8-2017

Immunological Strategies to Study GRP170 in Caenorhabditis elegans

Raven P. Baxter-Christian
State University of New York College at Buffalo - Buffalo State College, baxterrp01@mail.buffalostate.edu

Advisor
Gregory J. Wadsworth, Ph.D.
First Reader
Gregory J. Wadsworth, Ph.D.
Second Reader
Derek L. Beahm, Ph.D.
Third Reader
Daniel L. Potts, Ph.D.
Department Chair
Martha Skerrett, Ph.D.

To learn more about the Biology Department and its educational programs, research, and resources, go to http://biology.buffalostate.edu/.

Immunological Strategies to Study GRP170 in *Caenorhabditis elegans*

by

Raven Baxter-Christian

An Abstract of a Thesis
in
Biology

Master of Arts

August 2017

State University of New York
College at Buffalo
Department of Biology

Abstract of a Thesis

Immunological Strategies to Study GRP170 in *Caenorhabditis elegans*

Characterization of the ER protein folding chaperone GRP170 of *Caenorhabditis elegans* could be greatly facilitated by an antibody which recognized the chaperone. Antibodies have not been raised against nematode GRP170. Groups have prepared polyclonal antibodies against vertebrate forms of GRP170. My thesis goal was to investigate whether anti-vertebrate GRP170 antibodies can recognize the nematode homologue on a standard Western Blot assay. Sequences of antigens that groups used to generate anti-vertebrate GRP170 antibodies were analyzed. Peptides used by Ruan et al. (2013), which correspond to regions of human GRP170, shared greatest sequence similarity with nematode GRP170. I investigated whether the Ruan GRP170 antibody recognized nematode protein on Western Blots. I extracted mouse liver proteins and whole worm proteins from *C. elegans*, separated proteins by SDS-PAGE, transferred proteins to a PVDF membrane, and probed membranes with the Ruan anti-human GRP170 antibody. Although this antibody did recognize a high molecular weight mouse protein, it did not bind to an equivalent high molecular weight *C. elegans* protein. This antibody would have limited utility for studies of nematode GRP170. Isoform-specific antibodies generated against *C. elegans* GRP170 proteins would be valuable tools to differentiate the functions of the two GRP170 isoforms. I analyzed amino acid sequences of the two GRP170 isoforms of *C. elegans*. I identified two peptides in a highly-diverged region of *C. elegans* GRP170, possible isoform specific antigens. Antibodies raised against these peptides could show isoform specific recognition and be useful for characterizing the two-gene, two-protein GRP170 system in *C. elegans*.

Immunological Strategies to Study GRP170 in *Caenorhabditis elegans*

by

Raven Baxter-Christian

A Thesis
in
Biology

Master of Arts

August 2017

State University of New York
College at Buffalo
Department of Biology

Approved by:

Gregory J. Wadsworth, Ph.D.
Associate Professor in Biology
Thesis Adviser

Martha Skerrett, Ph.D.
Chair and Associate Professor in Biology

Kevin J. Miller, Ed.D.
Interim Dean of the Graduate School

THESIS COMMITTEE

Gregory J. Wadsworth, Ph.D.
Associate Professor in Biology

Derek L. Beahm, Ph.D.
Assistant Professor in Biology

Daniel L. Potts, Ph.D.
Associate Professor in Biology

ACKNOWLEDGEMENTS

I give thanks to Dr. Gregory Wadsworth, my advisor, for his invaluable leadership, patience, and support throughout my academic career at Buffalo State College. Dr. Wadsworth's part in my academic career is deeply appreciated. I thank Dr. Daniel Potts and Dr. Derek Beahm for serving on my thesis committee. I thank Dr. Potts for his insightful views of evolution from an ecologist's perspective. I also thank Dr. Beahm for lending his expertise in cell and molecular biology, and making helpful suggestions as I pursued my thesis questions.

I acknowledge the key contributions that Yuanyuan Li and Antonio Rockwell made to my thesis work. Their data provided the stepping stones for me to conduct my study. Without their data, there would have been much more to be understood about the GRP170 proteins in *C. elegans*.

I thank the Buffalo State faculty and my cohorts for their time and support. The Buffalo State Biology Department has provided an outstanding environment for me to achieve my goals. I am grateful for my education and experiences and as a student at Buffalo State College.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Introduction

Glucose-regulated protein 170 (GRP170) is a molecular chaperone found in the lumen of the endoplasmic reticulum (ER) of all eukaryotes. GRP170 appears to facilitate protein folding in the ER using two distinct mechanisms (recently reviewed in Behnke et al., 2015). GRP170 can act as a co-chaperone for the ER HSP70 chaperone homologue, BiP, also known as GRP78, by functioning as a nucleotide exchange factor (Andréasson et al., 2010). The nucleotide exchange factor activity of GRP170 removes ADP from BiP and replaces it with ATP. This cycling of adenosine nucleotides is central to BiP's chaperone activity (reviewed in Behnke et al., 2015). Additionally, GRP170 appears to function directly as a chaperone by binding to unfolded proteins in the ER (Behnke and Hendershot, 2013). GRP170 binds unfolded protein using its C-terminal domain in a mechanism like other members of the HSP70 superfamily.

GRP170 is a member of the HSP70 superfamily, a major class of molecular chaperones (Easton et al., 2000). Members of this superfamily have conserved structural domains: an N-terminal ATP binding domain and C-terminal substrate binding domain (SBD) (Bukau and Horwich 1998). The SBD has three conserved subdomains: a beta sandwich domain, an alpha helical domain and a short loop connecting the beta sandwich and alpha helical domains. Members of the HSP70 superfamily bind to hydrophobic residues of unfolded proteins via the SBD and prevent formation of protein aggregates within the cell (Tyson et. al., 1997). Disruption of GRP170's C-terminal domain does not interfere with its NEF activity but does prevent it from binding unfolded proteins (Park et al., 2003). GRP170 is considerably larger than other members of the HSP70 superfamily. Much of its extra size is due to a larger loop between the ATPase and SBD domains and an expanded alpha helical domain (Easton et al., 2000). The canonical

member of this superfamily, HSP70 and its ER paralogue BiP have been extensively characterized at the biochemical and genetic level (reviewed by Shaner and Morano 2007). However, studies of the GRP170 members of this superfamily have only recently begun to provide insight into their cellular roles in the ER (Behnke et al., 2015).

