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Truncated mortalin in animal cancer

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TRUNCATED MORTALIN IN ANIMAL CANCER

BY

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BS, University of New Hampshire, 2007

THESIS

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This thesis has been examined and approved.

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ABSTRACT

TRUNCATED MORTALIN IN ANIMAL CANCER

By
Katrina K. Olson

University of New Hampshire, May, 2010

Hematopoetic neoplasia or clam hemocyte cancer (a leukemia-like disease) has been studied in a number of bivalve molluscs for the last 20 years. Recent molecular studies of the hemocytes of the soft shell clam, Mya arenaria, have demonstrated an interaction between p53 and mortalin, the mitochondrial Hsp70. The former protein is intimately involved in the initiation of cell-cycle arrest, apoptosis, DNA repair, and cell differentiation. In cancerous clams, wild-type p53 is sequestered in hemocyte cytoplasm by mortalin and cannot be translocated to the nucleus. This is critical because although p53 is functions properly, it is unable to enter the nucleus and initiate cell-cycle arrest. This allows the immortality of cancerous clam hemocytes (CCH). In CCH, wild-type p53 can be induced to enter the nucleus and trigger apoptosis genotoxically following treatment with the topoisomerase II inhibitor, etoposide, or non-genotoxically with the dye, MKT-077. Full-length and truncated (missing exon 3) clam variants of human mortalin have recently been cloned.
Here I have generated and purified an antibody to the truncated variant of clam mortalin and have attempted to determine its location within cancerous clam hemocyte cytoplasm.

I point out problems with the transfection of the truncated mortalin antibody into clam hemocyte cytoplasm and in localizing antibodies in cancerous clam hemocytes embedded in water-soluble resins. The results of this study demonstrate localization of truncated mortalin near mitochondria of cancerous clam hemocytes.
INTRODUCTION

On the North Eastern coast of the United States, the soft shell clam, *Mya arenaria*, develops a fatal blood cancer in ≥1% of virtually all populations investigated\(^1\). This bivalve cancer was first reported in 1977 in clams from Maine\(^1\). The disease has been called a systemic, disseminated neoplasia by the now defunct Registry of Tumors in Lower Animals (formerly maintained by the National Cancer Institute), although the most common descriptors in the literature are hemopoetic, hematopoetic, or hemic neoplasia\(^2\). I will use the phrase cancerous clam hemocytes (CCH) to describe these malignant cells.

Amitotic normal clam hemocytes (NCH) phagocytize bacteria and other foreign particles from the hemolymph\(^1\). CCH undergo continuous mitosis and lose the ability to phagocytize\(^1\). CCH are immortalized cells, constantly replicating to accumulate in the blood sinuses within the gill, gonad, and digestive gland. Their increased numbers distort the size and function of these tissues\(^1\), increasing to a point where severe clam hemocyte cancer is fatal for clams\(^3\). The etiology of the disease has not been determined, although both viral agents and environmental pollutants have been suggested as potentially involved\(^4\).

Recently, the cellular mechanisms responsible for clam hemocyte cancer have been investigated for *Mya arenaria*\(^5,6,7\). In NCH, wild-type p53 is localized in both the cytoplasm and nucleus, while mortalin is only found in the cytoplasm\(^7\). In CCH, wild-type p53 co-localizes with mortalin in the cytoplasm when the latter is over-expressed\(^7\). P53 binds to the p53-binding domain of mortalin. Mortalin
retains wild-type p53 in the cytoplasm and, consequently, the transcriptional functions of p53 in DNA repair and/or apoptosis are negated. In humans, some patients with glioblastoma and colorectal carcinomas also display sequestration of wild-type p53 in the cytoplasm, although the mechanism for sequestering p53 is not understood\textsuperscript{8,9}. It is uncertain how the non-transcriptional, mitochondrial functions of p53 are affected in CCH by p53 sequestration.

Mortalin is the mitochondrial Hsp70 and is a member of the Hsp70 family of proteins. Mortalin is involved in many different cellular activities including mitochondrial protein import, intracellular trafficking (e.g. p53), and receptor internalization\textsuperscript{10,11}.

Both full-length and truncated variants of clam mortalin protein have been cloned and contain a p53 binding site that is 97% conserved with human mortalin\textsuperscript{7,12}. While the full-length variant of mortalin is over-expressed in cancerous clam hemocytes by 1,200-fold, this newly-discovered truncated variant is over-expressed 620-fold\textsuperscript{5}. The truncated version of mortalin is missing exon 3, consisting of 90 amino acids from the ATP/ADP binding site, which also encodes an ATPase. Figure 1 indicates the 90 amino acids (highlighted in red and representing exon 3) that are missing from truncated mortalin. When p53 is bound to mortalin, the ATPase is responsible for hydrolysis of ATP to ADP. ATP binding is required for the association of mortalin with p53, as the ADP-bound form has a very slow exchange rate. Once mortalin is bound to p53, the hydrolysis of ATP to ADP allows mortalin to change its conformation and lock in
p53, with a high binding affinity by closing the binding pocket, and stabilizing the interaction\textsuperscript{11}.

When Hsp70 proteins are bound to ATP, they cannot act as chaperones for proteins\textsuperscript{11}. It is uncertain how lacking the ATP/ADP binding domain will affect the chaperone activities of mortalin. Is the over-expression of truncated mortalin able to indefinitely bind p53, resulting in the inactivation of p53 in clam hemocyte cancer or does the loss of the ATPase domain, even with the over-expression of truncated mortalin, cause truncated mortalin to be unable to interact with p53\textsuperscript{5,11}.

