protein. Next, a bead containing a protein known to bind to the

constant region of IgG antibodies is added to the sample in order to complex with the antibody. Finally, the sample is centrifuged, allowing the heavier complex to precipitate while the aqueous layer is removed. The protein can later be recovered by releasing it from the beads using a combination of heat and dithiothreitol (DTT).

1D SDS-PAGE

The protein extracts along with a standard containing only antibody with protein G beads were separated for 45 minutes at 200V on a 12% pre-cast polyacrylamide gel (Biorad) using a 10% SDS/Tris/Glycine running buffer (Biorad). Proteins were stained for analysis overnight with Coomassie blue stain (Biorad), and the subsequent protein bands were anlalyzed using the Genomic Solutions gel imaging system.

In-Gel Digestion of Proteins

Protein bands of interest were excised from the gel and digested with trypsin (Promega). Bands were finely diced and placed into 1.5 mL eppendorf tubes. 100µl of 25mM ammonium bicarbonate diluted with 50% acetonitrile was used to wash the gel pieces three times, with a 10 minute period of vortexing and spinning at 10,000x between washes. The gel pieces were then lyophilized to dryness. A 10mM solution of dithiothreitol was then prepared in 25mM NH_4HCO_3, and 25µL was added to each sample before being vortexed, spun briefly at 10,000x, and then allowed to incubate at 56°C for one hour. The supernatant was discarded, and 25µL of a 55mM solution of iodoacetamide in 25mM NH_4HCO_3 was prepared and added to the gel pieces. The reaction was allowed to proceed for 45 minutes in the dark. The supernatant was discarded and the gel pieces were washed twice with 100µL of NH_4HCO_3 in 50% acetonitrile. The gel pieces were again lyophilized to dryness.

0.2ng/μL of trypsin was added to the gel pieces, and 25mM NH_4HCO_3 was added to completely cover the samples. The gel pieces were allowed to rehydrate on ice for 10 minutes before being spun and incubated on a heating block at 37°C overnight.

After the reaction was complete, the solution was then transferred to a clean 1.5mL eppendorf tube, and 30μL of a solution made of 50% acetonitrile and 5% formic acid was added to the gel pieces to stop the digestion with trypsin and extract the proteins from the gel. The gel pieces were vortexed for 10-20 minutes, spun at 10,000x, and then sonicated for 5 minutes. The solution was removed and added to the original solution. The extraction procedure was repeated and the solution was again added to the original solution. The protein solution was then lyophilized to 50% of the original volume to remove all acetonitrile prior to purification with a C18 ZipTip.

Purification

Samples were purified through the use of C18 ZipTips. Solutions of 100% acetonitrile were used to hydrate the tip, 70% acetonitrile to equilibrate the tip as well as to eject the sample, and 0.1% trifluoroacetic acid for washing the peptide solution on the tip. The sample was loaded onto the tip by aspirating 10μl of solution onto the tip ten times.

Analysis of Fragments

The tryptic peptides were processed and analyzed using a Bruker MALDI-TOF mass spectrometer which provided a molecular weight map of the peptide fragments.[46,47,48] The peptides were placed into a matrix of α-cyano-4-hydroxycinammic

acid in 50% acetonitrile and 1% trifluoroacetic acid on a 384 well sample plate. The molecular weight of the peptides was obtained and compared against an online database (MASCOT) in order to determine the identity of the parent protein as well as any post-translational modification sites that may have been present in the protein.

Denaturization of Cell Lysates

The proteins from some cell lysates were denatured prior to immunoprecipitation. A solution of 10mM dithiothreitol in 25mM ammonium bicarbonate was made, and 25µL was added to the precleared cell lysates in order to denature the proteins. After a one hour reaction at 56°C, a 110mM solution of iodoacetamide in 25mM ammonium bicarbonate was made, and 50µL was added to the samples. They were allowed to incubate at room temperature for 45 minutes in the dark, in order to covalently bind to the cysteine residues and prevent the protein from refolding, as well as to prevent the dithiothreitol from acting further when the polyclonal antibodies were added to the mixture.

Results

Initially the purpose of performing a 2D gel electrophoresis was to attempt to note any differences between the proteins present in B16 cells that had been treated with retinoic acid as opposed to those that had not. The gels appeared to have a few differences, such as a set of separated spots in the 30 kDa region that appeared to be focused into one much more intense spot in the samples reated with retinoic acid. The

protein spots were excised, and the protein prohibitin was positively identified using the MALDI-TOF mass spectrometer. The gels along with the indicated spot are shown below in figure 5.

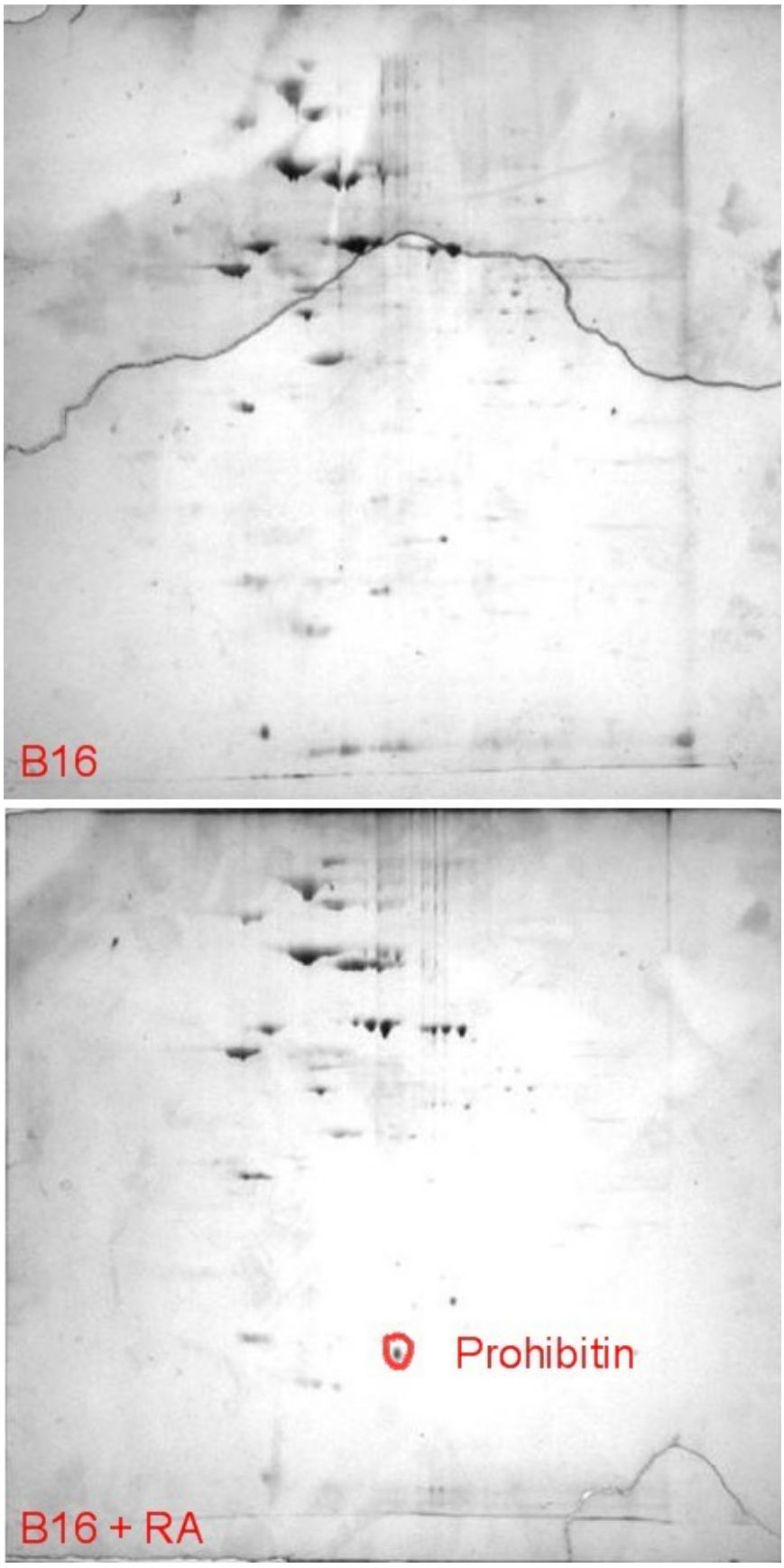

B16
B16 + RA
Prohibitin

Figure 5: 2D SDS-PAGE gel indicating the presence of prohibitin. Focused spot observed in the 30kDa region on the gel containing B16 cells treated with retinoic acid was excised, digested with trypsin, and identified by MALDI-TOF to be prohibitin.

Initially, the goal of this work was to isolate prohibitin from a cell lysate and analyze any differences present in the protein expression profile between treated and untreated B16 mouse melanoma cell lines using mass spectroscopy. As prohibitin is located in all cell types due to its proposed roles, initially rat liver tissue was used due to its availability, and cell lysates were fractionated between a nuclear and cytosolic extracts in order to attempt to obtain a better understanding of where prohibitin may be present in normal rat liver cells. One technique that proves very useful for this is immunoprecipitation, as it allows for a protein to be isolated for analysis in a cell lysate. The samples were run as a 1D SDS-PAGE, and an additional lane containing only the polyclonal antibody was loaded so that the positions of the heavy and light chains on the gel could be more easily identified. After several attempts at immunoprecipitation, it was noted that instead of only visualizing the one or two bands that had been predicted near the 34kDa region of the gel, a large array of proteins were being separated between 17kDa and 250kDa.