As a component of the ER chaperone network, GRP170 assists in protein folding, assembly, and transportation of secretory or transmembrane proteins (Wang et al. 2014). The physiological and pathological stress conditions that disturb the highly oxidizing and calcium-rich ER environment can trigger unfolded protein response (UPR) (Li et al. 2011). The UPR is a protein quality control mechanism that aims to limit ER stress and restore ER homeostasis, in part, by inducing the expression of ER chaperones and by targeting misfolded proteins to the ER-associated degradation (ERAD) pathway for degradation (Rutkowski et al. 2004). GRP170 is one of the chaperones induced by the UPR, however the exact role of GRP170 in the UPR is not currently known.

The roundworm *C. elegans* has been a model system for studying the protein folding machinery of the ER (Brignull et al., 2007) and may be especially well suited for investigating the role of GRP170. The nematode suborder Rhabditina (Clade V) which includes both non-parasitic and parasitic nematodes is unique among animal taxa by having not one but two loci encoding distinct isoforms of GRP170 (Harris et al. 2009). The two loci in *C. elegans*, T24H7.2 and T14G8.3, are also known as *grp170a* and *grp170b* respectively. Nei and Nikolaidis (2003) proposed that these two loci were generated by a gene duplication prior to the divergence of *C. elegans* and *C. briggsae* about 100 million years ago (Stein et al. 2003) The discovery that the

two loci structure is found in other *Rhabditina* suggest the divergence may predate even that (Harris et al. 2009).

The *grp170a* gene T24H7.2 is located on chromosome 2 and is part of an operon that encodes three other proteins and one snoRNA in addition to *grp170a*. The *grp170b* gene, T14G8.3, is encoded by a monocistronic operon located on the X chromosome (Harris et al. 2009). The GRP170A protein has a predicted mass of 104.2 kDa and is 925 amino acids long. Presumably the 170 kDa mass estimated from SDS-gel electrophoresis is due to the addition of glycosylation in the ER (Ikeda et al. 1997). The *grp170b* gene generates two protein isoforms, likely generated by alternative splicing at the beginning of the gene sequence. The two isoforms are nearly identical; isoform A codes for a protein that is 921 amino acids long, and isoform b codes for a protein that is 905 amino acids long.

Li (2015) developed isogenic strains of *C. elegans* lacking either *grp170a* or *grp170b* to investigate the genetic roles of the two isoforms. Worms that lacked *grp170a* showed delayed development compared to the control strain or worms that lacked *grp170b*. They took almost 30% longer, to mature from a hatched egg, develop into adults, and lay their first eggs. In contrast, the absence of *grp170b* was associated with no observable phenotype. Therefore, Li concluded that *grp170a* is more physiologically important in development because its absence results in developmental delay.

Rockwell (2015) demonstrated that the two *C. elegans grp170* mRNAs are differentially regulated during protein folding stress (Rockwell 2015). Worms induced for UPR by the

application of the glycosylation blocker tunicamycin showed strong induction of *grp170b* mRNA as measured by RT-qPCR. On the other hand, expression of *grp170a* mRNA was not induced during the unfolded protein response (Rockwell 2015).

It is not known if the regulation of grp170 at the mRNA level corresponds with the regulation at the protein level. It is commonly assumed that mRNA and protein levels correlate. However, the observed abundance of mRNA due to strong gene expression does not necessarily mean that the corresponding protein is also abundant or even active in the cell (Chen et al., 2002). Chaperone genes are often regulated at the translational level, controlling levels of protein synthesized from its mRNA. Studies in organisms such as *E. coli* also have shown that protein levels and mRNA copy numbers are sometimes uncorrelated at the cellular level (Taniguchi et. al., 2010). An antibody which recognizes nematode GRP170 could be used to determine if the expression of the protein GRP170 correlates with its mRNA expression levels.

The primary goal of my thesis was to investigate whether antibodies raised against mammalian GRP170, also known as HYOU1 or ORP150, could be used in protein studies of *C. elegans* GRP170. Although there are several antibodies raised against vertebrate GRP170 epitopes, none of these antibodies have been evaluated for the recognition of the nematode homologue of GRP170.

The current grp170 mRNA expression data does not provide insight into protein level functions such as post-translational modification or localization of corresponding proteins. GRP170 may be active in all parts of the worm or limited to a specific system, such as the

nervous or muscular system. An antibody against GRP170 can be used to localize GRP170 within the tissues of the worm using immunocytochemistry techniques. Antibodies can also be used to study protein interactions using co-immunoprecipitation and other approaches. Antibodies have been used to demonstrate that vertebrate GRP170 binds BiP as well as some unfolded proteins (Behnke and Hendershot 2013). It is not known if the two isoforms of *C. elegans* GRP170 interact differentially with other cellular proteins. It is possible that the two *C. elegans* isoforms have differentiated protein interactions where GRP170A may bind with BiP, and GRP170B may bind only unfolded proteins. It is also possible the two isoforms have different binding affinities for different proteins. Coimmunoprecipitation studies could be used to study the protein-protein interactions of the nematode GRP170s.

Antibody based protein-level studies like those briefly described above will be crucial for understanding the roles of the GRP170 two-gene, two-protein system in *C. elegans*. The goal of my thesis was to investigate whether anti-vertebrate GRP170 antibody can recognize and bind the nematode homologue on a standard western-immunoblot assay. In addition, I used bioinformatics to identify possible antigenic regions on the *C. elegans* GRP170 proteins to generate isoform specific antibodies in the future.

Materials and Methods

Worm Cultures

The standard laboratory strain of *C. elegans,* N2 was cultured on nematode growth media (NGM) (0.25% w/v tryptone, 0.3% w/v NaCl, 1.8% w/v agar, 1 mM CaCl2, 1 mM MgSO4, 5 µg/ml cholesterol in ethanol, 25 mM KPO4, pH 6) seeded with OP50 strain of *E. coli* as a food source (Brenner 1974). Nematode cultures were grown and maintained at 20°. New cultures were established by the chunking method wherein a sterilized spatula is used to remove a chunk of agar from an established plate worm culture and the chunk is placed on a fresh NGM plate seeded with OP50 *E. coli*. The worms crawl out of the chunk and spread out onto the bacterial lawn of the new plate to establish a new culture (Stiernagle 1999).