In this study, I have focused on two main objectives:

**Objective 1:** To generate and purify an antibody specific for the splice sites of exons 2 and 4 of truncated clam mortalin.

**Objective 2:** To use the antibody developed in Objective 1 to localize the truncated mortalin protein within cancerous clam hemocytes.

Figure 1. Amino acid sequence of full length clam mortalin compared with human mortalin. The red portion indicates exon 3, the 90 amino acids that are missing from truncated clam mortalin\textsuperscript{11}.
CHAPTER 1 – GENERATING AN ANTIBODY FOR TRUNCATED MORTALIN

I. Objective I: To generate and purify an antibody specific for the splice site of exons 2 and 4 of truncated clam mortalin.

Hypothesis I: An antibody can be generated for the sequence upstream and downstream of exon 3 that will identify truncated clam mortalin.

II. MATERIALS AND METHODS

Peptide Design and Generation

A peptide was designed to the last 7 amino acids in exon 2 (LLPSRLR) and the first 7 amino acids in exon 4 (METVPYK) of truncated clam mortalin protein, the 14 amino acids adjacent to the splice site for exon 3. The peptide consisted of seven amino acids upstream (exon 2), LLPSRLR, and seven amino acids downstream (exon 4), METVPYK, of the exon splice site (Figure 2). Based on this amino acid sequence, the peptide, NH₂-LLPSRLRMETVPYK-COOH, was ordered from New England Peptide LLC (Gardner, MA).

BSA Conjugation to the Peptide

In a 1.5 mL microcentrifuge tube, 10mg of Bovine Serum Albumin (BSA) were dissolved in 500 μL 0.2M sodium phosphate buffer (NaH₂PO₄) (monobasic, pH 7.5 with 1 M sodium hydroxide (NaOH)). In a second 1.5 mL microcentrifuge tube, a 30-fold molar excess of the truncated mortalin peptide relative to the BSA concentration was dissolved in 500 μL of distilled water. The peptide solution was neutralized by the addition of 1 M NaOH drop-wise until a pH of 7 was reached,
utilizing pH paper to determine the pH of the peptide solution. Both the peptide solution and the BSA solution were combined in a 15 mL Falcon tube.

While being vortexed, 1 mL 20 mM glutaraldehyde was added drop-wise to the peptide/BSA solution. The peptide/BSA solution was agitated at room temperature for 30 minutes. Conjugation of the peptide to BSA was terminated by first adding 250 µL of 1 M glycine to the solution, and then agitating the solution at room temperature for 30 minutes.

The peptide/BSA solution was placed into a 1 cm diameter boiled dialysis tubing (Spectrum Laboratories, California), and the tubing was sealed shut with a floating clip. Un-conjugated peptide and BSA were removed by exhaustive dialysis in 1X phosphate buffered saline (PBS). Exhaustive dialysis, ultrafiltration of the un-conjugated peptide/BSA, was performed by floating the peptide/BSA filled tubing in 1 L of 1X PBS (8 g NaCl, 0.2 g KCl, 0.2 g KH$_2$PO$_4$ monobasic, 2.17 g Na$_2$HPO$_4$·7H$_2$O dibasic), while stirring at 6°C for 24 hours. After 24 hours, the tubing was placed into a new solution of 1X PBS and stirred at 6°C for another 72 hours. The dialysate was emptied into a 50 mL Falcon tube and brought to a final volume of 20 mL with 1X PBS.

**Antibody Generation**

20 mL of conjugated peptide-BSA were sent to Pacific Immunology Corp (Ramona, CA). This solution, containing the 14 residue peptide-BSA complex, was used to immunize two rabbits, labeled 2273 and 2274. A pre-immune bleed was removed prior to immunization. Bleeds were removed approximately every
two weeks following initial injection, yielding six bleeds on 10/1/07, 10/15/07, 10/29/07, 11/12/07, 11/19/07, and 11/22/07.

Establishing Relative Antibody Concentrations

A 10 mg/mL solution of BSA was made in 10 mL 0.1 M sodium bicarbonate. Six μmoles of the 14 residue peptide were dissolved in 1 mL distilled water. The peptide solution was neutralized with 1 M NaOH to a pH 7, diluted in an equal volume of 0.2 M sodium bicarbonate, and chilled on ice, while Affi-Gel 10 beads (Bio-Rad Laboratories, California) were equilibrated to room temperature.

A sintered glass funnel (containing a glass mesh) and glass stir rod were washed sequentially in distilled water, 100% alcohol, and distilled water. The Affi-Gel 10 was placed into the funnel and stirred with the glass rod. After vacuum removal of excess liquid, the Affi-Gel was re-suspended in a small volume of 0.1 M sodium bicarbonate. Using a sterile glass rod, the Affi-Gel beads were mixed and placed in a 50 mL Falcon tube. The volume was adjusted to 50 mL with 0.1 M sodium bicarbonate to make a 50% suspension of the Affi-Gel 10 beads.