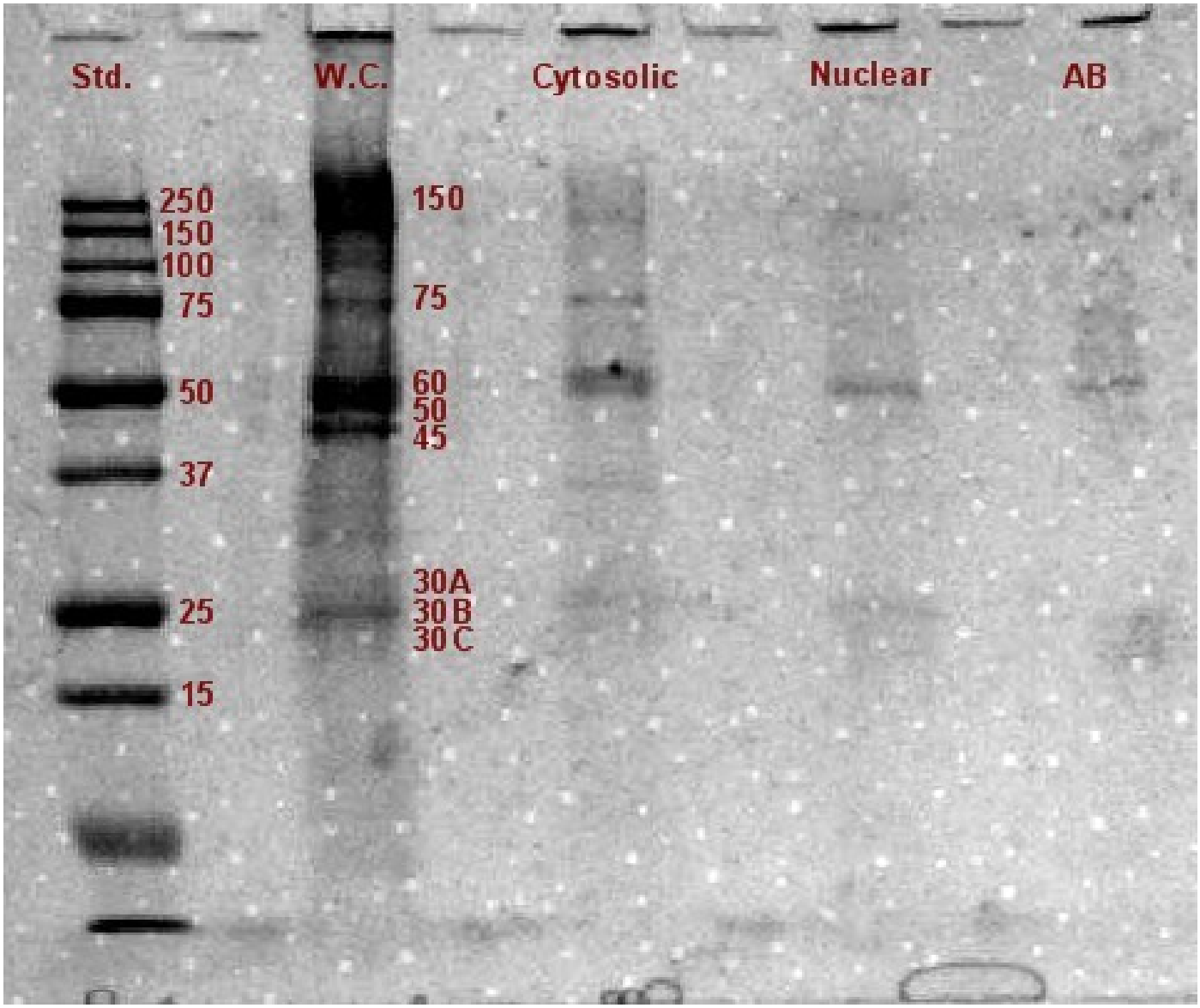
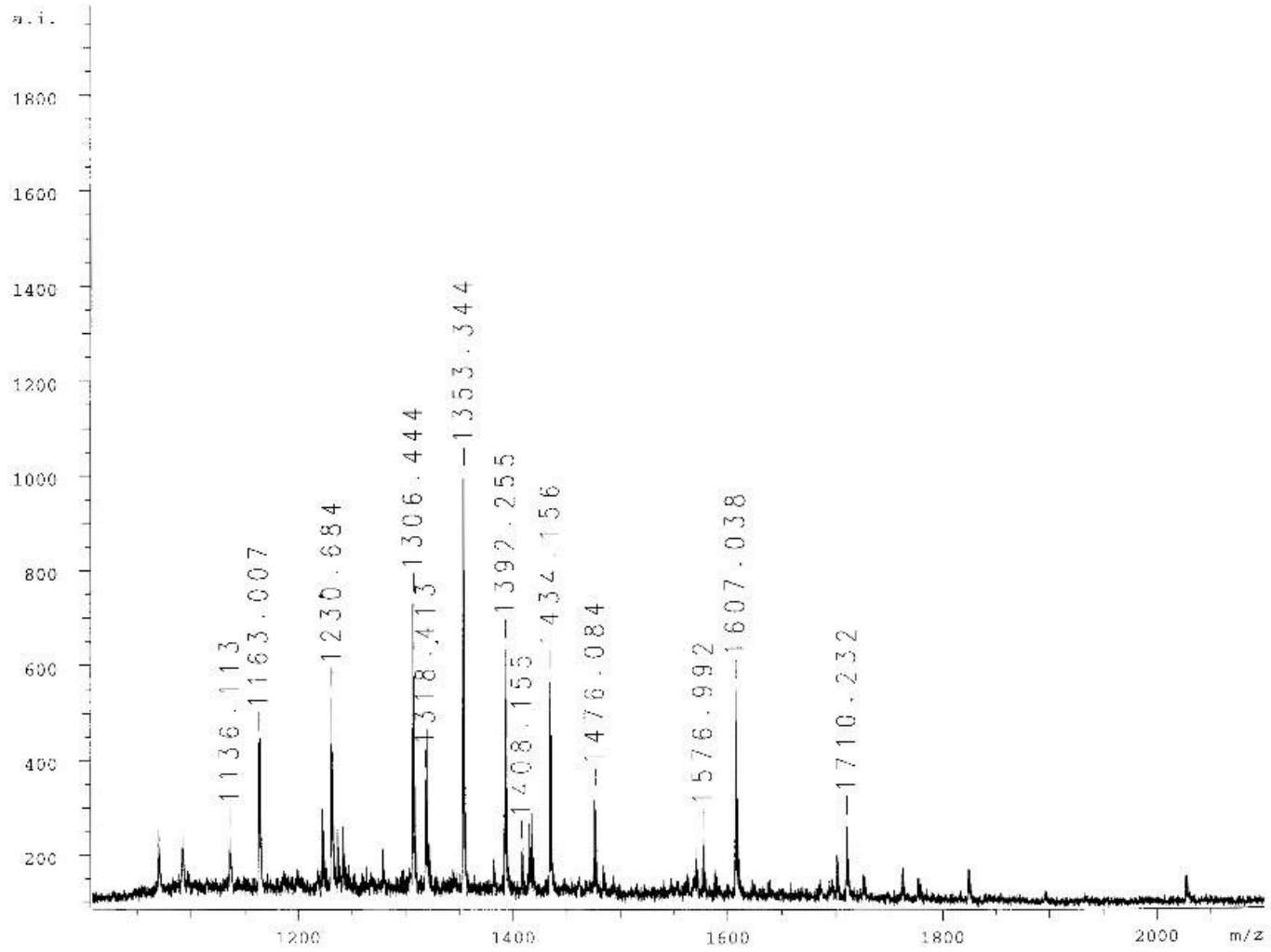


Figure 6: 1D-SDS-PAGE separation of rat liver cells. Labels on whole cell lysate lane indicate bands analyzed by mass spectroscopy. Other lanes present included nuclear and cytosolic fractions of the whole cell lysate, and an antibody control lane to note where the heavy and light chains would migrate during electrophoresis.

There were protein bands located at 150, 75, 60, 50, 45, and several near 30 kDa. These bands were excised and digested with trypsin for mass fingerprinting analysis using a Bruker MALDI-TOF mass spectrometer. The mass spectrum for the tryptic peptides from the protein at 150 kDa is shown in Figure 7. The masses were entered into the online MASCOT peptide search which compares these masses with the masses of tryptic peptides from known proteins in several protein databases. The results for the search are shown beneath Figure 7. The protein present was identified as carbamoyl phosphate synthase I (CPS I).



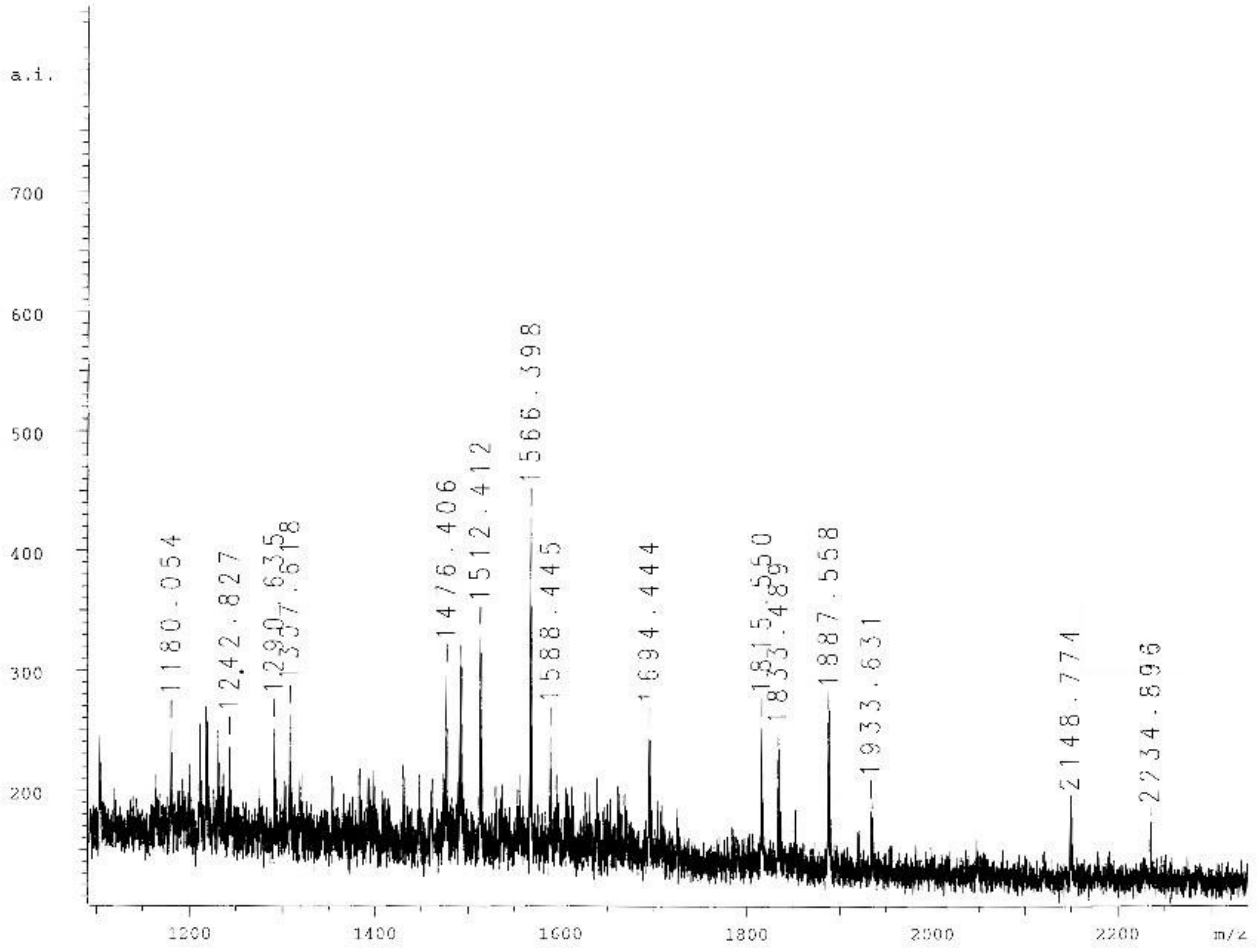
Start - End	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Sequence	
317 - 328	1353.3440	1352.3367	1352.7160	-0.3793	0	R.GQNQPVLNITNR.Q	
317 - 328	1434.1560	1433.1487	1432.6824	0.4664	0	R.GQNQPVLNITNR.Q	Phospho (ST)
403 - 419	1710.2320	1709.2247	1710.0040	-0.7792	0	K.GTTITSVLPKPALVASR.V	
533 - 547	1607.0380	1606.0307	1606.7872	-0.7565	0	K.VLGTSVESIMATEDR.Q	
548 - 560	1576.9920	1575.9847	1576.8096	-0.8249	1	R.QLFSDKLNEINEK.I	
576 - 587	1306.4440	1305.4367	1305.6751	-0.2384	0	K.AADTIGYPVMIR.S	
613 - 624	1392.2550	1391.2477	1391.7231	-0.4754	0	K.AFAMTNQILVER.S	
881 - 889	1136.1130	1135.1057	1134.5525	0.5532	1	K.MRDILNMDK.T	
1075 - 1085	1230.6840	1229.6767	1229.6438	0.0329	0	K.IMGTSPLQIDR.A	
1445 - 1453	1163.0070	1161.9997	1161.5931	0.4066	0	K.FVHDNYVIR.R	

Figure 7: MS analysis of 150kDa band. MASCOT search indicated the protein present to be Carbamoyl Phosphate Synthase I.

Next, the bands at 50 and 30 kDa were respectively identified as the heavy and light chains of the antibody used, by the presence of the bands on the gel in the antibody

lane, and this was next verified through mass fingerprinting data and the mass fingerprint MASCOT search as well. A mass fingerprint analysis of the band at 75 kDa (Figure 8) revealed that this protein was heat shock protein 70 (Hsp 70).

The interesting point about Hsp 70 being identified in the gel is that it primarily exists as a chaperone protein in the mitochondria. At first it was thought that perhaps it had somehow complexed with the prohibitin for the purpose of stability, however in light of the fact that prohibitin is also believed to possibly have a role in the mitochondria as a chaperone when complexed with prohibitone, it is possible that the antibody used instead had only recognized some sort of similar domain or structure within the protein, and it was this reason that caused it to be non-specifically targeted by the immunoprecipitation experiment.



Start - End	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Sequence
20 - 39	2234.8960	2233.8887	2234.0729	-0.1842	1	R.SPAAARHQDGWNGLSHEVFR.F
86 - 99	1476.4060	1475.3987	1475.7256	-0.3269	0	R.TTPSVVAFTPDGER.L
86 - 99	1556.3980	1555.3907	1555.6920	-0.3012	0	R.TTPSVVAFTPDGER.L Phospho (ST)
128 - 138	1337.6180	1336.6107	1336.6147	-0.0039	1	R.YDDPEVQKDTK.N
188 - 202	1694.4440	1693.4367	1693.8424	-0.4057	0	K.NAVITVPAYFNDSQR.Q
207 - 218	1242.8270	1241.8197	1241.6728	0.1469	0	K.DAGQISGLNVLR.V
395 - 405	1290.6350	1289.6277	1289.6728	-0.0451	0	K.VQQTVQDLFGR.A
395 - 409	1833.4890	1832.4817	1832.8223	-0.3406	1	K.VQQTVQDLFGRAPSK.A 2 Phospho (ST)
626 - 634	1180.0540	1179.0467	1179.3846	-0.3379	0	K.DSETGENIR.Q 2 Phospho (ST)
655 - 671	1887.5580	1886.5507	1886.6754	-0.1247	1	K.MASEREGSGSSSTGEQK.E 2 Phospho (ST)

Figure 8: MS analysis of 75kDa band. MASCOT search indicated the protein present to be Hsp 70.

Although prohibitin itself was not initially identified, several interesting things were noted about some of the first experiments performed. First, it was noted that the concentration of protein recovered after fractionation was relatively low, and therefore a

decision was made to focus effort onto the whole cell lysate rather than performing a differential lysis for every sample. Secondly, the extraction of carbamoyl phosphate synthase I in an immunoprecipitation of prohibitin initially introduced the possibility that there could be some interaction between the two proteins. Although CPS I primarily functions in liver tissue in the Urea Cycle, it is incredibly similar to CPS II, a protein involved in the formation of the pyrimidine structures used in DNA synthesis. Finally however, it was noted that prohibitin itself was not directly identified from the immunoprecipitation, whereas many other bands had been present in the gel. For this reason, it was decided to attempt to preclear the gels prior to performing the 1D SDS-PAGE, with the goal of eliminating as much background noise as possible before the immunoprecipitation of the protein.