Preparing Protein Extracts for SDS-PAGE

Mouse liver, used as a source of vertebrate protein, was a gift from Roswell Park Cancer Institute. It was harvested from adult mice and frozen at -80°C before being transported to Buffalo State College. A sample of 3.2g of liver tissue was physically ground to a liquid state in 15ml of deionized water using a mortar and pestle. The sample was centrifuged to eliminate solids, and the liquid supernatant was kept for protein determination. *Caenorhabditis elegans* N2 worms were harvested by washing the mixed larval and adult population from NGM plates with 5mL M9 buffer (42mM Na_2HPO_4, 22mM KH_2PO_4, 86mM NaCl, and 1mM $MgSO_4$) and collecting into a pre-weighed conical tube. The collected worms were pelleted by centrifugation at 2,500g and wet pellet weight mass was obtained by removing liquid from the pellet and measuring mass of pellet in pre-weighed conical tube. Worms were lysed in household bleach, releasing protein contents. Protein concentration of mouse liver and *C. elegans* extract was

obtained using the Bradford assay (Bradford 1976). Proteins were diluted to a final concentration of 2 mg/ml in SDS loading buffer (5% β-mercaptoethanol, 0.02% bromophenol blue, 30% glycerol, 10% sodium dodecyl sulfate, 250 mM tris-Cl, pH 6.8). Protein extracts were boiled for 10 minutes in SDS loading buffer.

SDS Electrophoresis

Denatured protein (10-20 μg/well) was loaded into sample wells of Mini-PROTEAN® TGX™ Precast Gradient Gels 4-12% (Bio-Rad, Hercules, CA) and run at 200V for 20 minutes. Precision Plus Protein™ Dual Color protein standards were used to estimate molecular weight on SDS-PAGE and Western Blots (Bio-Rad, Hercules, CA).

Antibody Selection

Three commercial sources of antibodies against GRP170 were identified from suppliers Abnova, Abbexa, and Bioss. All three were raised using synthetic peptides corresponding to different regions of human GRP170. The sequences of peptides used to raise the anti-GRP170 antibodies sold by Abnova was not provided, so this antibody was not used. The polyclonal rabbit antibody sold by Abbexa was raised against the amino acids #274-303 of human GRP170 (EMELRLRERLAGLFNEQRKGQRAKDVRENP), and the polyclonal rabbit antibody sold by Bioss was raised against AA# 400-450 of human GRP170 (VGKEELGKNINADEAAAMGAVYQAAALSKAFKVKPFVVRDAVVYPILVEFT).

To identify which anti-vertebrate GRP170 antibody had the greatest likelihood of cross-reacting with *C. elegans* GRP170, I determined which of the peptide antigens shared the greatest

sequence identity with the nematode GRP170 sequences. The peptide sequences were aligned to each *C. elegans* GRP170 using Clustal Omega (Lopez et. al 2014) (Nucleic Acids Research 2014). The Gap Penalty in the alignment algorithm was set to a high level (10.0) to minimize gaps.

Western Blot Analysis

After protein separation via SDS-PAGE, proteins were transferred to a PVDF membrane using the Trans-Blot® Turbo™ Transfer System (Bio-Rad, Hercules, CA) on the high molecular weight setting when preparing membranes for anti-GRP170 blotting, and the low molecular weight setting when preparing membranes for anti-actin blotting. To ensure that the proteins were evenly transferred from the gel to the PVDF membrane, post-transfer gels were stained using Coomassie stain (0.1% (w/v) Coomassie blue R350, 20% (v/v) methanol, and 10% (v/v) acetic acid) (data not shown). To block non-specific binding sites, membranes were incubated for 1 hour at room temperature in 3% Blotto (Santa Cruz Biotechnology, Santa Cruz, CA) in TBST (137mM NaCl, 2.7mM KCl, 19mM Tris base). After blocking, the membranes were incubated with their primary antibody for one hour at room temperature in 3% Blotto in TBST. Actin antibody was diluted 1:500 and used as a positive control (catalog #sc-1616-R, Santa Cruz Biotechnology, Santa Cruz, CA). Anti-ORP150 was diluted 1:500 and used as experimental antibody (Catalog number bs-4248R, Bioss Anitbody Inc, Worburn, MA) (Ruan et al. 2013). After incubation with the primary antibody, the membrane was washed for five minutes at a time with TBST, for five times. After washing, the membrane was incubated with the secondary antibody, goat anti-rabbit antibody conjugated to horseradish peroxidase (Thermo Fisher

Scientific, Grand Island, NY), which was diluted 1:1000, at room temperature for one hour. After incubation with the secondary antibody, the membrane was washed as previously described.

Image Analysis

After washing, the membrane was incubated with chemiluminescent substrate using the Pierce™ ECL Western Blotting Substrate Kit as per manufacturer's instructions (Thermo Fisher Scientific, Grand Island, NY). Imaging was done using the ChemiDoc™ MP System using the immunoblot program settings (Bio-Rad).

The prestained Precision Plus Protein™ Dual Color protein standards were visible on the blot after transfer. The blots with proteins standards were photographed under white light. Images of standards and chemiluminescent immune-detected proteins were compared to estimate molecular weight of immune-detected on Western Blots.

Computer-aided Sequence Analysis of GRP170 Proteins

Protein sequences of vertebrate and *Caenorhabditis elegans* GRP170 proteins were obtained from Uniprot, a database of protein sequence and functional information (UniProt Consortium 2017). Accession numbers corresponding to analyzed sequences were #Q9Y4L1 (Human GRP170), #Q9JKR6 (Mouse GRP170), and #Q22758 (*C. elegans* GRP170a) and #Q5WRQ0 (*C. elegans* GRP170b).

The boundaries for the four major structural subdomains of mouse GRP170 have been analyzed (Easton et al. 2000). The signal peptide corresponds to amino acid #1-3, the ATP

binding domain corresponds to amino acid #33-430, the beta sheet domain corresponds to amino acid #431-600, the alpha helical domain corresponds to amino acid #601-715. To determine the location of subdomains in the GRP170 proteins of other species, the amino acid sequences of the homologues (Mouse, Human, *C. elegans* GRP170a and *C. elegans* GRP170b) were aligned using the Clustal Omega multiple sequence alignment program (Lopez et. al 2014). The identities of boundary amino acids for the subdomains of each GRP170 homologue were predicted based simply on what amino acid of each homologue aligned to the boundary amino acid of the mouse protein. For example, the N-terminal boundary of *C. elegans* GRP170 ATP binding domain corresponds to the leucine at amino acid #32 in mouse GRP170. In the Clustal Omega alignment, the phenylalanine at amino acid #419 of *C. elegans* GRP170a aligned with the mouse leucine at #430. The subdomain boundaries of the GRP170A protein was predicted to be: AA#1-23 signal peptide, 24-419 ATP binding domain, 420-581 beta sheet domain, 582-672 acidic loop, 673-925 alpha helical domain. The subdomain boundaries of the GRP170B protein was predicted to be: AA# 1-26 signal peptide, 27-421 ATP binding domain, 422-562 beta sheet domain, 563-645 acidic loop, 646-921 alpha helical domain.