The slurry was mixed thoroughly, and 6 mL were poured into a 15 mL Falcon tube. The peptide solution was added to the slurry in the 15 mL Falcon tube, thoroughly agitated, and placed on Speci-Mix Rocker (Barnstead/Thermolyne, Dubuque, Iowa) overnight at 4°C to allow for adequate coupling. The BSA solution was treated the same way. The remaining Affi-Gel solution was combined with the BSA solution in a 50 mL Falcon tube, thoroughly agitated, and placed on the rocker overnight at 4°C.
Both Falcon tubes containing Affi-Gel beads were filled with 0.1 M sodium bicarbonate, and sequentially washed three additional times with the 0.1 M sodium bicarbonate. Between each wash, the Affi-Gel beads were centrifuged for 1 minute at 10,000 RCF and placed under a vacuum to remove the 0.1 M sodium bicarbonate. Once the last wash was centrifuged and the sodium bicarbonate removed, a 1:1 ratio of 0.1 M ethanolamine HCl: Affi-Gel slurry was added to the Affi-Gel beads and placed on the rocker for 1 hour at room temperature to block any un-coupled sites on the affi-gel.

Next, the beads were centrifuged for 1 minute at 10,000 RCF and the top aqueous layer was removed. The beads were washed in 0.1 M sodium bicarbonate containing 0.5 M sodium chloride (NaCl) followed by 0.1 M sodium acetate buffer (pH 4 with acetic acid) containing 0.5 M NaCl, and a final wash of distilled water. After each of these washes, the solution was centrifuged for 1 minute at 10,000 RCF and the upper aqueous layer was removed. This process of washing the beads, centrifuging, and removing the upper aqueous layer was repeated with 0.1 M sodium bicarbonate and three times with 1X PBS. Both sets of beads were stored in 3 M NaCl at 4°C overnight.

The following day, the beads were centrifuged at 10,000 RCF and the upper aqueous layer was removed. The beads were washed three times with 10 volumes each of 1X PBS, centrifuged at 10,000 RCF, and followed by removal of the upper aqueous layer. PBS (1X) was added to each tube to create a 1:1 slurry of peptide beads:1X PBS. In a 1.5 mL microcentrifuge tube, 100 μL of the peptide bead slurry were added to 200 μL of each antibody serum, including the
pre-immunization serum, and shaken on the rocker for one hour at room
temperature. The bead-antibody solutions were washed five times with 1 mL 1X
NP40 buffer (140 mM NaCl, 20 mM tris(hydroxymethyl)aminomethane-
hydrochloric acid (Tris-HCl), 1mM ethylenediaminetetraacetic acid (EDTA), 1%
Tergitol-type NP-40) with the top aqueous layer removed after each wash.

Fifty µL 5X sample buffer (250 mM Tris-HCl pH6.8, 10% SDS, 30%
Glycerol, 0.02% bromophenol blue) were combined with 2.5 µL β-
mercaptoethanol and added to each bead-antibody solution and boiled for three
minutes. Forty µL of each sample buffer/bead-antibody solution was added to the
well of a 4-15% Tris-HCl gel. Ten µL Precision Plus Dual Color Standards (Bio-
Rad Laboratories) were added to each of two wells on each gel. The gel was run
in a 1X running buffer of Tris/SDS/glycine (Bio-Rad Laboratories) at 20 mA/gel
for 90 minutes using a Power Pac Basic with Mini Trans Blot (Bio-Rad
Laboratories).

A polyvinylidene fluoride (PVDF) membrane (Bio-Rad Laboratories) was
wetted in 100% methanol for 1 minute, and then both the gel and the membrane
were equilibrated for 5 minutes in transfer buffer (22 mL 2M Tris, 168 mL 2M
Glycine, 350 mL 20% MeOH, 1210 mL de-ionized water). The gel was
transferred onto the PVDF membrane in transfer buffer for two hours at 75V on
ice. The membranes were incubated with anti-rabbit IgG (FC) AD Conjugate
antibodies (Promega, Madison, WI) at 1:5000 (1 part antibody to 5000 parts 1%
BLOTTO) (blotting-grade blocker, Bio-Rad Laboratories) in 1X Tween/Tris-
buffered salt solution (TTBS) (10X TTBS: 2 mL Tween 20, 12.1 g Tris, 17.4 g
NaCl, 200 mL de-ionized water) for 30 minutes at room temperature with agitation on a Red Rocker Model PR50 (Hoefer Scientific Instruments, San Francisco, CA). The membranes were washed two times in 5 mL 1X TTBS for 15 minutes each. Western blue (Promega Corp, Madison, WI) was added to each membrane and agitated until the color developed. The membranes were rinsed with water and air dried.

**Antibody Purification**

Two sera from each rabbit, containing the highest relative concentration of antibody and highest specificity for truncated mortalin, were chosen for purification. Antibodies from each serum were purified utilizing an identical apparatus (Figure 2). Prior to purification, small tubing and stop-cocks were washed sequentially with water, 95% ethanol, and water. A large ring stand was equipped with two clamps capable of holding a 3 mL syringe. Stop-cocks were added to the bottom of two 3 mL syringes where the needle is typically attached. A small amount of non-siliconized, glass wool in a loose ball was pushed to the bottom of the syringe with a transfer pipette (Figure 2).

Syringes in this apparatus were filled with 1X PBS to remove any air bubbles, and 1X PBS was allowed to pass through until just above the glass wool. The BSA-beads were added to the top syringe, and the peptide-beads were added to the bottom syringe (Figure 2). Sera from each of the four bleeds were purified separately, using two columns for each. 200 mL 1X PBS were run through the eight columns to wash the beads. The columns were stored at 4°C until use.
The apparatus was assembled with the columns as described above (Figure 2), and 200 mL 1X PBS were passed through the columns to check for leaks and to ensure that the beads were hydrated. Fifteen mL of the antibody serum were passed through first the top and then the bottom columns, and the column void (liquid passing through the bottom column) was collected. The columns were washed with 10 mL 1X PBS. The tubing between the top and bottom columns was removed from top column. Fifty mL 1X PBS were passed through the bottom column to wash away loosely-associated antibodies. The peptide column was washed with 20 mL 0.5 M NaCl (20 column washes). Dialysis tubing was boiled, and the remaining NaCl in the column was removed with a transfer pipette.