B16 cells were obtained from stocks harvested by Dr. Richard Niles, and after some preliminary tests that showed the same results as those observed with the rat liver tissue, a protocol for preclearing the protein A beads prior to immunoprecipitation was used. The attempts at preclearing the samples had mixed results. Many of the bands disappeared as had been expected, however all of the bands present in the general location of where prohibitin should have been present vanished. In most trials, the antibody bands were still faintly visible, indicating that the heavy and light chains were again recovered in the experiment, and the series of heavier bands between 250kDa and 100kDa were still present, however the rest of the gel appeared to be clear. Other gels still showed the heavier bands at the same relative intensity as before, however also showed a drastic reduction in the intensity of lower bands, if the bands were visible at all.

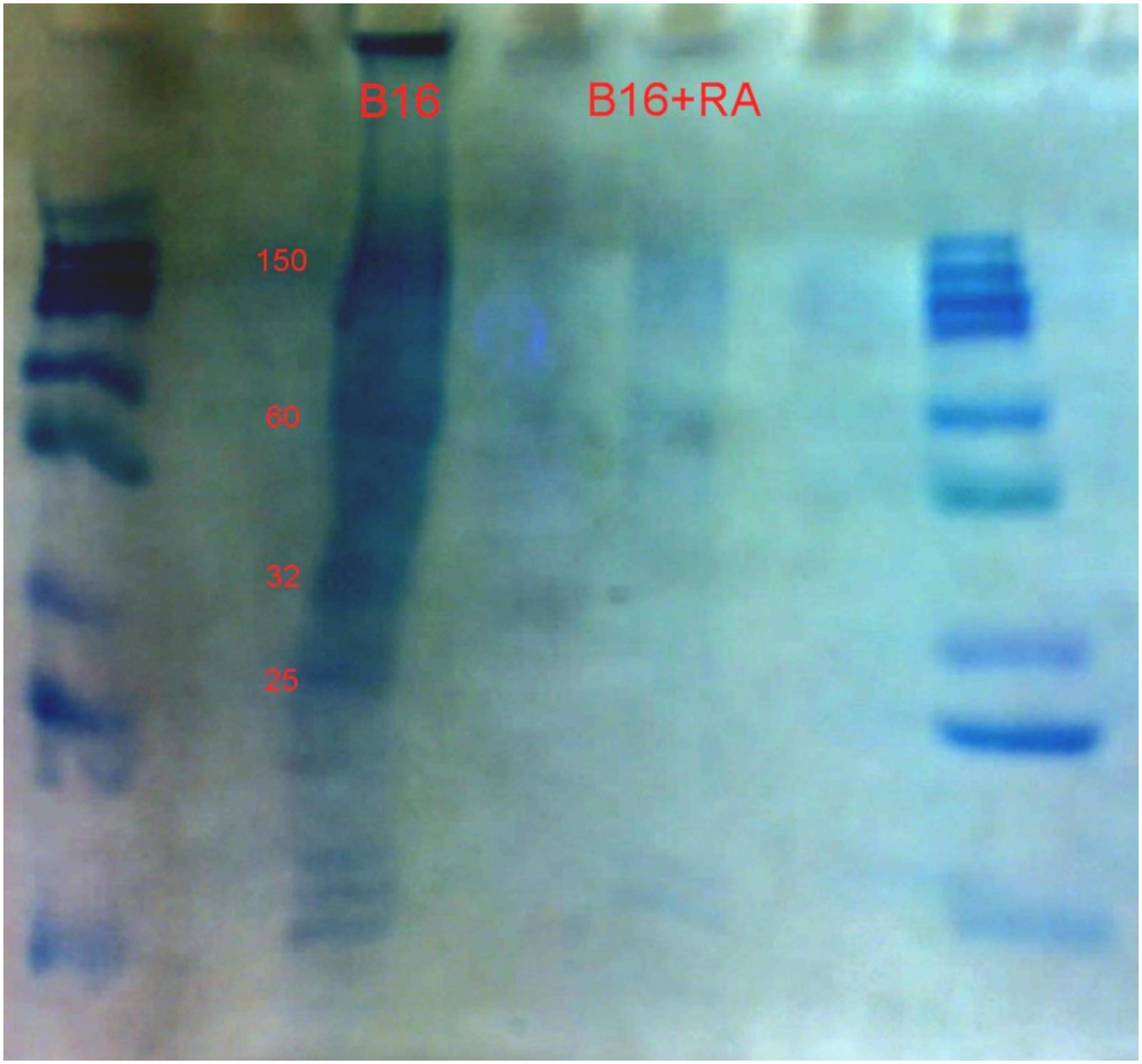


Figure 9: 1D SDS-PAGE of Precleared B16 Whole Cell Lysates. Both the leftmost and rightmost lanes are composed of a standard molecular weight marker, while the two lanes labeled at the top of the figure contain the untreated B16 control cells, and the B16 cells treated with 10µl of retinoic acid for 48h. Labeled bands indicate proteins that were excised for analysis.

After attempts to preclear the samples again failed at recovering prohibitin, we decided to go back to the fundamental basis of the project and prove that not only was

prohibitin present in the samples being tested, but also that it was present in a different concentration between B16 cells that had been treated with retinoic acid versus those that had received no treatment. In order to do this, work on the experiment was taken back to the tissue culture level to ensure that all samples had received the same treatment, were placed in a lysis buffer conductive to recovery of potentially membrane bound proteins, and carried through a western blot to indicate presence of prohibitin, as well as prove that the antibody being used could target the protein.

When the protocol was modified to begin examining the B16 cells, tissue culture work was performed under the guidance of Linda Eastham and Dr. Richard Niles at the Marshall University School of Medicine. The cells were split from flasks that were being maintained within the lab, however before long, a problem arose. B16 cells undergoing retinoic acid treatment typically show drastic morphological changes including increased melanin production as noted by the darker coloration when collected, lower cell density, and a more rounded appearance indicating that the cells are at least to an extent, lifting off of the plate. The initial batches of cells did not show any of these expected characteristics, and after treatment, appeared to have mutated to become resistant to the treatment. Fresh cells were obtained from frozen stocks after passage number 16, and were again plated and the cell culture protocol was repeated. Cells began to exhibit the behavior that was expected of them, and were harvested in four day cycles over the following four weeks to obtain samples for the western blotting and immunoprecipitation procedures.

The samples used in generating a Western blot were quantified using the Bradford assay, then underwent 1D SDS-PAGE.

µl BSA	Absorbance	B16 Samples	Absorbance
2µl	0.220	2µl Control 1	0.812
5µl	0.436	2µl Control 2	0.504
10µl	0.656	2µl RA 1	0.858
20µl	1.300	2µl RA 1	0.276
30µl	1.458		
40µl	1.550		
50µl	1.676		

Table 1: Sample Raw Data from Bradford Assay for Two B16 Samples. Absorbance readings were used to generate a linear plot for determining the amount of µg of protein in each sample.

From the data presented in the table above, a plot was made of the concentrations of bovine serum albumin used against the absorbance recorded at 595nm. Since the standard used contained 1µg of protein per 1µl, it could be assumed that the same would be true of any protein mixture recorded at the same wavelength. It was found that above the 20µl measurement, the linearity of the plot was compromised, however since the B16 samples recorded all contained absorbance readings far below the readings corresponding to the higher BSA concentrations, it was safe to assume that the higher data points could be eliminated in order to gain more precision for the lower concentrations. The corrected graph is shown below.

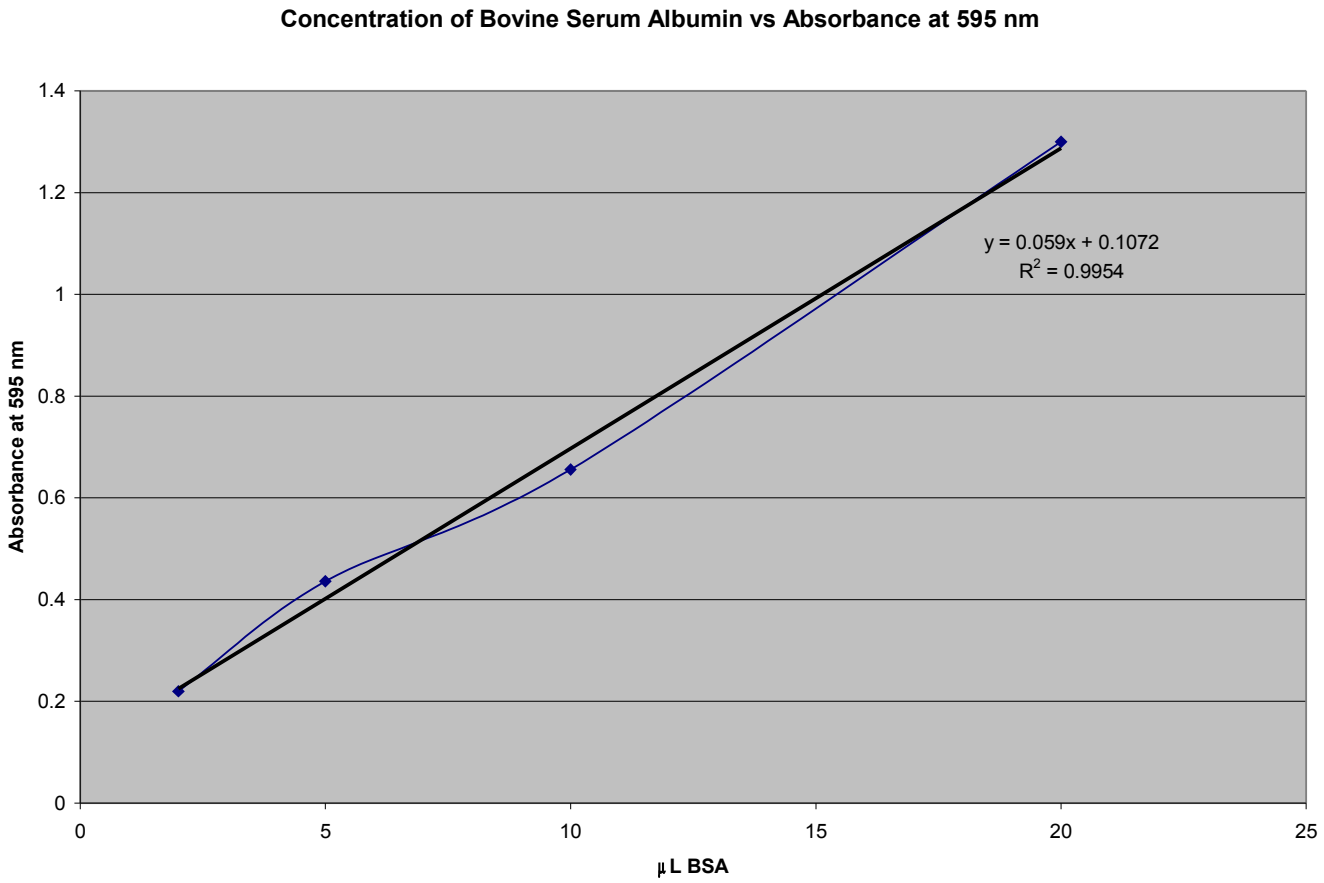


Figure 10: Corrected plot of BSA absorbance readings. Plot was generated over a range of 2-20µg of protein due to loss of linearity in more extreme regions of the graph

As can be seen from the graph above, the best fit curve to the data was highly linear, and allowed for an equation to be generated to approximate the amount of protein contained within the B16 samples. The results of the plot were used in calculating the amount of protein to load onto the gels for the western blot, to ensure that an equal amount of protein was loaded between the samples.

The Western blot followed a fairly typical protocol involving a 24 hour hybridization with the primary anti-prohibitin antibody, followed by a one hour incubation with the secondary antibody. Initial attempts at performing the Western blot

proved unsuccessful, as after developing the x-ray films with a 1:1 mixture of the luminol enhanced chemiluminescence (ECL) solutions, the background noise generated caused the resulting film to be overdeveloped. The membranes were stripped according to the protocol detailed in the materials and methods, washed with TBST, and reprobed for a second attempt at developing the film. It was found that the ECL solution used was heavily concentrated, and needed to be diluted beyond a 1:1 ratio in order for a good signal to be obtained. After several trials, the solutions were diluted by a factor of 40 and the film exposure time was reduced to 15 seconds. Several examples of the resulting images are shown below.