To identify which subdomains within the *C. elegans* GRP170's had the greatest level of sequence divergence, the sequences of the subdomains of the two *C. elegans* GRP170's and the human GRP170 were aligned against in pairwise alignments using the Clustal Omega multiple sequence alignment program. Clustal Omega uses a substitution matrix that generates scoring matrices that are designed to identify distant evolutionary relationships by generating values such as sequence identity and similarity (Pearson 2014). The program calculates "sequence identity" as the percentage of identical amino acids conserved between aligned sequences. The

Amsterdam University Profile Alignment (PRALINE) multiple sequence alignment application alignment program was I used to evaluate residue hydrophobicity (Simossis and Heringa 2016).

Results

Immunological Analysis

The initial goal of my thesis project was to determine if antibodies raised against vertebrate GRP170 could cross-react with GRP170 from *Caenorhabditis elegans*. I used the Western Blot assay which is routinely used to evaluate antibody specificity and can be used to determine cross species reactivity (Michaud et al. 2003). If an antibody cross reacts with a homologous protein of another taxa, then when tested against total cellular proteins from that taxa, it should generate a single band on the Western Blot corresponding to the size of the target protein. Failure to generate a positive signal would indicate the antibody did not cross react with the homologous protein.

As a positive control for my Western Blot protocol, I used a rabbit antibody raised against the C terminus of human actin (Santa Cruz Biotechnology 2017). The antibody against human actin had previously been reported to cross-react with its nematode protein homologue. The molecular weight of actin in both mouse and *C. elegans* is 42 kDa (Whalen at. al 1976)

The actin antibody bound to two bands of peptides in my mouse extracts (Figure 1). Using whole worm protein extracts in a Western Blot, the anti-vertebrate actin antibody recognized a single 40 kDa protein in *C. elegans* (Figure 1). This was consistent with the manufacturer's report that this antibody cross reacted with *C. elegans* actin (Budde and Roth 2009). I believe the smaller band recoginized in the mouse extract was proteolytic degradation products of the mouse actin – possibly associated with the freezing and thawing of tissue associated with transport extracting of the liver protein. The mouse liver had to be frozen for

transport to Buffalo State. Freezing and thawing may have damaged the liver cells resulting in proteolytic degradation. Additionally, the mouse tissue was ground in pure water with no attempt to inactivate proteases until the sample was boiled in SDS-PAGE loading buffer. In hindsight, a protease inhibitor may have solved this issue; however, it was not used in the experiment. The worms, however, were harvested alive and directly from their culture plates and boiled in SDS-PAGE loading buffer. The lack of detection of smaller proteolytic fragments *in C. elegans* demonstrated that direct extraction of nematode proteins by boiling live worms directly in SDS sample buffer limited proteolysis. This positive control demonstrated that my protein extraction technique, electrophoresis, blotting, and immunodetection strategy could detect cross-phylum recognition of a *C. elegans* protein by an antibody raised against the vertebrate homologue.

There were several commercially available sources of rabbit anti-GRP170 antibodies. Each of these antibodies was generated in research laboratories and then licensed to commercial antibody suppliers to make them available to the scientific community. These GRP170 antibodies were all raised using synthetic polypeptides corresponding to subdomains of human GRP170 protein.

An anti-GRP170 antibody raised against a peptide that shares the greatest similarity to *C. elegans* GRP170 would be the most likely to cross-react with the *C. elegans* proteins. I analyzed the peptides used to raise these antibodies to determine which shared the greatest similarity with nematode GRP170's. The peptide with the greatest similarity to nematode GRP170 corresponded to the rabbit antibody Anti-ORP150 was the antibody used in the Ruan (2013) study (Catalog number bs-4248R, Bioss Antibody Inc, Worburn, MA). The antibody is generated against a KLH

conjugated synthetic peptide derived from human ORP150. The immunogen range is against amino acids 400-450 of the HYOU1 sequence (Figure 2-3) . This immunogen shares 3.0% and 2.9% sequence identity with the *C. elegans* GRP170A and GRP170B proteins respectively. There are several contiguously conserved amino acids in the alignments. GRP170A and the Ruan (2013) antibody share seven identical amino acids between numbers 401 and 409 in the sequence alignment (Figure 2). The GRP170B protein shares 13 identical amino acids between numbers 404-419 in the sequence alignment (Figure 3). The Abbexa antibody immunogen only shares 0.9% and 1.1% sequence identity with the *C. elegans* GRP170A and GRP170B proteins, respectively.

The Ruan (2013) antibody has been demonstrated to cross-react with human, mouse, and rat HYOU1. This antibody was used in a Western Blot experiment to determine the sensitivity of the antibody to mouse and *C. elegans*. To test whether the Ruan antibody raised against mouse GRP170 would cross react with *C. elegans* GRP170 I used the Western Blot analysis to test its specificity. My initial experiments yielded unexpected results. Prior to acquiring the donated mouse liver tissue, the experiment was ran using ground beef extract. The antibody appeared to bind to a high molecular weight protein in beef extract and nematode extracts however major bands of the western blot were the detection of proteins of about 70 kDa. (Figure 4). On internet forum postings, researchers have reported other antibodies cross reacting with a smaller than expected protein (Drupaz 2016). The speculation was that the recognition of a 70 kDa protein might be a cross-reaction with HSP70 proteins or some other 70 kDa protein such as an albumin.

A technical solution suggested on the Research Gate forum to detect larger molecular weight proteins such as the GRP170 protein was to probe just the region of the blot corresponding to proteins greater than 70 kDa (Drupaz 2016). This was achieved by physically cutting off the region of the PVDF membrane corresponding to the lower molecular weight protein and just probing the region of the blot corresponding to higher molecular weight proteins with the antibody (Figure 5). The signal on the blot was faint but the antibody clearly recognized a mouse protein in in the 170 kDa range. This band is presumptively the mouse GRP170. However, the antibody did not react with similar sized proteins in the nematode extracts. This suggests that the antibody against vertebrate GRP170 did not cross react with nematode GRP170. However, there may be alternative explanations for the lack recognition of a high molecular weight protein in *C. elegans*. It is possible that there was some level of cross reaction but it that it was too low to be detected by standard Western Blot procedures. I cannot rule out the possibility that there is simply much less GRP170 in nematodes and there was just too little to detect by Western Blotting.