Three mL 3.5 M magnesium chloride (MgCl$_2$) were added to the column. The first 5 mL 3.5 M MgCl$_2$ voided from each column were collected in clean 15 mL Falcon tubes and labeled Elute 1. A knot was tied in the dialysis bag and the Elute 1 flow through was added to the dialysis bag, which was closed with a floating clip. An air bubble was kept in the tubing to ensure the bag would float, and the bag was placed in 1 L 1X PBS. Another 5 mL 3.5 M MgCl$_2$ were collected, labeled Elute 2, and the dialysis process repeated. MgCl$_2$ (4.5 M) was passed through the column and the first 5 mL and second 5 mL voided and labeled as Elutes 3 and 4. These were also placed in separate dialysis bags. The four elutes were placed in 1 L 1X PBS at 4°C overnight with magnetic stirring. The next day, the 1X PBS was changed for fresh PBS, and the elutes
were left at 4°C overnight with continued stirring. The four elutes were placed into separate 15 mL Falcon tubes.

An Ultrospec 2000 (Pharmacia Biotech, Uppsala, Sweden) was used to determine the concentration of each of the purified antibodies'. At 280 nm, a reading of 0.1 equaled approximately 0.1 mg/mL of antibody. The purified antibody solutions were labeled and frozen at -20°C.

Protein Extraction

An NE-PER protein extraction kit (Pierce, Rockford, Illinois) was used to isolate the cytoplasmic and nuclear protein fractions from CCH. In a microcentrifuge tube, 1.5 mL of CCH were centrifuged at 2,000 RCF for 10 minutes. This was repeated a second time to increase the amount of CCH collected, removing the supernatant prior to addition of CCH and after the second centrifugation. 200 μL ice-cold CER I (from the NE-PER kit) were added to the CCH and the mixture was vortexed to resuspend the pellet. The tube was incubated on ice for 10 minutes. Eleven μL CER II (from the NE-PER kit) were added to the tube, vortexed, and incubated for an additional sixty seconds. The tube was centrifuged at 18,000 RCF for 5 minutes. The supernatant was placed in a clean 1.5 mL tube and labeled as the cytoplasmic protein fraction.

The pellet was resuspended in 100 μL ice-cold NER by vortexing, and incubated on ice for 40 minutes while vortexing the tube every 10 minutes. The sample was centrifuged at 18,000 RCF for 10 minutes, and the supernatant was collected. Both the cytoplasmic and nuclear protein fractions were stored at -20°C.
**Antibody Verification**

Two hundred μL 5X sample buffer were combined with 10 μL β-mercaptoethanol in a 1.5 mL tube. Thirty-two μL cytoplasmic CCH protein and 8 μL sample buffer were combined for each antibody sample tested. This protein solution was denatured in boiling water for three minutes. Ten μL precision plus dual color protein ladder (Bio-Rad Laboratories) were added to every other well in a 4-15% Tris-HCl gel. 40 μL of the protein/buffer solution were added to the remaining five wells. The gel was run for two hours at 20 mA/gel in 1X running buffer. The resulting gels were transferred to a PVDF membrane overnight at 90 mA at 4°C.

The resulting PVDF membrane was cut to yield one protein sample and one ladder for each section. Each membrane was placed in 1% Blotto in 1X TTBS with a 1:100 dilution of one of the four first elutes of each purified antibody. The fifth membrane was placed in 1% Blotto in 1X TTBS with a 1:1000 dilution of MAMOT (*Mya arenaria* mortalin protein) fraction 2 antibodies, antibodies previously purified, for a positive control. MAMOT fraction 2 is more concentrated than the purified truncated mortalin antibodies, allowing for a larger dilution factor in 1% Blotto. The MAMOT fraction 2 antibody recognizes a region downstream of the splice site of mortalin, making it possible to identify both truncated and full-length mortalin. Membranes were agitated for 3 hours at 4°C. Each membrane was washed two times with 5 mL 1X TTBS for 15 minutes each. Western blue was added to each membrane and agitated until the color developed at which point the membranes were rinsed with sterile water and air dried.
Figure 2. Diagrammatic representation of the peptide column used for purification of each serum. After the serum was run through the two columns, the tubing was removed from the bottom of the top syringe filled with BSA-beads.
III. RESULTS

Establishing Relative Antibody Concentrations

Sera from the seven bleeds from each rabbit (2273 and 2274) and the pre-immune bleed were evaluated for the highest antibody titer. The bands on the PVDF membranes were the heavy chain of the antibodies within each serum from rabbit 2273 (Figure 3) and rabbit 2274 (Figure 4) that was tested. As a result of heat and detergent treatment, the heavy and light chains had separated. Once separated, the light chain ran off the gel, leaving the heavy chain to be visualized by the secondary anti-rabbit antibodies.