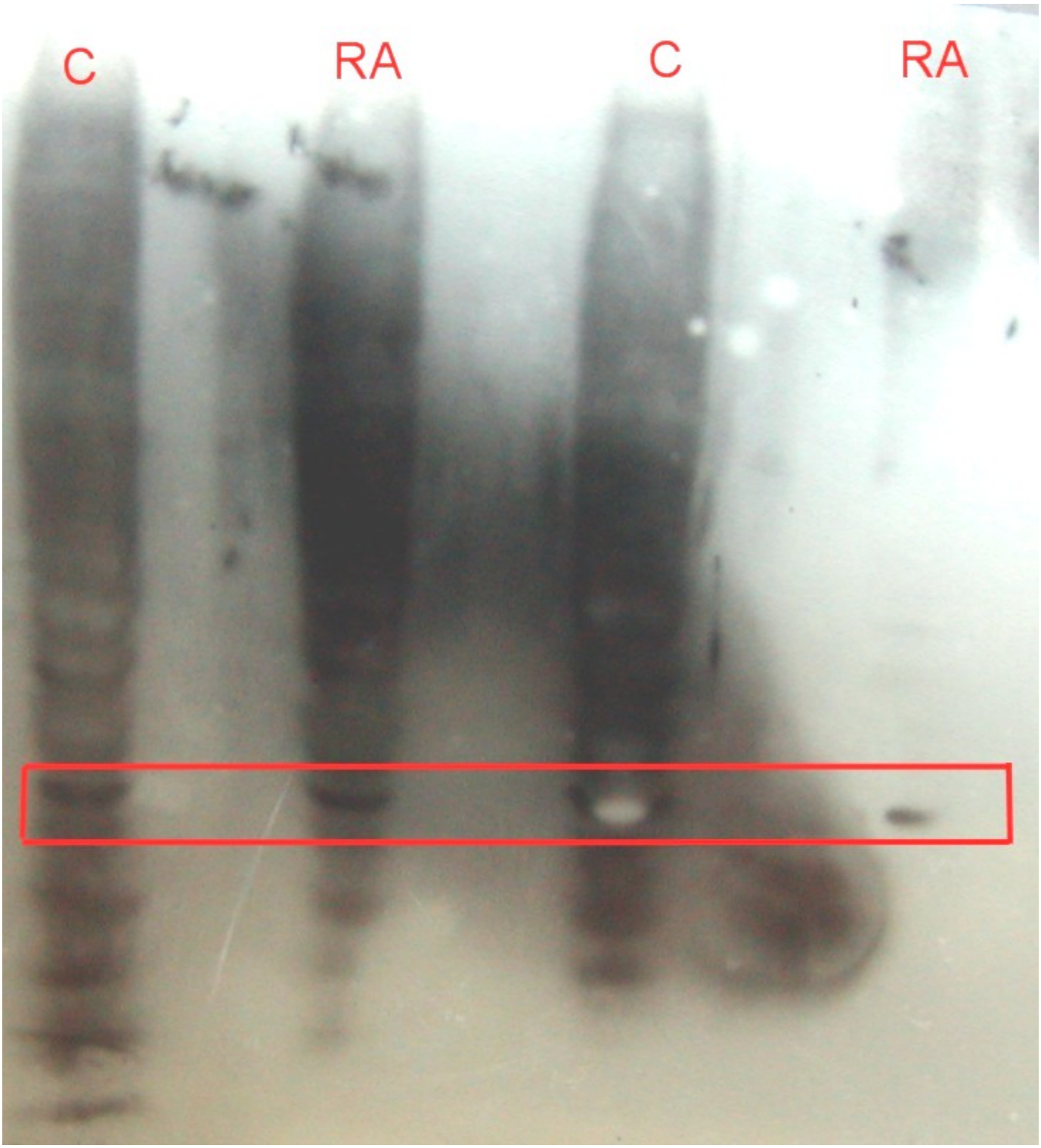


Figure 11: Western Blot of B16 cells using 15s exposure time. Rabbit polyclonal anti-prohibitin antibody used along with a 1:40 dilution of the luminol reagents. The four lanes are composed of control B16 cells as well as B16 cells treated with retinoic acid

across two trials. The bands indicated by the red box contain the protein prohibitin in all four samples.

The above film was developed at a longer exposure time than was needed, as the development time was still being tested. The strong bands present below the 32kDa weight marker indicated that prohibitin had been successfully tagged, however a reduced exposure was needed in order to attempt to see any real differences between the bands. A membrane from a previous trial was used, only with a 10 second exposure to the luminol reagent instead of the 15 second exposure used in the previous trial. The lanes on the right half of the membrane represent the B16 samples in which the mutation occurred causing the cells to become resistant to retinoic acid. The lanes on the left represent samples in which the B16 cells responded positively to the treatment with retinoic acid as indicated by the presence of increased melanin, inhibition of cell growth, and appearance of cells on the culture dishes. The resulting film from this trial is shown below.

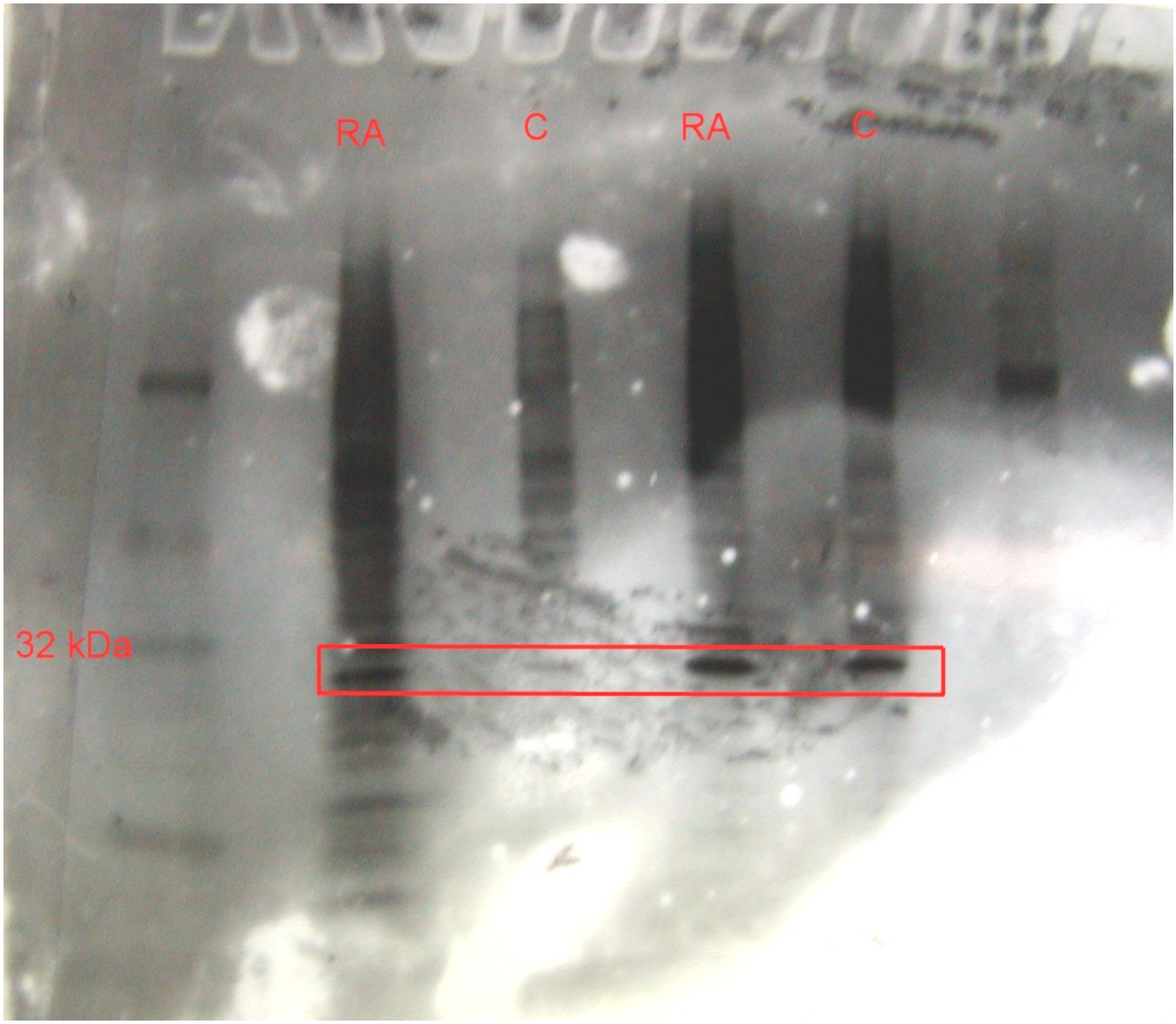


Figure 12: Western Blot of B16 cells using 10s exposure time. Image of second blot performed under a 1:40 dilution of the luminal reagents at a 10 second exposure time to account for amount of signal generated. The four lanes are composed of control B16 cells as well as B16 cells treated with retinoic acid across two trials. The bands indicated by the red box contain the protein prohibitin in all four samples.

The results of the second western blot indicated several things. Primarily, the antibody obtained from Abcam was able to bind to and label prohibitin, and secondly that the samples used contained enough prohibitin to be visualized on a gel. In addition to

this however, the western blot also showed the array of bands present in the background as seen in the initial experiments performing the immunoprecipitation. This seemed to indicate that the epitope for the antibody being used was perhaps not as specific as one would hope for purifying the protein. Further reading into the antibody indicated that it is capable of identifying the protein in both its native and denatured states, implying that the epitope could in fact be more generalized than one would hope. Since the antibody had shown a good affinity for the protein in a denatured gel however, the decision to stop running the native 1D SDS-PAGE, and the protocol was modified to include an optional treatment with dithiothreitol followed by iodoacetamide to denature the proteins and keep them in that state until the immunoprecipitation was performed.

The final, and perhaps most important result of the western blot was that prohibitin did in fact show an increased concentration in the lane treated with retinoic acid than the lane the represented the control sample. This confirmed the results that had been seen in previous trials performed at Marshall University.

Following the western blot, several more immunoprecipitation trials were set up using the B16 samples that had been treated with retinoic acid and harvested. As the antibody used was listed by Abcam as being able to recognize prohibitin in either its native or denatured conformation, the concept of the western blot was followed and the proteins were all denatured by treatment with ditheithreitol and iodoacetamide prior to being run through 1D SDS-PAGE. The resulting gels again showed the higher molecular weight band present in the gels, but little to no detectable bands present in the lower weight regions. One possibility that could explain this is that the antibody used may have a similar affinity for the higher weight proteins, and therefore, if they are present in a

larger amount, may be binding to more of those proteins than the prohibitin that it is designed to target. The amount of antibody used had been increased several times, however perhaps this was still not enough to prevent more of the antibody from binding to proteins other than prohibitin. Although there appeared to be some shadowing indicating possible bands in the lower regions of the gel, it was uncertain if there were indeed spots present. Regardless, the suspected spots were excised, digested, and prepared for analysis using MALDI-TOF mass spectrometry.

The analysis of the digests by the MALDI-TOF was inconclusive, as either no solid matches were found when comparing the peaks observed with the MASCOT database, or as was the case many times, no peaks were observed at all in the samples.

After several more trials of the immunoprecipitation procedure in which no conclusive results were found, another set of B16 cells were subjected to treatment using varying concentrations of camptothecin. A preliminary experiment was performed in which one plate each of 5μM, 10 μM, 20 μM, 30 μM, and 40μM concentrations were used over a period of 48 hours to observe the level of effect on the cells. Although the data for this experiment is not shown, it was learned that the chemical induced such a large degree of DNA damage that the cell numbers were drastically reduced. Because of this, a second experiment was set up in which 5 control dishes, 5 5μM dishes, 10 10μM dishes, and 10 30μM dishes were plated with 250,000 cells and allowed to grow for 24 hours before being subjected to a 48 hour exposure to camptothecin. Below is an image taken of the control cells, so that the morphological changes between the treated and untreated samples may be observed.

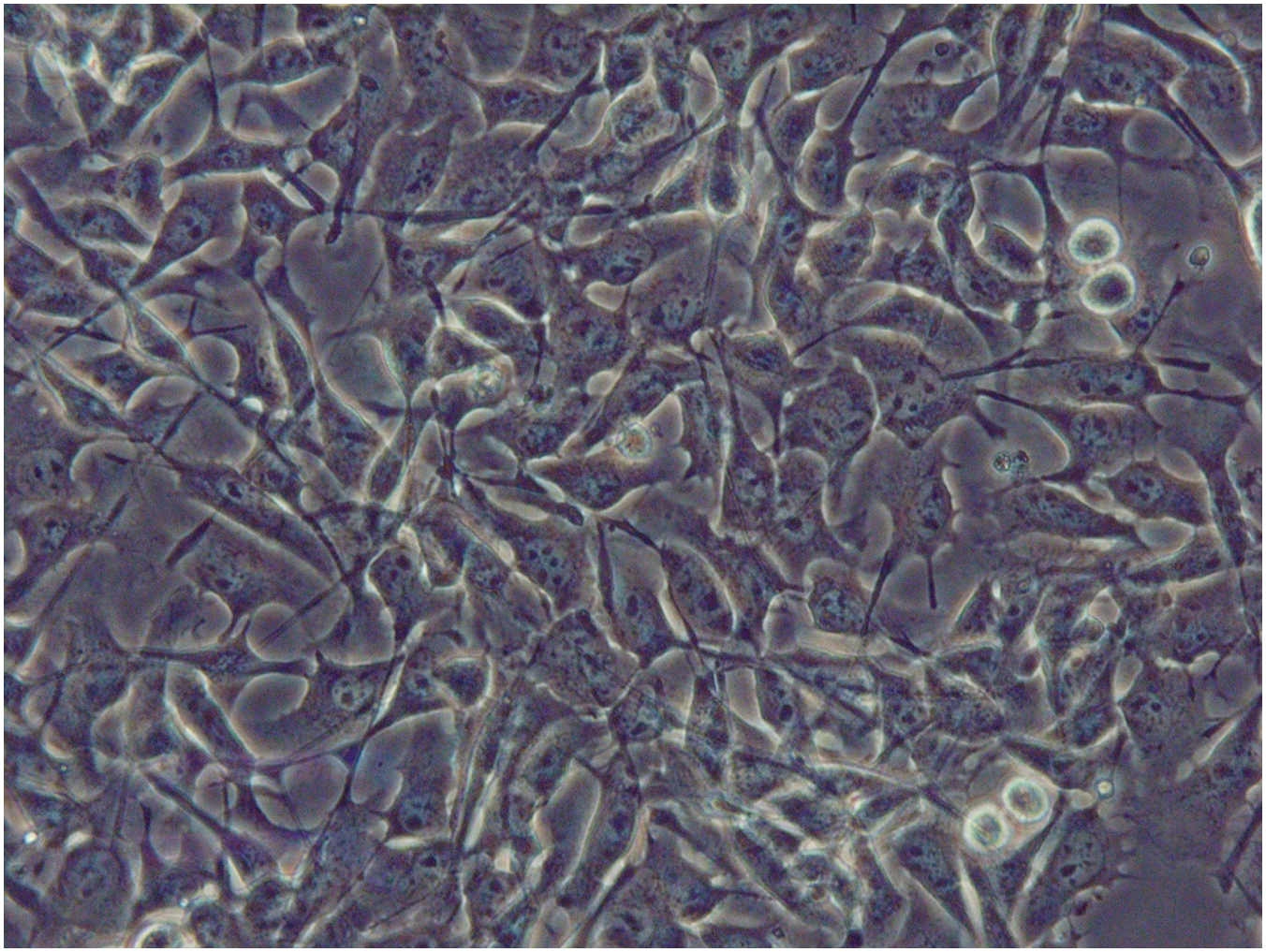


Figure 13: Representative image of untreated B16 cells. Image of cells magnified to 400x after being allowed 72 hours of uncontrolled growth.