Analysis of Potential GRP170a and GRP170b Antigens

Based on my Western Blot results it seems unlikely that antibody raised against vertebrate GRP170 will be useful for studying the nematode protein. Therefore, immunological analysis of nematode GRP170 may require producing antibodies directly against the nematode protein. In principle, the same strategy used to generate the vertebrate GRP170 antibody (Hancock and O'Reilly 2005) could be used to generate a nematode GRP170 antibody. Oligo-peptides corresponding to regions of the nematode GRP170 could be synthesized, cross linked and then used as an antigen for production of polyclonal antibodies in rabbits. One advantage of

generating antibodies directly against nematode GRP170 peptides is that it should be possible to generate isoform-specific antibodies. An isoform-specific antibody would only bind to epitopes of one isoform (GRP170a or GRP170b) but not the other isoform. Because isoform specific antibodies could distinguish between the two GRP170's, they would be powerful tools to characterize and differentiate the functions of the two isoforms. The two *C. elegans* isoforms have diverged almost as much from each other, 37% sequence identity, as the nematodes GRP170's have diverged from the vertebrate protein, 33% sequence identity (Table 6). With this degree of sequence divergence, it should be possible to identify peptides within of the nematode proteins that will provide isoform specific epitopes for antibody generation/recognition.

Sequence Analysis

I analyzed the sequences of the two *C. elegans* GRP170 isoforms to identify peptide regions within the proteins with high levels of sequence divergence. My strategy was initially to analyze the individual functional domains within the larger protein. The rational is that the specific function of each domain would differentially constrain sequence divergence. This had been already documented for the subdomains of the HSP70 chaperone superfamily and GRP170 (Easton et al. 2000). The major subdomains of GRP170 include the signal peptide, alpha helical domain, ATP binding domain, beta basket, and the acidic loop domain. The signal peptide was not included in this analysis because it is cleaved from the protein after import into the ER and is not part of the mature GRP170. Bracher and Verghese (2015) had previously determined the boundaries of each functional domain within the mouse protein. These subdomain boundaries within the mouse protein were used to identify boundaries in the *C. elegans* GRP170's. Each of the four subdomains of the *C. elegans* GRP170 isoforms were individually aligned and the

percent sequence identity was determined (Table 2) (Lopez et. al 2013). Percent sequence identity represents the percent of identical amino acids shared between two aligned sequences. Of the four functional domains of the nematode proteins, the ATP binding domain was the most conserved with 54% sequence identity and the loop domain was the least conserved with only 22% sequence identity. These data suggest that the loop domain might provide the most likely peptide sequence for isoform specific antigens, however the two proteins are very diverged from one another and an isoform specific antigen may very well be found in another domain of the protein. Upon further investigation, sequence alignment indicated there were unique insertions in the beta sheet domain that could serve as epitopes for isoform-specific antibody recognition. These are the peptide regions that I recommend as antigens for isoform-specific antibodies.

Hydrophilic peptides are much more antigenic than hydrophobic ones (Hopp and Woods, 1981). Fortunately, the beta sheet domain was rich in hydrophobic amino acids (Figure 6). Additionally, insertion/deletion regions represent especially good targets isoform specific epitopes (Scherer et al. 1995). I identified corresponding peptides from the *C. elegans* beta sheet domains that were composed of mostly hydrophilic amino acids. For GRP170A, the region corresponded to AA 498-528 (SVALKYGKIESFTKQQVQEIG. This peptide shares 0.8% identity with GRP170B) (Table 3) (Figure 6). For GRP170b the region corresponded to AA 535-550 (KDAIEKEVTDENSVLKGVKTT) (Table 3) (Figure 6). This peptide shares 0.9% identity with GRP170A (Table 3.)

Discussion

Using a standard Western Blot assay I could not detect any cross reaction between an anti-vertebrate GRP170 antibody and nematode GRP170. The simplest explanation for this result was that the nematode GRP170s have diverged so significantly that they did not share the epitopes that the antibody recognized on the vertebrate protein. The Ruan (2013) antibody was chosen for this analysis because it was generated by a peptide that shared greater sequence identity with the nematode GRP170's compared to peptides used to generate other antibodies (Table 3). The Ruan (2013) antibody shared 3.0% and 2.9% sequence identity with the GRP170A and GRP170B proteins, respectively. However, this level of sequence conservation may be insufficient to include shared epitopes. Antibodies that were not chosen included antibodies that were generated against the N-terminus of the human GRP170 protein, as these antibodies shared most of their sequence homology with the worm protein in the leader signal sequence, which is cleaved by proteases (Wolfe et al. 1983).

An alternative explanation for my negative results was that there were insufficient levels of GRP170 protein in *C. elegans* to detect by Western Blotting. The signal on the Western Blot for the positive control mouse liver GRP170 was very faint. If the level of GRP170 protein was significantly lower in *C. elegans*, than the worm GRP170 may not be detectable under these Western Blot conditions. There have been no reported attempts to measure levels of GRP170 in nematodes, so it is possible that levels of the chaperone are low. One characteristic of GRP170 across taxa is that its protein levels are induced by stress associated with the Unfolded Protein Response (Lee 2001). Although GRP170 protein induction has not been assayed in nematodes,

at the mRNA level the gene encoding GRP170b is reported to be induced greater than six-fold during the UPR (Rockwell 2014). It might be worthwhile to repeat these Western Blot assays using protein extracted from worms induced for the UPR to see if induction of GRP170 would allow it to be detected by Western Blotting.

A third explanation for my negative results was that the *C. elegans* GRP170 protein was exposed to proteolytic degradation during extraction. I did not include a mixture of protease inhibitors in the extraction solution that some labs include during extraction (Bhaskaran et al. 2011). If the *C. elegans* protein had been proteolytically cleaved, it might not have been detected on the high molecular weight region of the blot. There was evidence that the mouse samples had suffered some proteolytic degradation. In the control actin blot, several smaller peptides were detected by the anti-actin antibody which were presumably proteolytical fragments. The proteolysis may have occurred during the freeze thawing of the tissue or because of its direct extraction into water. It is noteworthy that no similar proteolysis of *C. elegans* actin was observed. This is likely because the worms were not frozen prior to extraction nor were they extracted into water. Instead the nematode proteins were extracted directly from live worms into 100°C denaturing SDS buffer. These harsh conditions would have denatured the proteases before they could have cleaved of worm proteins. The single 40kDa band detected by the actin antibody on the Western Blot in the worm proteins suggest this extraction technique effectively minimizes proteolysis for the worm proteins. Therefore, I do not believe that lack of detection of GRP170 in the worm samples was due to proteolysis of the worm GRP170.

Proteolysis could be the explanation for the lower molecular weight proteins detected by the anti-GRP170 antibody. However, these lower molecular weight proteins were observed both in the mouse and the worm samples. If the strategy for extracting the worm proteins prevented proteolysis, then the bands associated with lower molecular weight bands are more likely to be due to cross reaction of the antibodies with other proteins.