Figures 3 and 4 indicate the results of the seven different bleeds from rabbits 2273 and 2274. Lanes with the most intense staining contained higher relative antibody concentrations. The pre-immune sera contained no antibodies that recognized truncated mortalin (Figures 3 and 4). It is possible that the slight shading observed within the pre-immune sera was unspecific binding of antibodies to mortalin prior to purification. The bleed from 10/1/07 for rabbit 2273 had the highest relative concentration of antibody against truncated clam mortalin (Figure 3). The bleed from 10/15/07 for rabbit 2274 had the highest relative concentration of antibody against truncated clam mortalin (Figure 4). All the other bleeds, except the pre-immune serum, had similar concentrations of antibodies against truncated clam mortalin (Figures 3 and 4).

Antibody Purification

Quantification of antibody concentration for the four purified antibody washes is shown in Table 1. With the exception of 2273 10/1/07, the first elute
had the highest antibody concentration (Table 1). The fourth elute had the lowest antibody concentration (Table 1).

*Antibody Verification*

Immunoblots of the CCH cytoplasmic protein showed proteins at approximately 28 and 45 kD in lanes 1 and 3 (Figure 5). Lanes 2 and 4 contained a single protein at approximately 68 kD (Figure 5). Lane 5 contained two proteins at approximately 68 kD and 74 kD (Figure 5).
Figure 3. Immunoblot of each serum from rabbit 2273. Lane 1: 11/22/07; Lane 2: 11/15/07; Lane 3: ladder; Lane 4: 11/12/07; Lane 5: 10/29/07; Lane 6: 10/15/07; Lane 7: ladder; Lane 8: 10/1/07; Lane 9: pre-immune.
Figure 4. Immunoblot of each serum from rabbit 2274. Lane 1: 11/22/07; Lane 2: 11/15/07; Lane 3: ladder; Lane 4: 11/12/07; Lane 5: 10/29/07; Lane 6: 10/15/07; Lane 7: ladder; Lane 8: 10/1/07; Lane 9: pre-immune.
Table 1. The concentration in mg/ml of antibodies in each purified sera. Absorbance 280 nm.
Figure 5. Immunoblot of the CCH cytoplasmic proteins; each segment contains first the ladder and second the protein extract. Segment 1: 2273 serum 10/1/07; Segment 2: 2274 serum 10/15/07; Segment 3: 2273 serum 11/22/07; Segment 4: 2274 serum 11/22/07; Segment 5: MAMOT fraction 2.
IV. DISCUSSION

Immunoblotting indicated which of the six bleeds should be purified for each rabbit. The immunoblot for rabbit 2273 showed that the 10/1/07 serum had the highest concentration of antibodies out of the six 2273 sera. The 10/15/07 serum had the highest concentration of antibodies out of the six 2274 sera. The other sera for each rabbit had similar, but lesser concentrations. The pre-immune serum had no antibodies for truncated clam mortalin.

Based on higher concentrations of antibodies and antibody specificity, only two sera from each rabbit were purified. The first to be chosen were sera 10/1/07 from 2273 and 10/15/07 from 2274 based on the higher antibody concentrations. The sera from 11/22/07 of both 2273 and 2274 were also purified. Although the later sera contained lower concentrations of antibodies, they should contain antibodies with higher specificity for the peptide resulting from more precise configurations for the peptide conjugate administered. Time allows for more precise B cell differentiation towards the antigen, in this case our peptide, generating high specificity antibodies.

The second immunoblot of the newly purified antibodies showed that rabbit 2273 had produced antibodies with some specificity for the truncated mortalin splice site, but not specific enough to recognize truncated mortalin. Two proteins were recognized by the purified antibodies from rabbit 2273, neither of which were truncated mortalin. Both purified antibodies from 2274 (10/15/07 and 11/22/07) recognized truncated mortalin only (located at 68 kDa). MAMOT fraction 2 (an antibody generated for the region downstream of the splice site of
mortalin), recognized both truncated mortalin, at 68 kDa, and full-length mortalin, at 74 kDa. These results show that rabbit 2274 11/22/07 first elute antibody is specific for the truncated clam mortalin and has the highest relative concentration (Table 1, Figure 5).
Objective II: To use the antibody developed in Objective I to localize truncated mortalin protein within cancerous clam hemocytes.

Hypothesis II – Truncated mortalin is located near the nuclear pores in cancerous clam hemocytes.

Hypothesis III – Truncated mortalin antibodies can be transfected into cancerous clam hemocytes where they will block mortalin from tethering of p53, followed by apoptosis.

II. Materials and Methods

Generation of CCH Cytospins

Clams were classified as normal (0% round, non-motile CCH; 100% attached, motile clam hemocytes), early incipient cancerous (1-50% round, non-motile CCH), late incipient cancerous (50-99% CCH), or fully cancerous (100% CCH). CCH were only withdrawn from clams classified as fully cancerous. CCH were withdrawn from the pericardial sinus of clams with 100% cancerous hemocytes using a 21 gauge needle attached to a 3 mL syringe. Sea water was sterile filtered using a .22 µm mesh. A cell suspension of $2.2 \times 10^6$ CCH/mL of sea water was prepared. 100 µL diluted CCH were placed onto poly-lysine coated
slides and spun at 133 RCF for eight minutes by a Shandon Cytospin 3 (Thermo Scientific, Rockford, Illinois) for optimum coverage and spread.

**Fixation and Embedding of CCH**

Hemolymph, CCH, was removed from the pericardial sinus of clams and centrifuged at 14,000 RCF for 15 seconds with a Beckman Coulter Microfuge 22R Centrifuge (Fullerton, CA). The supernatant was removed and 1 mL 2.5% glutaraldehyde in filtered sea water was added to the tube. The solution was vortexed until the pellet floated and placed on a Glas Col mini-rotator (Terre Haute, IN) at 35 rpm for 1 hour. The tube was centrifuged again. The supernatant was removed, and 1 ml of distilled water was added. The solution was vortexed until the pellet floated and placed on a mini-rotator at 35 rpm for 10 minutes. The tube was centrifuged again and the supernatant removed.