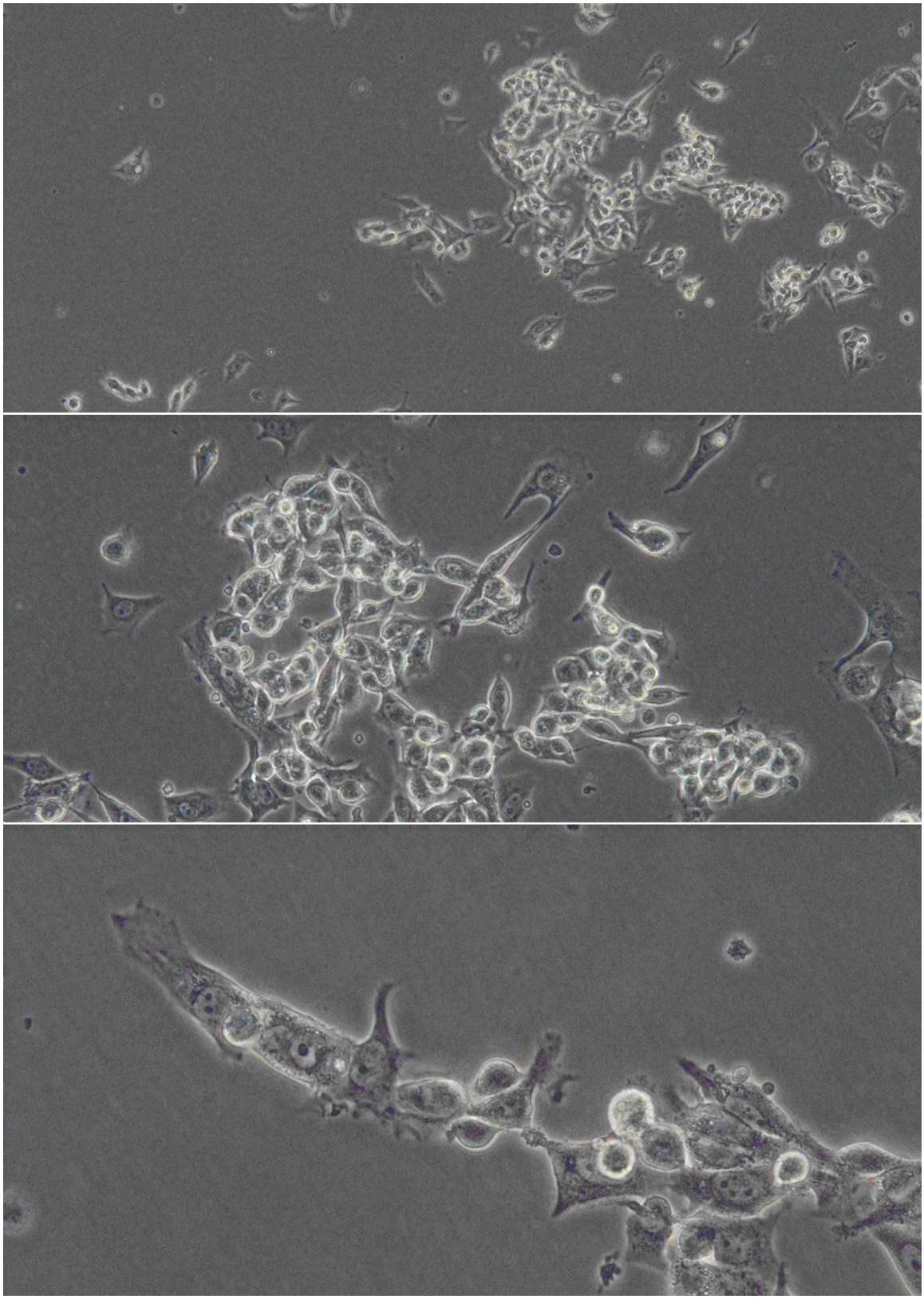


Figure 14: Representative images of 5μM camptothecin treatment. Cells were exposed to 5μM of camptothecin for a period of 48 hours. The topmost section of the figure was taken at 100x magnification, while the middle and lower sections were at 200x and 400x respectively.

The topmost portion of Figure 14 shows the B16 cells under 100x magnification. The second portion shows the cells under 200x, and nicely shows that even with a 5μM treatment, the cells still show a decent amount of growth, and tend to cluster together, indicating that although growth has obviously been inhibited from the control samples, the cells are still dividing. In the final section of the image, the B16 cells are shown at a 400x magnification. The bottom image best shows the morphological changes that the cells are undergoing. In the control sample, the cells were growing to the extent that as they spread out on the dish, they were overlapping, and appeared to be branching out in several directions. In this treated sample, it can be seen that the cells are not sufficiently packed onto the plate for the clusters to be truly dense, and that although the cells are spread out on the plate, they do not show the branching pattern seen in the control dishes. It should also be noted that even at the lowest concentration of camptothecin tested, significant amounts of dead cells were still present, indicated by the circular objects floating off of the plate, and other cellular debris present in the samples.

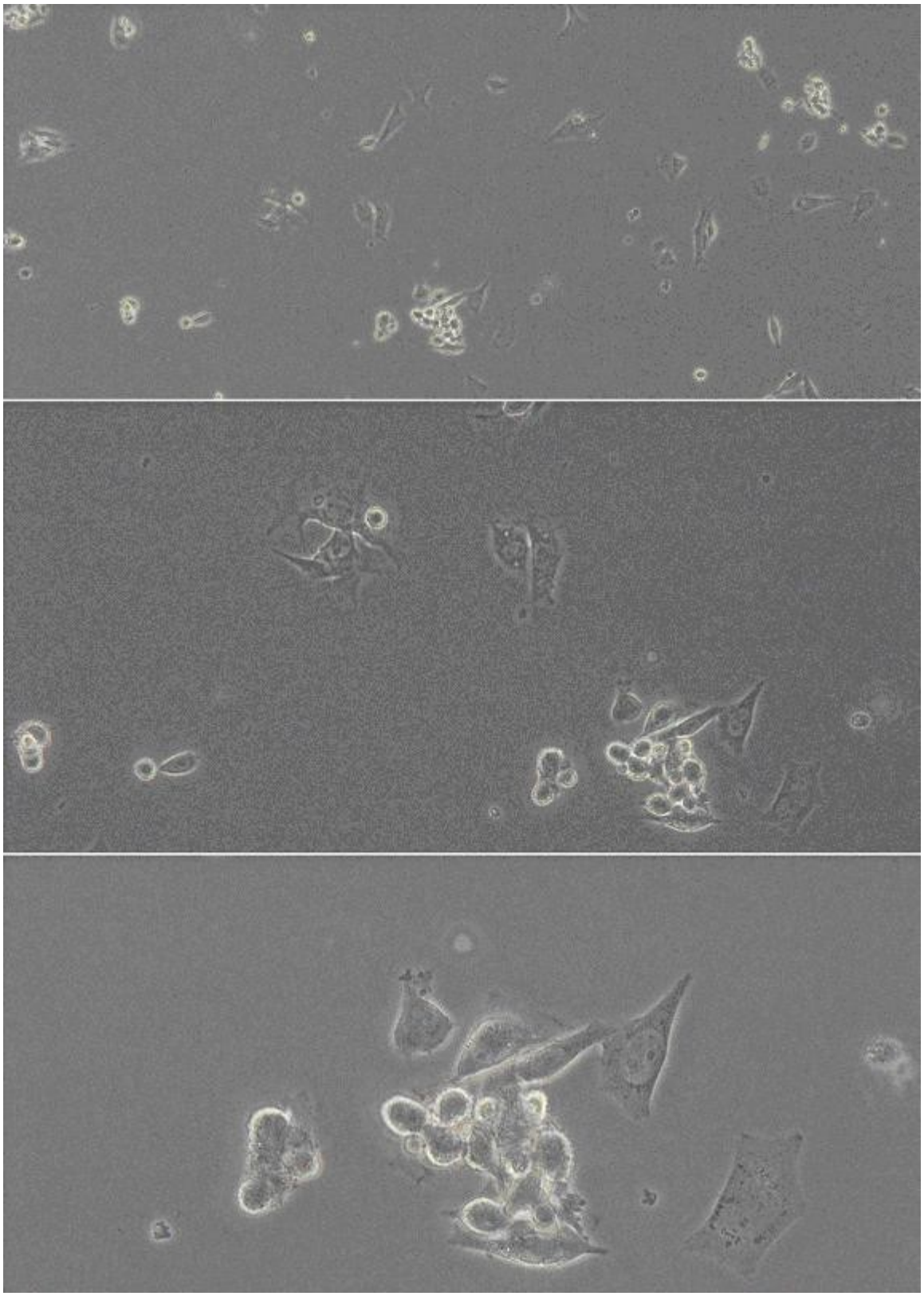


Figure 15: Representative images of 10μM camptothecin treatment. Cells were exposed to 10μM of camptothecin for a period of 48 hours. The topmost section of the figure was taken at 100x magnification, while the middle and lower sections were at 200x and 400x respectively.

The topmost portion of Figure 15 shows the B16 cells under 100x magnification, and indicates that although some cells still appear to be viable, their count has been drastically reduced, as cell clusters are very sparse. The second portion shows the cells under 200x, and is zoomed in on a portion showing a cluster of some inhibited cells, as well as some cells that have already died and lifted from the plate. In the final section of the image, the B16 cells are shown at a 400x magnification. As was the case with the 5μM samples, the bottom image best shows the morphological changes that the cells are undergoing. In the 30μM sample, it can be seen that the cells no longer show any resemblance to the branching pattern observed in the control cells, and although they may be viable, the extent of just how fragile they are can be seen in the fact that the cells no longer form large clusters that spread out over the place. Cell density was greatly reduced, and a large number of cells again were taking a more rounded morphological appearance, before lifting from the plate and floating as cellular debris. The results were drastic, even for being the highest concentration of camptothecin tested, as these images were taken only 48 hours after treatment.