An important control in my experimental design was the use of an anti-actin antibody. This control was chosen because the antibody raised against human actin had been previously reported to cross react with the nematode protein (Budde and Roth 2009). As expected, this antibody specifically recognized a single protein of the approximate molecular weight of actin in the nematode (43kDa). This result confirms that the standard Western Blot conditions I used could detect cross-phylum protein homologues. Taxa cross-reaction of anti-actin antibody is not surprising given the high degree of amino acid identity between vertebrate and nematode actin. Rabbit and nematode actin have sequence homology of 93.6% sequence identity. With such a high sequence homology, it is likely that there would be cross-taxa antibody binding

It appears the anti-vertebrate GRP170 will not be useful to study the two-isoform chaperone system in nematodes. However, given the potential value of immunological assays for investigating the roles of these proteins, I propose it is worth producing antibodies directly against nematode GRP170. These antibodies will be of even more valuable if they are specific to individual isoforms of GRP170. Antibodies are generated using antigens that trigger an immune response in the source animal. The blood serum is then collected from the animal and the antibodies purified (Hanly et. al, 1995). To generate anti *C. elegans* GRP170 antibodies,

source animals could be immunized with the full-length GRP170 as the antigen. However, nematodes are not an ideal system for isolated large quantities of purified protein. A more cost effect route would be to synthesize peptides based on the predicted amino acid sequence of the GRP170 isoforms. Using a synthetic *C. elegans* GRP170 peptide would be cheaper and require less effort to obtain than purifying the native protein. Therefore, this is the approach to GRP170 antibody generation I would recommend.

When using synthetic peptides to generate antibodies, selecting the sequence of the peptide antigen is the most critical step. There are several characteristics of the peptides that should be considered to maximize the likelihood of success in producing antibodies (Van Regenmortel and Muller 1999). If the protein is going to be studied in its native state, it would be wise to choose a peptide sequence that is found on the surface of the native protein. If the protein is going to be denatured, one should select a sequence that is found on the linearized protein. These sequences should contain both hydrophilic and hydrophobic residues, and preferably have antigenic amino acids such as cysteine, leucine, and valine (Van Regenmortel and Muller 1999).

Future Research Prospects

GRP170A and GRP170B have diverged so significantly that it should be possible to generate isoform specific antibodies. I have identified two potential peptide antigens. The next step in studying nematode GRP170 should be to generate isoform specific antibodies. There are commercial providers of peptides. The antibodies could be generated at Buffalo State College, or by one of several outside vendors specializing in antibody production such as GenScript or Pacific Immunology. Upon obtaining antibodies, it should initially be tested to confirm that it

recognizes GRP170. More importantly, the antibodies should be tested to see if they recognize isoform specific epitopes. Li (2014) mutant strains lacking *grp170a* or *grp170b* could be used to confirm that the antibodies are isoform specific. If the antibodies are isoform specific, then the antibody raised against GRP170a peptides should not recognize a protein in the strain lacking the grp170a and the antibody raised against GRP170b peptides should not recognize a protein in the strain lacking the grp170b.

Tables

Table 1: Sequence Conservation within Subdomains of Mouse and *C. elegans* GRP170a and GRP170b

GRP170 Subdomains	Mouse GRP170 vs. *C. elegans* GRP170A	Mouse GRP170 vs. *C. elegans* GRP170B	*C. elegans* GRP170A vs. *C. elegans* GRP170B
Signal Peptide	30%[1]	19%	24%
NBD	47%	46%	54%
B-basket	29%	27%	32%
Acidic Loop	22%	20%	26%
Alpha Helical	24%	24%	22%

[1] Percent sequence identity. The sequences of individual subdomains of Mouse GRP170 and *C. elegans* GRP170A and GRP170b were aligned and percent sequence identities were calculated using EMBOSS stretcher (Hancock and Bishop 2004).

Table 2: Comparison of Amino Acid Sequence Conservation between Mouse and *C. elegans* Actin and GRP170.

	Mouse Actin	Mouse GRP170	*C. elegans* GRP170A
C. elegans Actin	96%[1]	-	-
C. elegans GRP170A	-	33%	-
C. elegans GRP170B	-	33%	37%

[1] Percent sequence identity. Mouse and *C. elegans* Actin and GRP170 sequences were aligned and percent sequence identities were calculated using the ClustalW algorithm (Lopez et al. 2014).

Table 3: Comparison of Amino Acid Sequence Conservation between Vertebrate Peptides used to Raise GRP170 Antibodies and the *C. elegans* GRP170 Isoforms.

	Abbexa Antibody Immunogen	Bioss Antibody Immunogen
GRP170A	0.9%[1]	3.0%
GRP170B	1.1%	2.9%

[1] Percent sequence identity. *C. elegans* whole protein sequences and antibody peptide sequences were aligned and percent sequence identities were calculated using the EMBOSS needle algorithm (Hancock and Bishop 2004).

Table 4: Sequence Comparison of Selected Peptides for Isoform-Specific Antibody Production for GRP170a and GRP170b and the Entire Protein Sequences of *C. elegans* GRP170a and GRP170b.

	C. elegans GRP170A peptide	*C. elegans* GRP170B peptide
C. elegans GRP170A	2.3%[1]	0.8%
C. elegans GRP170B	0.9%	2.3%

[1] Percent sequence identity. *C. elegans* whole protein sequences and selected peptide sequences were aligned and percent sequence identities were calculated using the EMBOSS needle algorithm (Hancock and Bishop 2004).