The pellet was dehydrated in increasing concentrations of ethanol (20%, 50%, 70%, 95%, and 100%), rotated at 35 rpm for 10 minutes, centrifuged at 14,000 RCF for 15 seconds, and the supernatant removed. The pellet in 100% ethanol was centrifuged at 10,000 RCF for 30 seconds. The supernatant was removed and 500 µL of a 1:1 solution of 100% ethanol to Pelco Acrylate Resin (Ted Pella Inc., Redding, CA) were added to the pellet.

The pellet was left at room temperature for 1 hour and agitated every 15 minutes with a transfer pipette. The solution was centrifuged at 10,000 RCF for 30 seconds and the supernatant was removed. 250 µL Pelco Acrylate Resin were added to the pellet and incubated for 1 hour, agitating every 15 minutes.
with a transfer pipette. The solution was centrifuged and the supernatant
removed. This process was performed twice to remove any ethanol residue.

Finally, 250 µL Pelco Acrylate Resin were added to the pellet. The pellet
and acrylate resin were placed into a gelatin capsule (EMS, Hatfield, PA),
centrifuged at 10,000 RCF for 30 seconds, and allowed to polymerize for
approximately 12 hours at 60°C. The gelatin capsule was filled to the top with the
acrylate resin and incubated at 60°C for 12 hours. Five µm sections were cut
using a Reichert OMIU3 ultramicrotome with glass knives and heated in a drop of
water on poly-lysine coated slides until dry.

Localization of Truncated Mortalin within CCH Embedded in Plastic and as
Cytospins

Sections of CCH on poly-lysine coated slides were placed in a Coplin jar
containing de-ionized water for 10 minutes to start the hydration process of the
CCH sections. Cytospins of CCH prepared as described above were fixed and
permeabilized in cold acetone for 10 minutes. Both sets of slides were placed in
a Coplin jar with 0.3% H₂O₂ in methanol to initiate permeabilization of the CCH
sections and rocked for 30 minutes on a Red Rocker Model PR50 (Hoefer
Scientific Instruments, San Francisco, CA). The slides were rocked in Vectastain
buffer (1.2 g mono-basic NaPO₄, 9 g NaCl, 1 ml Triton X-100, and final volume 1
liter with de-ionized water) for 10 minutes.

A Vectastain ABC kit for rabbit IgG and a DAB substrate kit, 3,3’-
diaminobenzidine (Vector laboratories Inc, Burlingame, CA) provided the normal
serum, secondary-antibody, ABC solutions, DAB, and DAB enhancer. In the end,
the two kits combined attach a substrate to the IgG antibody and allow
visualization via a colorimetric reaction. The slides were incubated with dilute normal serum (3 mL Vectastain buffer and 1 drop of concentrated normal serum from kit) in a hydration chamber for 20 minutes. The hydration chamber consisted of a Petri dish with a wet thin sponge on the bottom, two thin, round plastic tubes at either end of the dish (upon which slides rested), and a second Petri dish as a lid. Both Petri dishes were covered with tin-foil on the outside to protect the slides from light during incubation.

The slides were incubated overnight (24 hours) in the hydration chamber with 50 μL of a 1:4 solution of the clam polyclonal antibody MAMOT fraction 2 (specific for both truncated and full-length mortalin) or 2274 11/22/07 elute 1 (specific for truncated mortalin): dilute normal serum. A negative control slide with sections of CCH was incubated with 50 μl dilute normal serum. The following day, another 50 μl of 1:4 solution of antibody:dilute normal serum or just dilute normal serum (negative control) was added to the CCH slides in the hydration chamber and incubated for a second night in the chamber for maximum delivery into the CCH. The CCH cytospin slides were continued to the 10 minute Vectastain buffer step below.

The CCH slides were incubated in Vectastain buffer for 30 minutes, while the CCH cytospin slides were incubated in Vectastain buffer for 10 minutes. The embedded CCH slides were incubated overnight while the CCH cytospin slides were incubated for 1 hour in the hydration chamber in secondary goat anti-rabbit antibody solution (2.5 ml of vectastain buffer, 1 drop of dilute normal serum from
kit, and 1 drop of concentrated secondary antibody from kit, a biotin-labeled goat antibody to rabbit IgG heavy chain).

The following day for embedded CCH slides (or an hour after having incubated in the secondary antibody solution for CCH cytopsins), the slides were rocked in Vectastain buffer for 10 minutes. The ABC solution contained avidin, which binds multiple sites on biotin at the site of the primary antibody/protein of interest and was made 30 minutes prior to use (2.5 mL of vectastain buffer, 1 drop of A from kit, Avidin DH solution, and 1 drop of B from kit, biotinylated enzyme). The slides were incubated with the ABC solution for 30 minutes in the hydration chamber. The slides were rocked in Vectastain buffer for 10 minutes to remove excess ABC solution while maintaining permeability of the CCH sections.