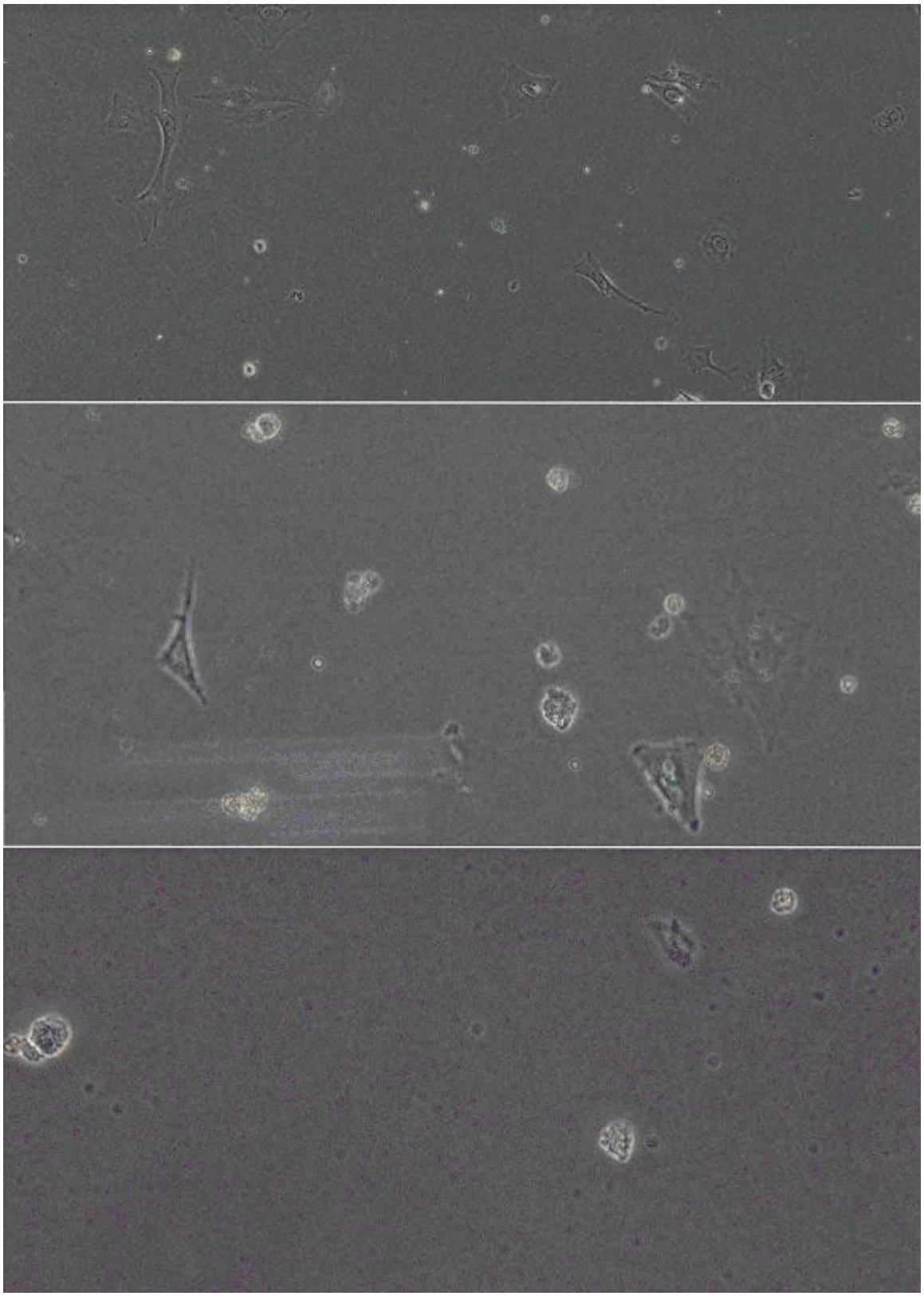


Figure 16: Representative images of 30μM camptothecin treatment. Cells were exposed to 30μM of camptothecin for a period longer than 96 hours. The topmost section of the figure was taken at 100x magnification, while the middle and lower sections were at 200x and 400x respectively.

The most extreme examples of what treatment with camptothecin caused with the B16 cells can be seen in the figure above, where the cells were exposed to camptothecin for longer than 96 hours. By the 400x magnification, the only thing that can be observed was that the cells appear to only exist as debris floating in the sample. The 100x and 200x magnifications in this case give a much better picture of the extent of the damage present. In these images, you can see that what cells are present are either severely weakened if not non-viable, and the amount of cellular debris floating in the dish is extreme. As camptothecin acts by inducing DNA damage in rapidly dividing cells, this result is not all that surprising, however the level of the damage is beyond what was initially expected.

The cell lysates were taken through the procedure for 2D SDS-PAGE detailed above, and then run on precast 4-16% polyacrylamide gels**.** The resulting gels were stained overnight in Coomassie Blue, and then imaged using a Genomic Solutions gel imaging system. The images taken of each concentration are shown below, and have been recolored to make the spots more visible.

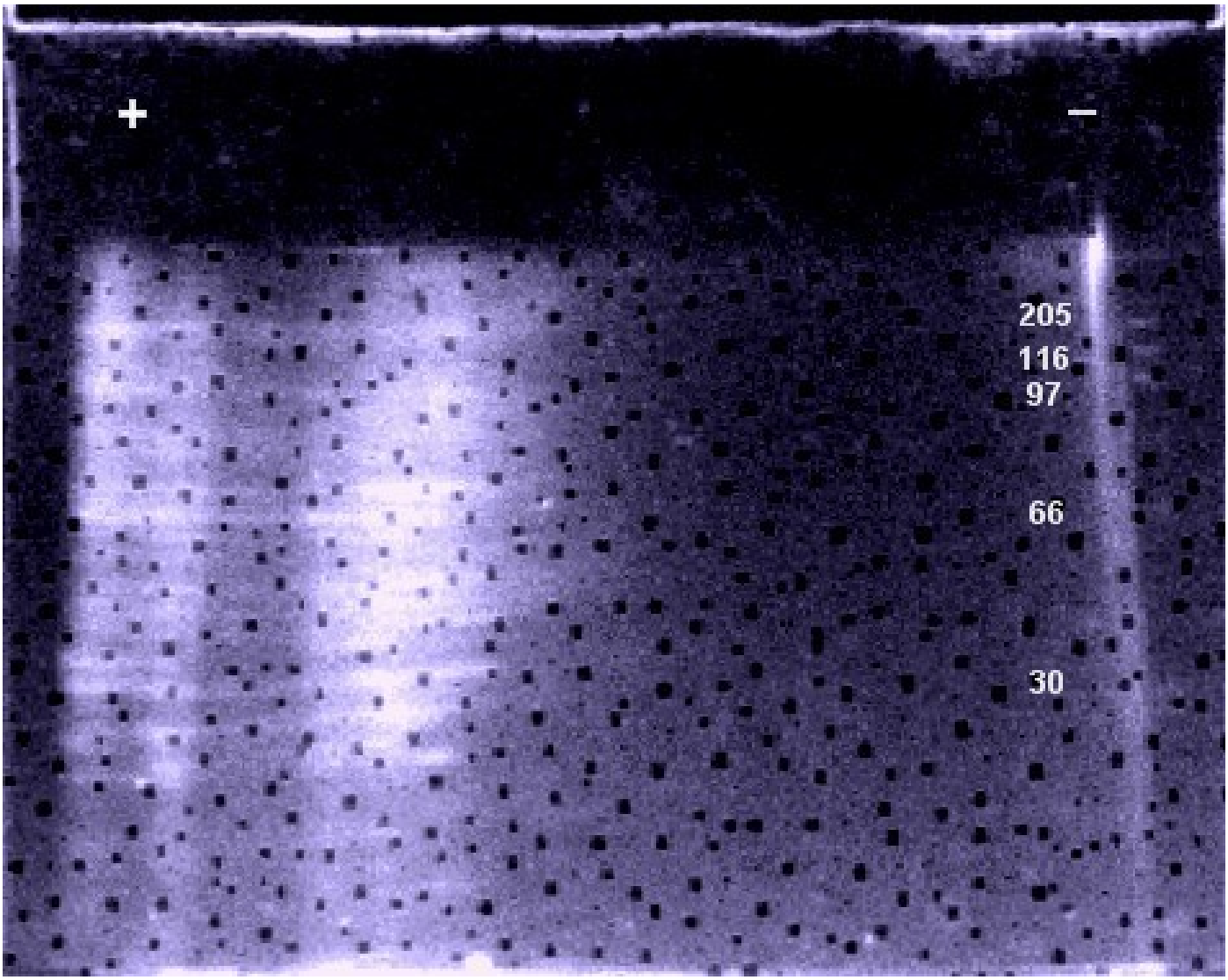


Figure 17: 2D SDS-PAGE Results of Control B16 Cells. Significant streaking prevented extraction of protein spots for analysis, however weight markers were still visible as indicated on the right of the figure. Charges are indicated at the top of the image, showing the direction of separation in the first dimension.

The control gel appeared to have some streaking present during the separation of the first dimension, resulting in poorly resolved protein spots. Bands were still able to be observed for comparison with the other samples, however the streaking present introduced the possibility that multiple proteins could be contained within a single spot, and prevented any good samples from being extracted.

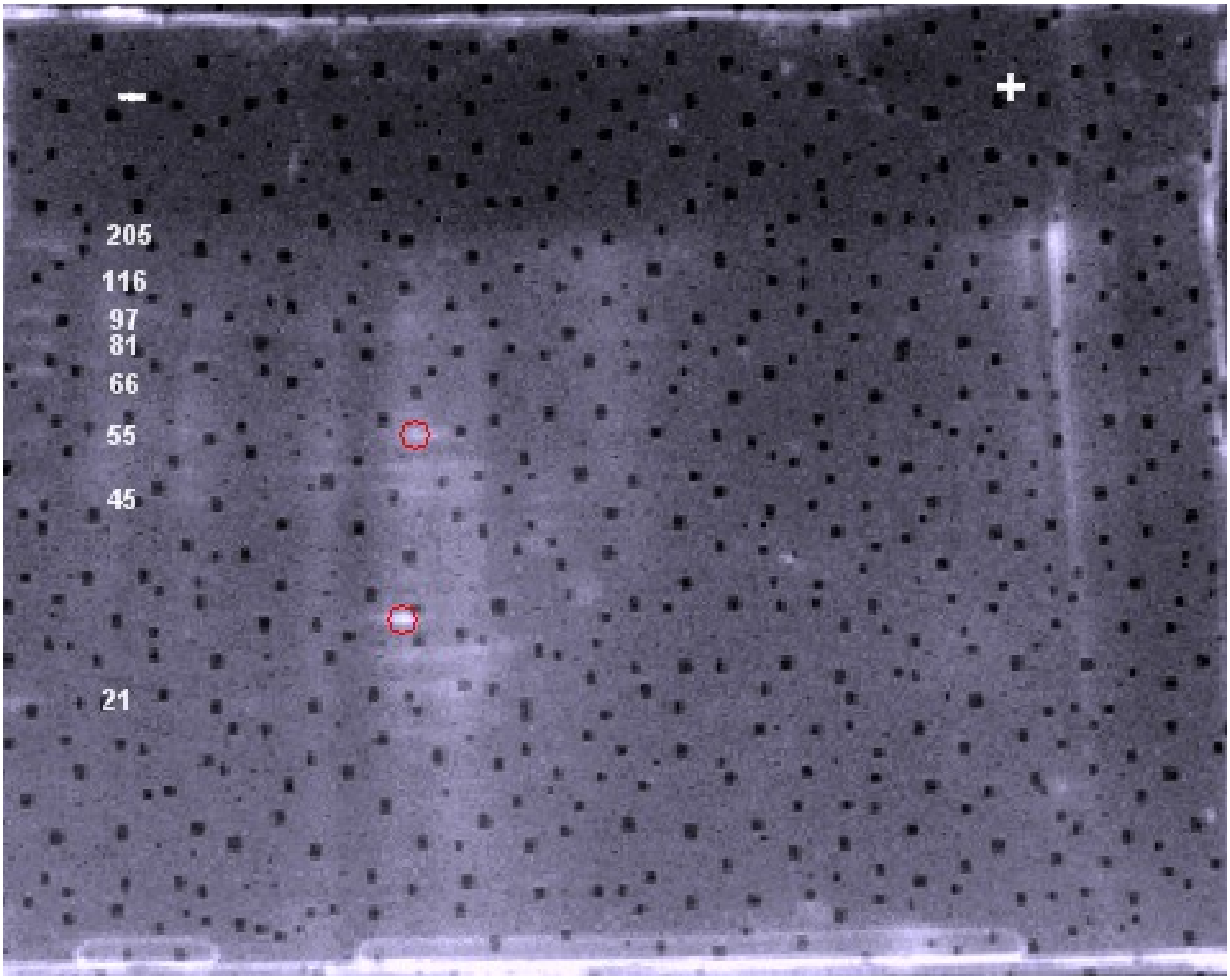


Figure 18: 2D SDS-PAGE Results of B16 Cells Treated With 5µM of Camptothecin. Circled spots indicate proteins excised for digestion with trypsin. Charges are indicated at the top of the image, showing the direction of separation in the first dimension.

The 5µM sample turned out much better, as there was minimal streaking, and there were a few proteins present in a higher concentration than the others, as indicated by the spots. As seen in the other gels, the molecular ladder did not stain as well as was hoped, however it was possible to make estimations based on the markers that were

present, and use them as a guideline for comparing and excising protein spots from the gels.