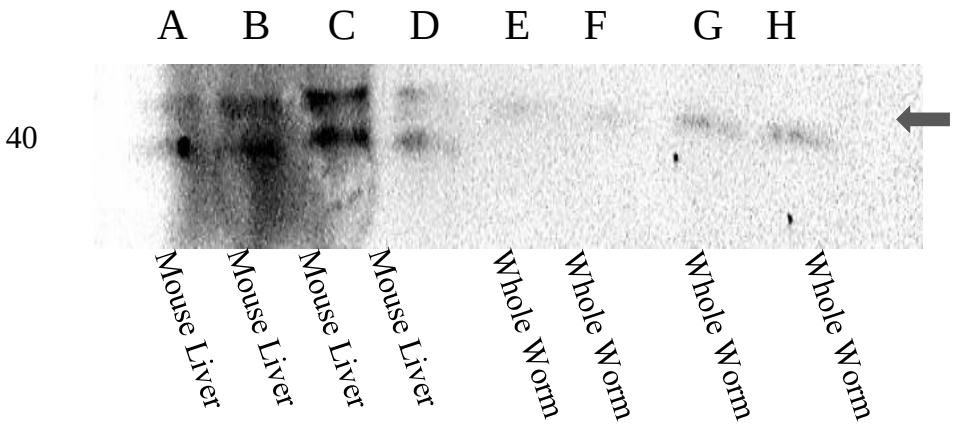


Figure 1: Anti-vertebrate Actin Antibody Cross Reacts with *C. elegans* Actin. Protein extracts of Mouse Liver (Lanes A-D) and mouse liver (Lanes E-F) were analyzed by Western Blot analysis. Two quantities of protein extracts were loaded on the gel, 10 μg (lanes A, B, E and F) and 20 μg (Lanes C, D, G and H). Proteins were separated by SDS electrophoresis and transferred to PVDF membranes. Membranes were probed with a rabbit anti-human GRP polyclonal antibody followed by a horseradish peroxidase conjugated secondary antibody. Position of 40 kDa molecular weight standard is indicated on the left. Antibody binding was detected by chemiluminescence. The arrow indicates the binding of the anti-vertebrate actin antibody to 40 kDa worm actin.

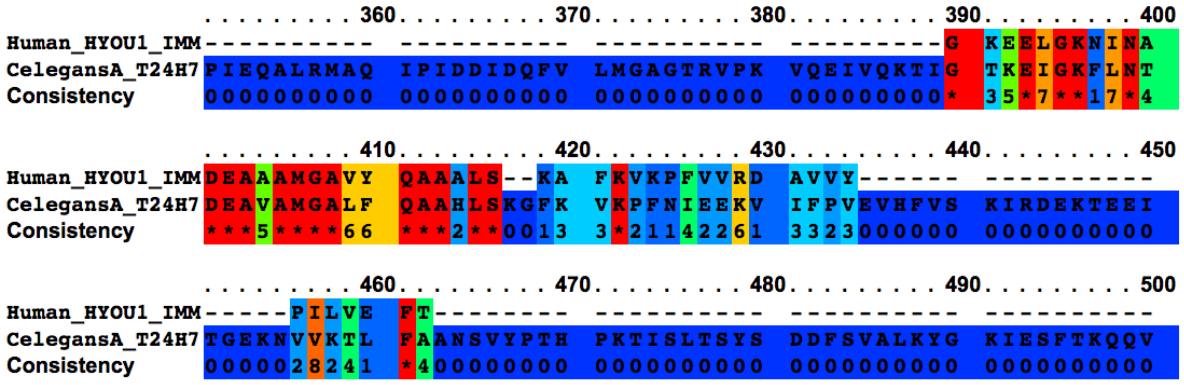


Figure 2: Alignment of the peptide sequence used to make the Ruan anti-vertebrate GRP170 antibody with the sequence of *C. elegans* GRP170a. Sequences were aligned using the PRALINE algorithm. Color indicates the chemical similarity of the aligned amino acid residues with Red indicating identity and blue indicate chemical dissimilarity. Consistency values 0-9 also indicate degree of chemical similarity with 0 being most dissimilar and 9 being most similar. A region of approximately 15 residues of the Ruan peptide showed a high degree of similarity.

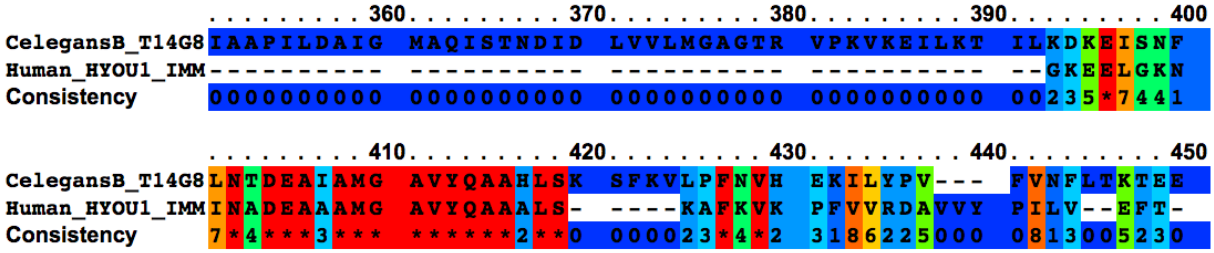


Figure 3: Alignment of the peptide sequence used to make the Ruan anti-vertebrate GRP170 antibody with the sequence of *C. elegans* GRP170b. Sequences were aligned using the PRALINE algorithm. Color indicates the chemical similarity of the aligned amino acid residues with Red indicating identity and blue indicate chemical dissimilarity. Consistency values 0-9 also indicate degree of chemical similarity with 0 being most dissimilar and 9 being most similar. A region of approximately 15 residues of the Ruan peptide showed a high degree of similarity.

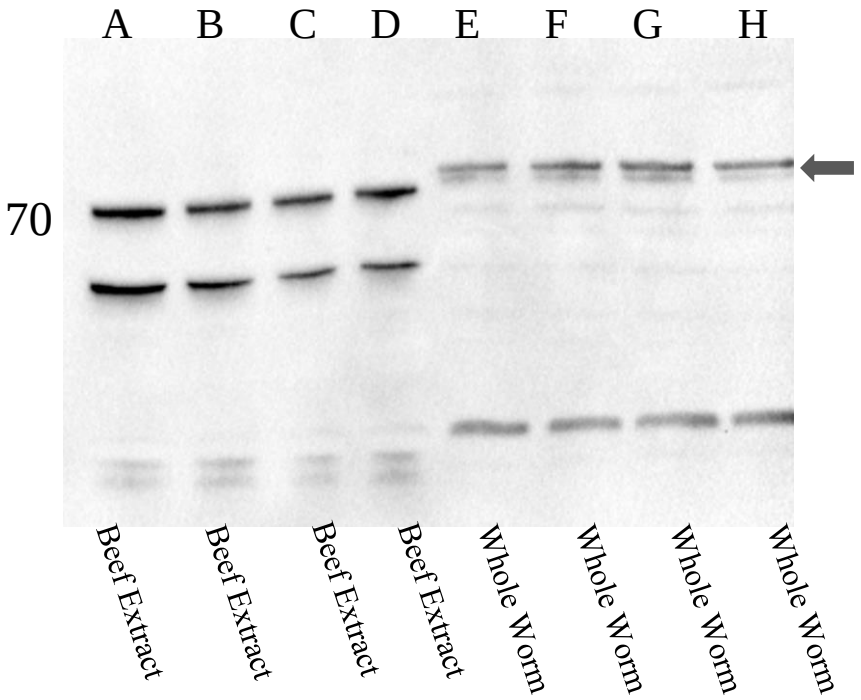


Figure 4: Anti-vertebrate GRP170 detects proteins in *C. elegans* and beef extract on a Western Blot. Protein extracts of beef extract (Lanes A through D) and whole worm protein extract (Lanes E through H) 10 μg were loaded on the gel, analyzed by Western Blot analysis. Proteins were separated by SDS electrophoresis and transferred to PVDF membranes. Position of molecular weight standards (kDa) is indicated on the left. Membranes were probed with a rabbit anti-human GRP polyclonal antibody followed by a horseradish peroxidase conjugated secondary antibody. Antibody binding was detected by chemiluminescence. The arrow indicates the binding of the anti-human GRP170 antibody to a high molecular weight protein in the whole worm protein extracts.