The DAB Substrate Kit 3,3'-diaminobenzidine was used to react with the avidin substrate previously attached to the slides from the ABC solution. The slides were incubated in the hydration chamber with 400 µL DAB (2.5 mL water, 1 drop buffer from kit, 2 drops DAB from kit, and 1 drop H₂O₂ from kit) until a tan color developed, about 60-90 minutes depending on color development. The slides were rinsed in water and placed in 0.05 M sodium bicarbonate, pH 9.6, for 10 minutes. The slides were incubated with DAB Enhancing Solution (Vector Laboratories Inc, Burlingame, CA) for 20 seconds. The DAB Enhancing Solution enhances the reaction product from DAB to increase the sensitivity of the kit.

The CCH slides were rinsed in de-ionized water and air dried. The CCH cytopsin slides were dehydrated for 1 minute each in an increasing ethanol series followed by xylene (50%, 70%, 95%, and 100% ethanol). The slides were
removed from xylene and immediately mounted under a 2x2 cm coverslip using Permount (Fisher Scientific). The slides were examined at 63X bright field (BF) on a Zeiss Axioplan II MOT equipped with epifluorescence, an AxioCam MR camera, and AxioVision 4.3 software (Carl Zeiss, Inc., Thornwood, NJ).

**Transfection of Antibodies into CCH using Chariot**

MAMOT fraction 2 (positive control), rabbit 2274 11/22/07 elute 1, and cytochrome C antibodies were each transfected into a set of CCH. For each set, and for a negative control, 2 µL of Chariot Protein Delivery Reagent (Active Motif, Carlsbad, CA) were combined with 50 µL of nuclease-free water. Chariot delivers antibodies into live cells by one of two possible ways: 1) by creating a pore in the cell membrane which allows the antibody to flow into the cell or 2) by attaching to the antibody on the outside of the cell and flipping it to the inside of the cell. Of the two methods, the one responsible for transfection has not been verified. Because there were four different treatments (3 antibodies, MAMOT, rabbit 2274, and cytochrome C, and a negative control), 8 µL of Chariot were added to 200 µL of nuclease-free water (Chariot Protein Delivery Reagent kit).

1.5 µg antibody (MAMOT fraction 3, rabbit 2274 11/22/07 elute 1, or cytochrome C rabbit polyclonal IgG antibody (Santa Cruz Biotechnology, Santa Cruz, CA) were brought to a final volume of 50 µL with 1X PBS. The 50 µL antibody solution was combined with 52 µL of the Chariot-water solution. For the negative control, 52 µL of the Chariot-water solution were combined with 50 µL 1X PBS. The antibody-Chariot solution was incubated at room temperature for 30 minutes.
1.5 mL of CCH were removed from the pericardial sinus of a 100% cancerous clam as described above and placed in a microcentrifuge tube on ice. The concentration of CCH was determined using a hemocytometer. CCH (0.1x10^6 cells) were added to each of the three chariot-antibody solutions as well as to the negative control. The volume was brought to 150 μL with filtered sea water. The four solutions containing CCH were incubated at 4°C for 90 minutes. A first set of cytospins was created for each cell solution with 72 μL of the cell solution per slide. The cytospins were spun at 133 RCF for 8 minutes. The remainder of each CCH solution was left to incubate for an additional 90 minutes at 4°C. A second set of cytospins was spun at 133 RCF for 8 minutes with 72 μL of the cell solution.

Localization of Truncated Mortalin within Chariot-Treated CCH

Chariot-treated CCH cytospins were incubated in cold acetone for 10 minutes. The slides were rinsed with Vectastain buffer and placed in 0.3% H₂O₂ in methanol for 30 minutes. The slides were incubated in Vectastain buffer for 10 minutes followed by incubation in the hydration chamber with dilute normal serum for 20 minutes.

To detect truncated mortalin antibody, chariot-treated CCH were incubated in the hydration chamber with secondary antibody solution for 1 hour. Preparation of these slides was as in the above section Localization of Truncated Mortalin within CCH as Cytospins. The slides were examined at 63X bright field (BF) on a Zeiss Axioplan II MOT equipped with epifluorescence, an AxioCam MR camera, and AxioVision 4.3 software.
III. RESULTS

Localization of Truncated Mortalin within CCH Embedded in Plastic

These preparations were used to determine whether perinuclear localization of clam mortalin was attributable to full length, to truncated mortalin, or to both. Neither the truncated nor the full-length antibody detected any variant of mortalin within the CCH embedded in plastic (Figure 6).

Localization of Truncated Mortalin in CCH cytopsins

Results depended on which primary antibody was initially transfected into CCH. Full length clam mortalin antibody (MAMOT), which recognizes both full length and truncated variants of clam mortalin, indicated that clam mortalin was evenly distributed in the cytoplasm (Figure 7-1). Truncated clam mortalin was restricted to small spots within the cytoplasm and not in the nuclei of CCH (Figure 7-2). Truncated clam mortalin was not detected in control CCH (Figure 7-3).