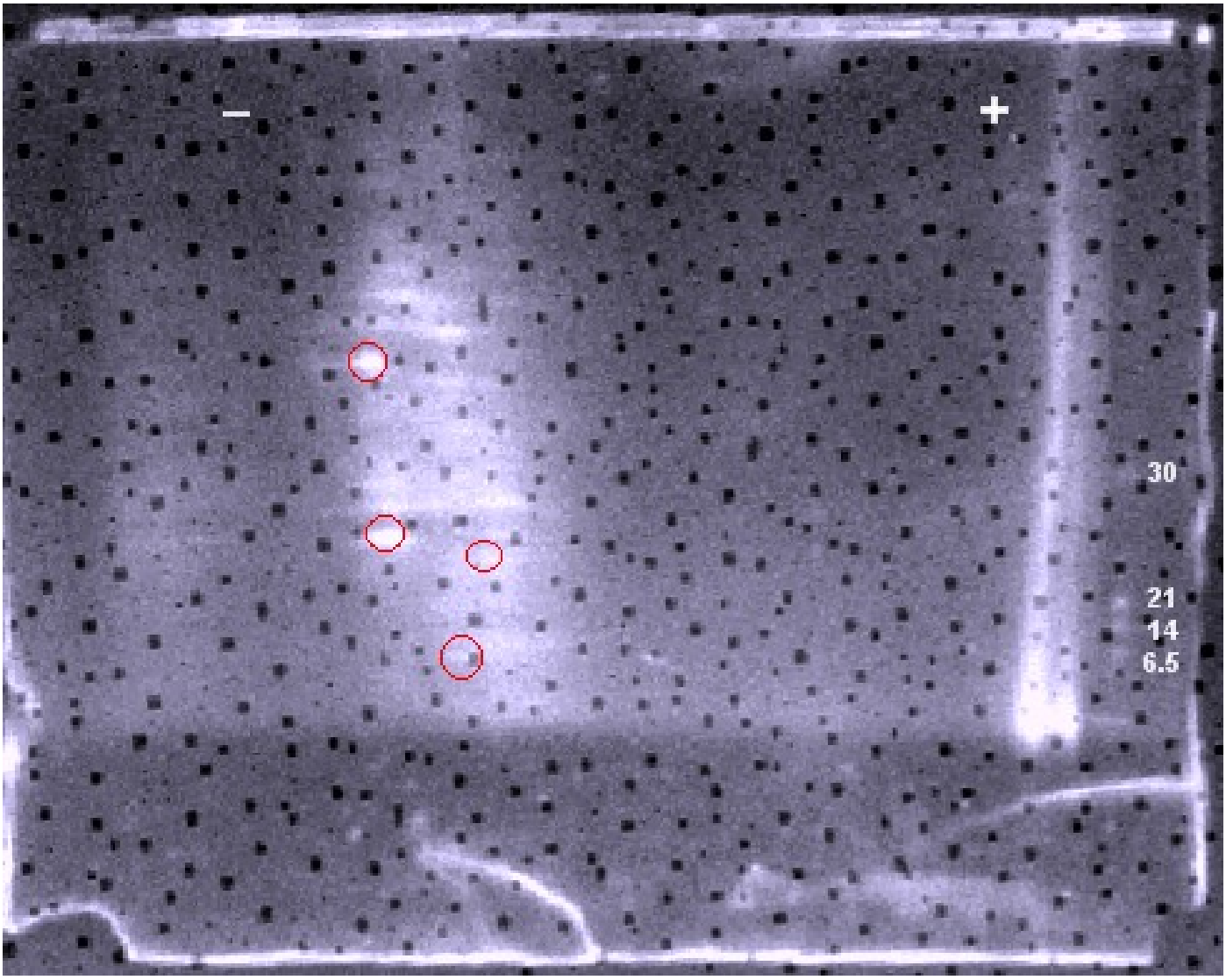


Figure 19: 2D SDS-PAGE Results of B16 Cells Treated With 10µM of Camptothecin. Circled spots indicate proteins excised for digestion with trypsin. Charges are indicated at the top of the image, showing the direction of separation in the first dimension.

The 10µM gel showed perhaps the best results of all the samples processed, as it definitely indicated an up regulation of some of the proteins present, and the spots were easily resolved, and therefore easily excised as well. The 10µM was used as a basis for

comparison of the other gels, and had the most protein removed as indicated in the figure above.

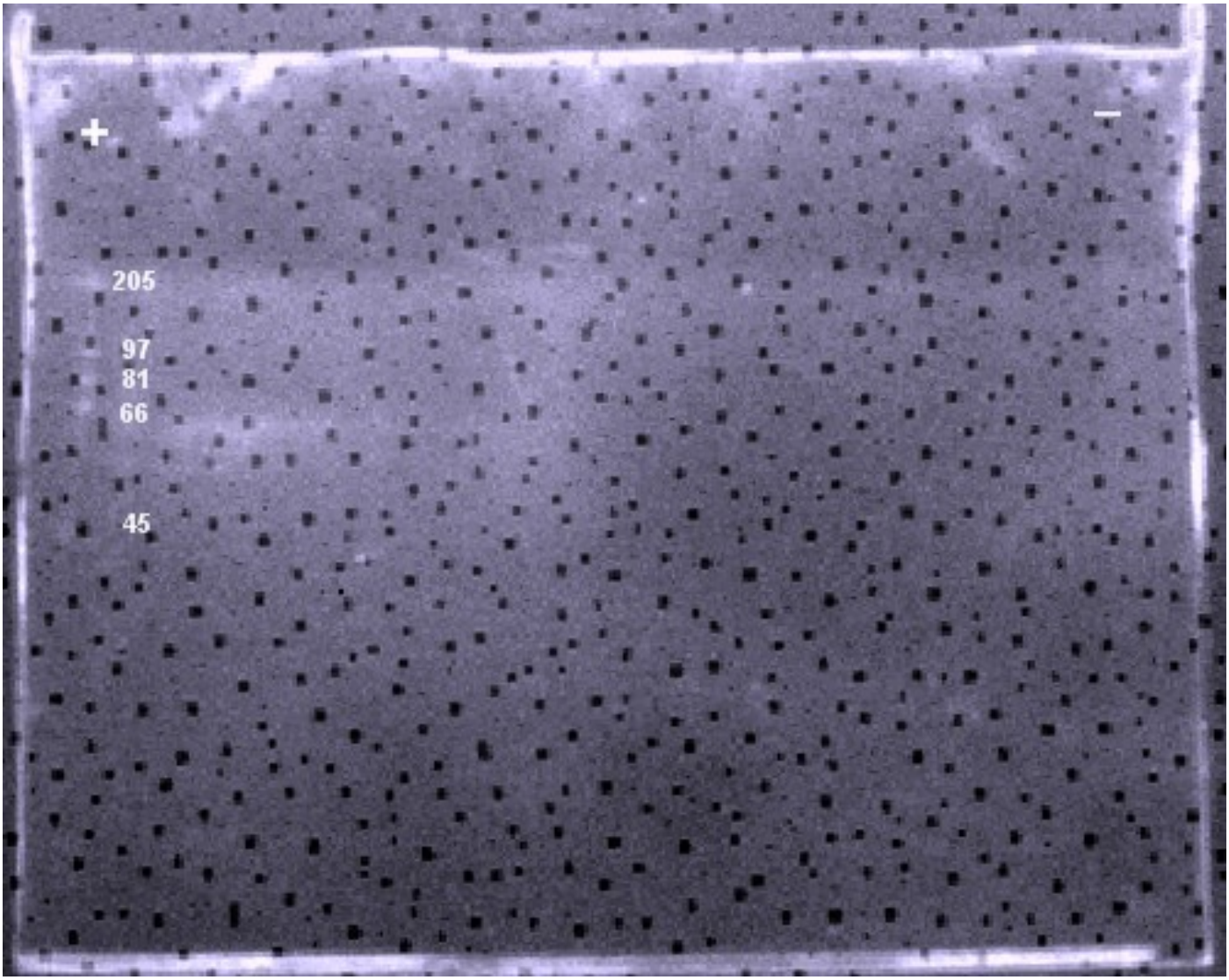


Figure 20: 2D SDS-PAGE Results of B16 Cells Treated With 30μM of Camptothecin. Low amount of protein loaded onto the gel prevented distinct spots from appearing, so no protein could be excised and digested for analysis. Charges are indicated at the top of the image, showing the direction of separation in the first dimension.

No protein was able to be recovered from the cells treated with 30μM of camptothecin. This result however, was not a surprise based upon the amount of cellular damage that had been observed following the treatment. From the image it can be seen

that there was some protein present, however it was so low in concentration that it would have been fruitless to attempt to remove and digest it with any certainty.

The excised spots were digested with trypsin as outlined in above, and were again processed for analysis using MALDI-TOF mass spectrometry. Although many of the spots yielded peaks, only one search yielded results that matched when performing a MASCOT search. The protein excised near 30kDa on the 5µM sample was reported to be an excellent match for Annexin A5. Below is the resulting match obtained from the MASCOT search engine, showing by just how little the observed peaks differed from those expected to be seen. For the search, the results were restricted to proteins found in *mus musculus*, with possible carbamidomethyl and phosphorylation modifications present.

Start - End	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Sequence
5 - 16	1268.3000	1267.2927	1267.5834	-0.2906	0	R.GTVTDFPGFDGR.A
28 - 43	1703.6000	1702.5927	1702.8737	-0.2810	0	K.GLGTDEDSILNLLTSR.S
125 - 149	2915.0000	2913.9927	2914.2417	-0.2490	0	K.QVYEEEYGSNLEDDVVGDTSGYYQR.M
192 - 199	954.3000	953.2927	953.5335	-0.2407	0	K.FITIFGTR.S
211 - 225	1802.5000	1801.4927	1801.8556	-0.3629	0	K.YMTISGFQIEETIDR.E
275 - 283	1106.4000	1105.3927	1105.5768	-0.1840	0	R.SEIDLFNIR.K

Figure 21: MASCOT Result of 5µM 30 kDa Protein Spot. Low delta values along with zero missed cleavages indicated a very good match for Annexin A5.

As can be seen by the proximity of the delta values to zero, the peaks searched were a very close match for Annexin A5, which the MASCOT database reported to have a mass of 35.7 kDa, further indicating that the correct protein was matched.

Discussion

Throughout the course of this experiment, prohibitin was never successfully isolated, although it was identified when performing the western blots. In the immunoprecipitations that were performed, it is likely that the main problem occurred with the antibody being used in that it may not have been specific enough for prohibitin. Evidence for this can be found in the vast number of bands that were present in the sample prior to preclearing. It is also possible that in the preclearing step, some of the prohibitin may have stayed within the protein A beads, further reducing the possibility that it could be extracted. After the preclearing steps however, there were still several bands present that showed a very strong signal after staining. One possibility for this is that the proteins showed some affinity for the antibody, and were present in a much higher concentration than prohibitin, and therefore bound to most of the antibodies present before they could find their desired target. A problem such as this could be corrected by incubating the samples with higher concentrations of the anti-prohibitin antibody, and this in fact was tried in order to troubleshoot the immunoprecipitation, however the results remained the same.

This seems to conflict somewhat however with the results of the western blot, in which a very small dilution of the antibody was all that was necessary to identify prohibitin. It was believed at first that the difference between the two procedures existed in that while the incubation phase up until that time had been performed under native conditions, the incubation for the western blot was performed under denatured conditions. Several trials were performed using denatured conditions, however the results remained the same. The results of the western blot however did show a similar result to the immunoprecipitation in that a vast quantity of bands were present, and it was

only after the reaction of the enhanced chemiluminescence solution with the horseradish peroxidase attached to the secondary antibody that a stronger signal was observed. It may be possible that while the primary antibody was able to bind to a vast number of proteins, it may have had more binding sites on prohibitin, resulting in a stronger signal even if the overall protein concentration was lower. A final possibility exists in that perhaps although the antibody was listed by the manufacturer as working in both western blotting and immunoprecipitation techniques, it may have in fact worked far better for only the western blotting technique. Without a second antibody from a different manufacturer to validate the results against however, this possibility will remain unknown.

It was rather unfortunate that after so many attempts, prohibitin still could not be isolated for analysis using mass spectrometry, however over the course of the experiments, several other interesting proteins were identified. Both the heavy and light antibody chains were recovered in the immunoprecipitation experiments, indicating that the protein A beads had indeed recovered the antibody molecules contained in the cell lysates. After this however, Hsp70 was identified in the initial experiments. Hsp70 is found in the mitochondria as a chaperone protein, which is interesting since one of the proposed roles for prohibitin is to function in a complex as a chaperone within the mitochondrial membrane. It may be that the antibody targeted this due to some similarity in structure where the antibody is expected to bind with prohibitin.

Also identified in the initial immunoprecipitation experiments, another one of the protein spots that was positively identified was carbamoyl phosphate synthase I. Carbamoyl phosphate synthase I is a 164kDa protein that is also known to reside in the

mitochondrial membrane, which makes it of considerable interest as it fit both the observed weight in the gel as well as a known location for prohibitin within the cell.

Carbamoyl phosphate synthase (CPS) is an enzyme generally associated with the rate limiting step of the Urea Cycle in which HCO^-_3 is eventually reacted in a multi-step process to yield carbamoyl phosphate. CPS is actually a family of proteins and the enzyme identified in this study, CPS I is the enzyme that functions directly in producing the starting reactants in the urea cycle by following the reaction below.

$$2ATP + HCO_{3^-} + NH_3 \longrightarrow H_2NC(O)OPO_3^{2-} + 2ADP + P_i$$

Although not specifically identified in this study, CPS II is an interesting enzyme that was speculated about based on its membership in this family of proteins. CPS II carries out the same initial reactions as CPS I, however it further functions in the synthesis of pyrimidine structures for DNA synthesis. One interesting possibility existed in that CPS I and CPS II could have had similar enough structures and sites for binding to show similar binding affinities for a protein such as prohibitin. The thought was that perhaps this was one method for prohibitin to have an effect on progression of the cell cycle and proliferation, as well as explain some of the observation that the prohibitin mRNA was able to stop the progression of the cell cycle during the S phase, when new DNA is synthesized.

Initially, this appeared to be a promising result, however it was observed that CPS I was being separated on gels when lysates were analyzed without the presence of antibody. It was later found that CPS I was being trapped by the protein beads rather than the antibody, and therefore was a result completely unrelated to the immunoprecipitation of prohibitin, but rather just an effect of the beads used as part of

the protocol. This also served as both an example and a reminder of the fact that although the theory behind immunoprecipitation is very good, the practice of the technique is made more difficult by additional interactions in the lystate beyond just the antibodies, such as interactions between the analytes and the protein beads. The samples in some trials were precleared prior to immunoprecipitation to eliminate some of the extra bands that were being observed during the SDS-PAGE, however although this technique works to reduce the effect of some of these interactions with the protein beads, it also introduces the possibility of losing vital signal for proteins such as prohibitin which may also react with the protein bead.

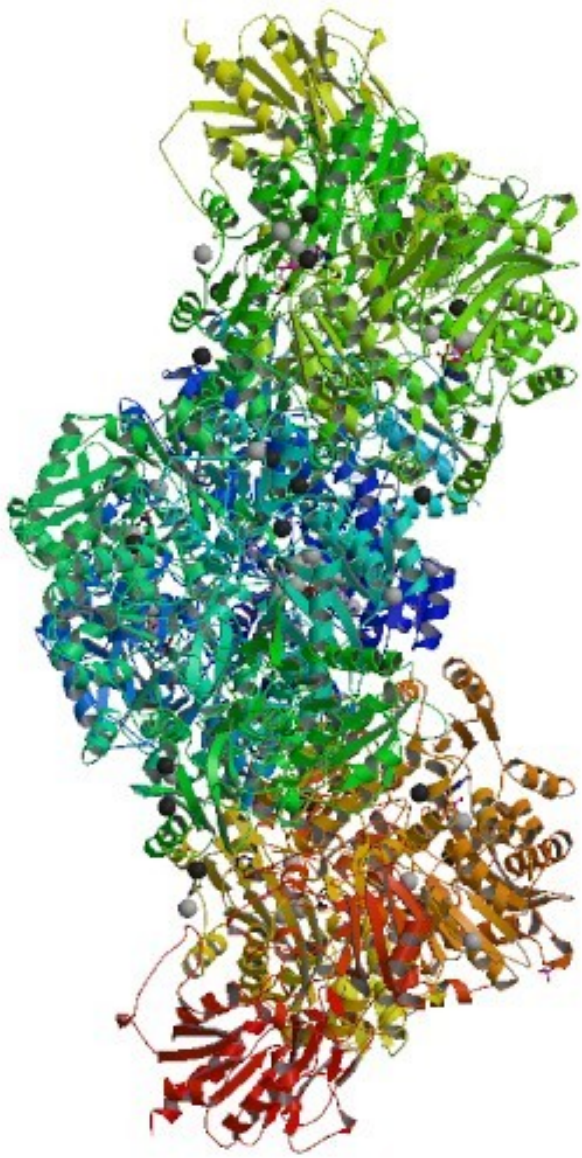


Figure 22: Structure of Carbamoyl Phosphate Synthase as Found in *Escherichia coli* (Structure 1JDB, www.rcsb.org, 2007)

Finally, annexin A5 was identified in the 2D gel for the camptothecin treated B16 cells by mass spectroscopy. Annexin A5 is important because it is a well studied protein that among other roles, can serve as a marker for apoptosis. In one common assay, annexin A5 is labeled using either radioactive or fluorescent tags and then observed as an

indicator of cells that are in the early stages of apoptosis. As cells enter the early stages of apoptosis, annexin A5 may bind to the phospholipid membrane and form a non-covalent trimer of proteins. As these trimers form, a two dimensional crystal lattice begins to form on the exterior of the cell, increasing the likelihood of a cell being targeted by a phagocyte for removal from the tissue.

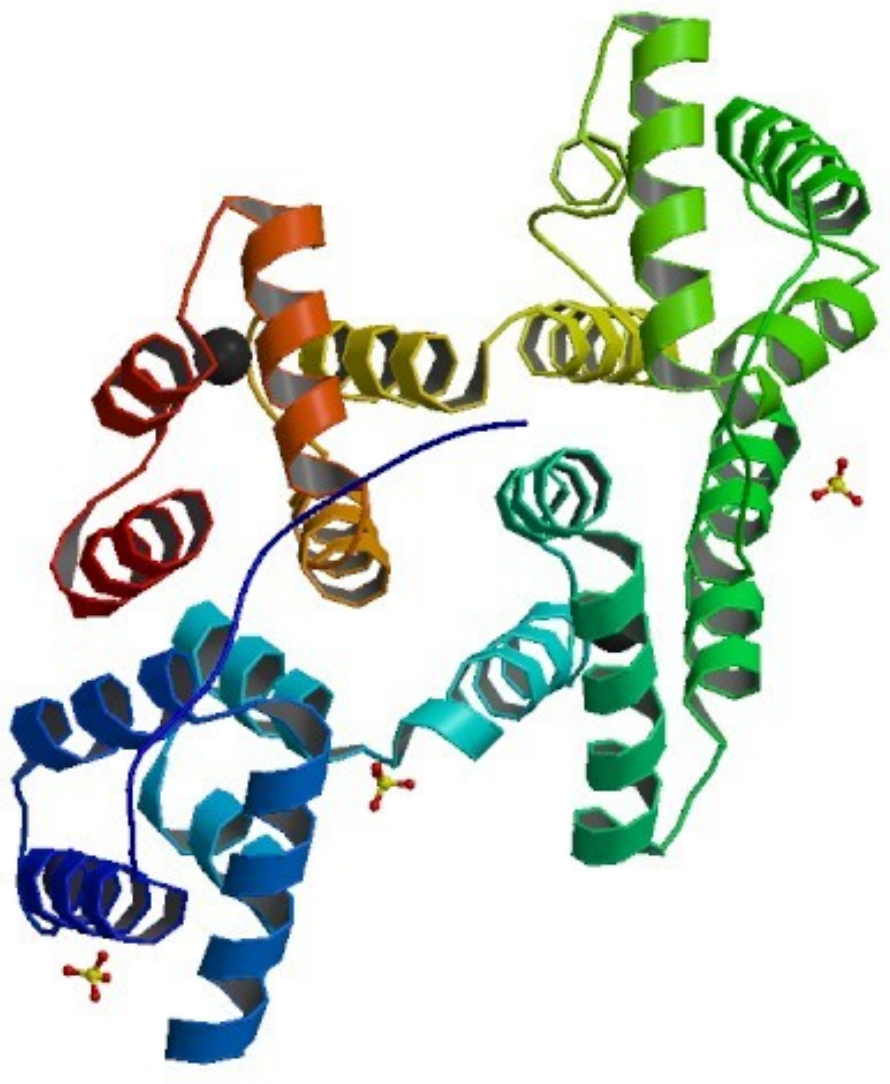


Figure 23: Structure of Annexin A5 as Found in *Gallus gallus* (Structure 1YII, www.rcsb.org, 2007)

The distinct spot visualized on the gel corresponding to annexin A5 indicates that the B16 cells had been increasing production of the protein prior to their death, indicating that they were preparing for apoptosis. The literature involving studies observing prohibitin in camptothecin induced apoptotic conditions indicated that the condition would facilitate movement of prohibitin as well as several other proteins known to take part in apoptosis from the nuclear region of the cell directly to the mitochondria.[10] It was hoped that perhaps an up regulation would be seen in prohibitin similar to what was observed in annexin A5, however this result was not found, and in fact remains inconclusive, as prohibitin was still unidentified in the samples as of the final stages of the experiment.

The most likely cause as to why prohibitin was not identified remains lack of an appropriate antibody to target the protein. Perhaps in time, an antibody will be available for purchase which can better and more efficiently isolate the protein, so that further work into its analysis may be completed, allowing for a clearer understanding of the modifications present during its activity within the living cell.

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Appendix I

Recipes listed in Materials and Methods:

2D Rehydration Buffer:

8M urea to keep the proteins denatured, 2% CHAPS to maintain protein solubility, 50mM dithiothreitol to eliminate disulfide bonds, and 0.2% of a solution of Bio-Lyte ampholytes obtained from Bio-Rad for conductivity

SDS-PAGE Equilibration Buffer I:

6M urea, 0.375M Tris-HCl, pH 8.8, 2% SDS, 20% glycerol, and 2% DTT

SDS-PAGE Equilibration Buffer II:

6M urea, 0.375M Tris-HCl, pH 8.8, 2% SDS, 20% glycerol, and 2.5% Iodoacetamide

Lysis Buffer:

2.5% 1M Tris (pH 7.5), 0.2% 0.5M EDTA, 10% glycerol, 1% Triton X, and the Complete, Mini Protease inhibitor cocktail obtained from Roche.

Hypotonic Lysis Buffer

10mM HEPES pH 7.9, 1.5mM $MgCl_2$, and 10mM KCl, 0.1M dithiothreitol, and 5µL of a protease inhibitor cocktail containing 4-(2-Aminoethyl) benzenesulfonyl fluoride, pepstatin A, bestatin, leupeptin, aprotinin, and trans-epoxysuccinyl-L-leucyl-amido(4-guanidino)-butane

Differential Cell Lysis Extraction Buffer:

20mM HEPES pH 7.9, 1.5 mM $MgCl_2$, 0.42 M NaCl, 0.2 mM EDTA, and 25% glycerol, 0.1 M dithiothreitol, and 1µL of the protease inhibitor cocktail used in the hypotonic lysis buffer.

Blocking Buffer:

1X TBS, 5% dry milk powder, and 0.05% Triton X-100

Stripping Buffer

62.5 mM Tris pH 6.7, 2% SDS, and 100mM beta mercaptoethanol

Cell Lysis Buffer:

2.5% 1M Tris (pH 7.5), 0.2% 0.5M EDTA, 10% glycerol, and 1% NP40

Appendix II

List of abbreviations

BAP32 – B-cell Associated Protein 32, another name for prohibitin

BSA – Bovine Serum Albumin – Serum albumin often used in the Bradford assay

cMyc – Protooncogene that increases cell proliferation when over-expressed

CPS I – Carbamoyl Phosphate Synthase I – Protein which produces the starting reactants in the Urea cycle.

CPS II – Carbamoyl Phosphate Synthase II – Protein related to CPS I which completes the reaction in producing ring structures to be used in DNA synthesis

DTT – Ditheithreitol – Chemical agent used to eliminate disulfide bridges in a protein

E2F – Group of transcription factors found in eukaryotic cells

E2F1 – Transcription factor that functions in control of the cell cycle as well as has some tumor suppressing functionality

Hsp70 – Heat Shock Protein 70 – Protein found within the mitochondria which functions as a chaperone protein in stabilizing and assisting the proper folding of proteins

IP – Immunoprecipitation – Technique for the isolation of proteins through the use of forming complexes with IgG antibodies

MALDI-TOF – Matrix-assisted laser desorption/ionization time of flight mass spectrometer

p53 - Protein 53 - Transcription factor that is known for its function as a tumor suppressor, and is known to act in the regulation of the cell cycle.

PBS – Phosphate Buffered Saline

PHB – Prohibitin, also known as B-cell Associated Protein 32

RARE – Retinoic Acid Response Element

Rb – Retinoblastoma protein – Tumor suppressor protein that normally prevents the cell from replicating damaged DNA through inhibition of E2F transcription factors

TBST - Tris-Buffered Saline Tween-20 – Solution used during Western blotting procedures

Appendix III

Explanation of Techniques

2D Gel Electrophoresis – Separates and isolates proteins based on two dimensions across two different stages. In the first dimension of the separation, the proteins in the lysate are subjected to a charge which causes them to migrate along a pH gradient. The pH at which they focus is equal to the isoelectric focusing point (pI) of the protein, which is the point at which the overall charge on the amino acids throughout the protein becomes equal to zero. After the proteins have been separated by their pI values, they are run through a traditional SDS-PAGE which then separates them by their molecular weight through a polyacrylamide gel. Using this method, proteins may be isolated into distinct spots as opposed to larger bands which may contain multiple proteins, and thus complicate the final analysis of the proteins contained within.

MALDI-TOF Mass Spectrometry – Functions by desorbing a solid analyte contained within a solid matrix into a gaseous ion phase through the use of a laser. The matrix absorbs the majority of the energy imparted by the laser, preventing the analyte from fragmenting, thus providing just enough energy to impart a +1 charge on the analyte. This charge allows the analyte to be focused using a series of voltage gates, and the directed kinetic energy of the gaseous ion carries it along a predefined flight path. The

ion strikes a detector at the end of its path and its mass to charge ratio (m/z) is recorded. Since the m/z is determined by the time it takes the ion to reach the detector, which is then determined by the velocity at which the ion travels, and thus its mass, a fingerprint may be generated for each protein based on the masses of its individual peptides that are ionized and detected.

Western Blotting – Method of identifying the presence of an analyte in a solution. The solution to be identified is first subjected to SDS-PAGE to separate the components inside by their molecular weight, and the gels are then transferred using electricity onto a membrane through a blotting technique. The membranes are incubated with a primary antibody designed to target the analyte of interest, and then incubated with a secondary antibody that is targeted to the primary antibody. The secondary antibody is covalently linked to an enzyme which when in the present of its substrate, produces a chemiluminescent signal which may be detected. The membrane with the bound antibodies is allowed to react with this substrate, and is then exposed to a piece of x-ray film. The luminescence produced causes the film to be exposed, so that it may be developed and the presence of the target protein may be identified within the sample by looking at the banding pattern that is produced in the final film.