Localization of Truncated Mortalin within Chariot-Treated CCH

When CCH were treated with MAMOT (Figure 8-1), truncated mortalin antibody (Figure 8-2), and cytochrome C (Figure 8-3) similar distributions of protein were observed in cytoplasm. None of these proteins was detected in control CCH (Figure 8-4). Following all three treatments, the structure of CCH differed from that of untreated CCH. The plasma membranes of CCH, independent of treatment, were ruffled (Figure 8-1 to 8-3).
Figure 6. CCH embedded within Pelco Acrylate Resin: 1) clam full-length mortalin antibody (MAMOT) (fraction 2) and 2) control; N = nucleus; Scale bars = 10 μm.
Figure 7. Cytospins of CCH with treated with: 1) MAMOT (fraction 2), 2) clam truncated mortalin antibody (2274 11/22/07 elute 1) and 3) control; N = nucleus; Scale bars = 10 μm.
Figure 8. CCH transfected with: 1) MAMOT (fraction 2), 2) clam truncated mortalin antibody (2274 11/22/07 elute 1), 3) cytochrome C antibody and 4) control; N = nucleus; Scale bars = 10 µm.
IV. DISCUSSION

Localization of Truncated Mortalin within CCH Embedded in Plastic

In CCH embedded in Pelco Acrylate Resin, there was no evidence that proteins had been localized using MAMOT (full-length and truncated mortalin) antibodies (Figure 6). Truncated and full-length mortalin proteins are both found in high concentration within CCH as demonstrated by results from western blotting\(^7\). CCH treated with full-length antibody (MAMOT fraction 2) should have identified both of these proteins as indicated in Walker et al., 2006 and did not do so in these preparations. This suggests that the antibodies we used did not penetrate the hydrophilic acrylate resin.

Localization of Truncated Mortalin within CCH as Cytospins

Control CCH that were not incubated with either primary antibody did not stain (Figure 7-3). Localization of clam full-length and truncated mortalin within CCH is illustrated in Figures 7-1 and 7-2 respectively. Their cytoplasmic localization within CCH is unique. Clam full-length mortalin is evenly distributed in the cytoplasm (Figure 7-1). Clam truncated mortalin is localized to small spots at discrete locations in the cytoplasm (Figure 7-2). While some of these spots surround the nucleus, it is likely that truncated mortalin is associated with mitochondria, which have similar cytoplasmic distribution near the nucleus and also throughout the cytoplasm. In humans, mortalin is transported to and ultimately located in the mitochondrial matrix. My results seem to refute Hypothesis II which proposed that truncated mortalin would be localized exclusively around the nuclear pores.
Localization of Truncated Mortalin within Chariot Treated CCH

Control CCH that were not incubated with antibody did not stain (figure 8-4). Figures 8-1 through 8-3 demonstrated a generalized darkening of all cellular components following transfection with all three antibodies (truncated and full-length mortalin antibody and cytochrome C) in Chariot treated CCH. Such darkening is often observed under the conditions outlined in this study and is interpreted here as a failure to successfully transfect CCH in all cases. These results indicate that transfection with the Chariot system under the conditions outlined in the Materials and Methods above did not deliver truncated mortalin antibodies into CCH.
CONCLUSIONS AND FUTURE DIRECTIONS

Previous research indicates that mortalin is co-sequestered with p53 near the centriole or microtubular array in the cytoplasm of CCH, but not NCH\textsuperscript{7}. The same study demonstrated that competitive inhibition of mortalin by the cationic inhibitor MKT-077 promoted access of wild-type p53 to the nucleus and in some cells induced apoptosis\textsuperscript{5,7}. Genotoxic-stress to CCH, resulting from etoposide treatment, increases the levels of p53 mRNA as shown by QPCR and suggests that increased levels of p53 can lead to apoptosis\textsuperscript{5}.

Recently a splice variant of full-length clam mortalin, missing exon 3 was cloned\textsuperscript{12}. My study was designed to develop an antibody that could differentiate truncated from full-length clam mortalin and to demonstrate the localization of truncated mortalin within the cytoplasm of CCH using the newly designed antibody. Based on the results in Walker et al., 2006, I hypothesized that truncated mortalin would be localized exclusively near or surrounding the nuclear pores in CCH. The results of this study demonstrate that truncated mortalin was localized at both the centrioles and the mitochondria as well as near the nucleus, although not uniformly localized near the nuclear pores as was hypothesized.

Mortalin is a member of the Hsp-70 family of proteins that has been localized at multiple sites in transformed human cell lines. Although it is unclear how mortalin is delivered in different cell lines, multiple subcellular sites have been identified including, mitochondria, endoplasmic reticulum, cytoplasmic
vesicles, and cytosol. The principle site for mortalin's function is mitochondria.

In an earlier study of CCH, mortalin was localized both perinuclearly and at mitochondria, respectively. The perinuclear localization of mortalin was documented in Walker et al (2006) where mortalin and p53 were co-localized near the nucleus in CCH, both in vivo and in vitro. In the current study of CCH, it appears that truncated mortalin is localized both in the perinuclear regions of the CcH and at mitochondria, although this needs to be tested in conjunction with FITC labeled WGA and cytochrome C antibodies respectively.

The transfection of antibodies into CCH using Chariot resulted in the darkening of all cellular components. All attempts at transfecting antibodies used in this study using Chariot methodology indicated that more studies need to be carried out to successfully transfect antibodies into CCH.

Walker et al (2006) demonstrated the co-localization of p53 and mortalin within CCH but not NCH. When this paper was published, the truncated variant of mortalin had not been detected.

Although the current study indicates that truncated mortalin is confined to two main locations within CCH, perinuclear and mitochondrial, its role in p53 sequestration has not been resolved. Since previous studies indicated p53 at perinuclear localization, it is possible that truncated mortalin will be found co-localized with p53 in subsequent studies at perinuclear locations. If truncated mortalin is associated with mitochondria and with the nucleus, it is possible that it could be involved in both genotoxic and non-genotoxic stress related induction of
p53 when cytoplasmic sequestration of wild-type p53 is defeated in CCH. Both of these possibilities are dependent on the idea that truncated mortalin is able to associate with p53 despite the lack of the ATPase domain in the non-existent exon 3.
Literature Cited