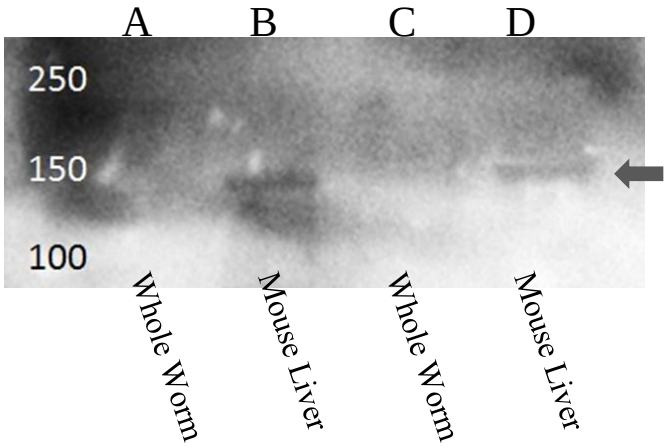
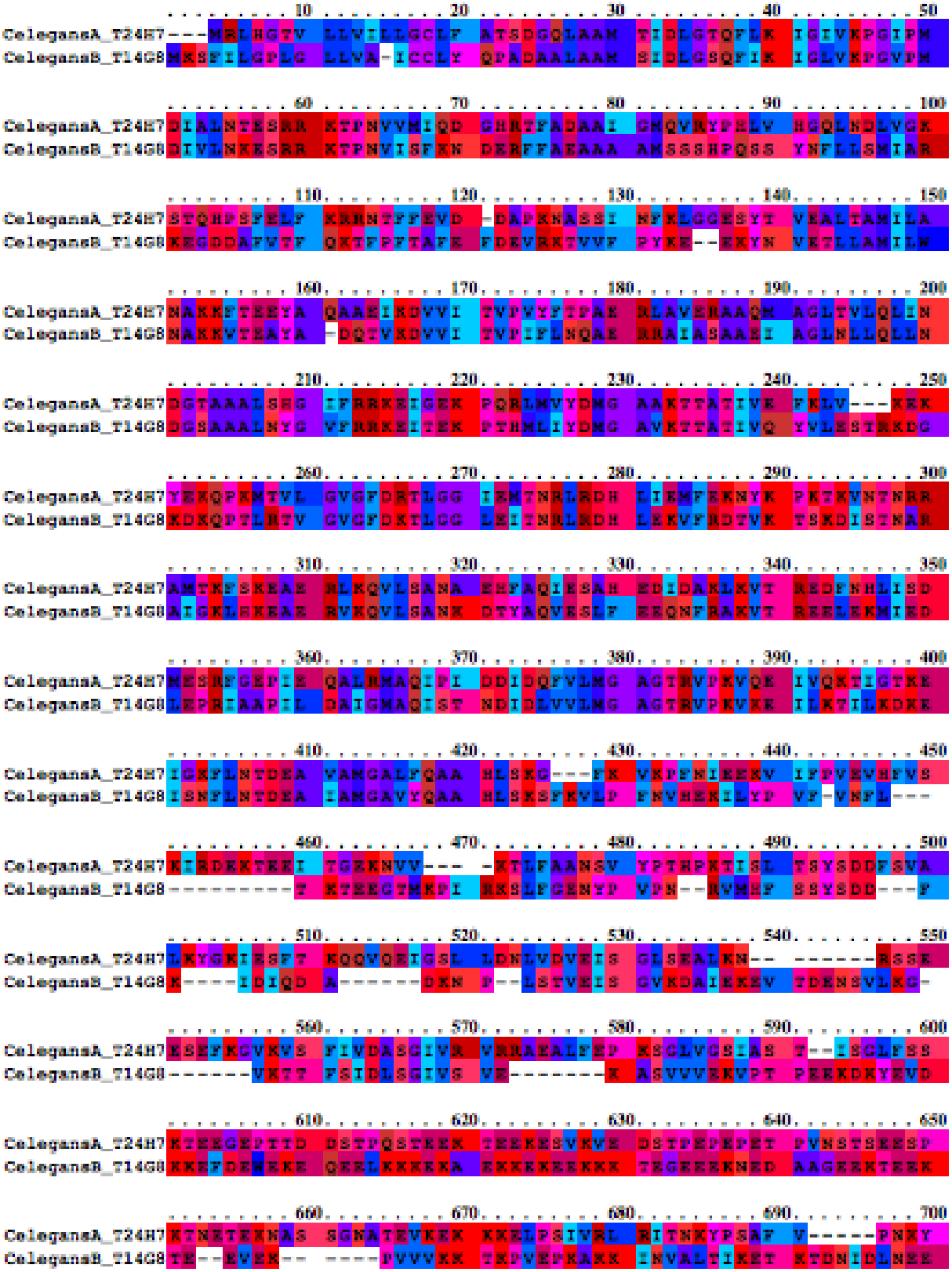


Figure 5: Anti-vertebrate GRP170 antibody fails to detect *C. elegans* GRP170 on a Western Blot. Protein extracts of whole worms (Lanes A and C) and mouse liver (Lanes B and D) 10 μg were loaded on the gel, analyzed by Western Blot analysis. Proteins were separated by SDS electrophoresis and transferred to PVDF membranes. Position of molecular weight standards (kDa) is indicated on the left. Membranes were probed with a rabbit anti-human GRP polyclonal antibody followed by a horseradish peroxidase conjugated secondary antibody. Antibody binding was detected by chemiluminescence. The arrow indicates the binding of the anti-human GRP170 antibody to a high molecular weight protein in the mouse liver extracts. Binding of anti-human GRP170 antibody to a large molecular weight protein in whole worm extracts was not detected.


CelegansA_T24H7
CelegansB_T14G8

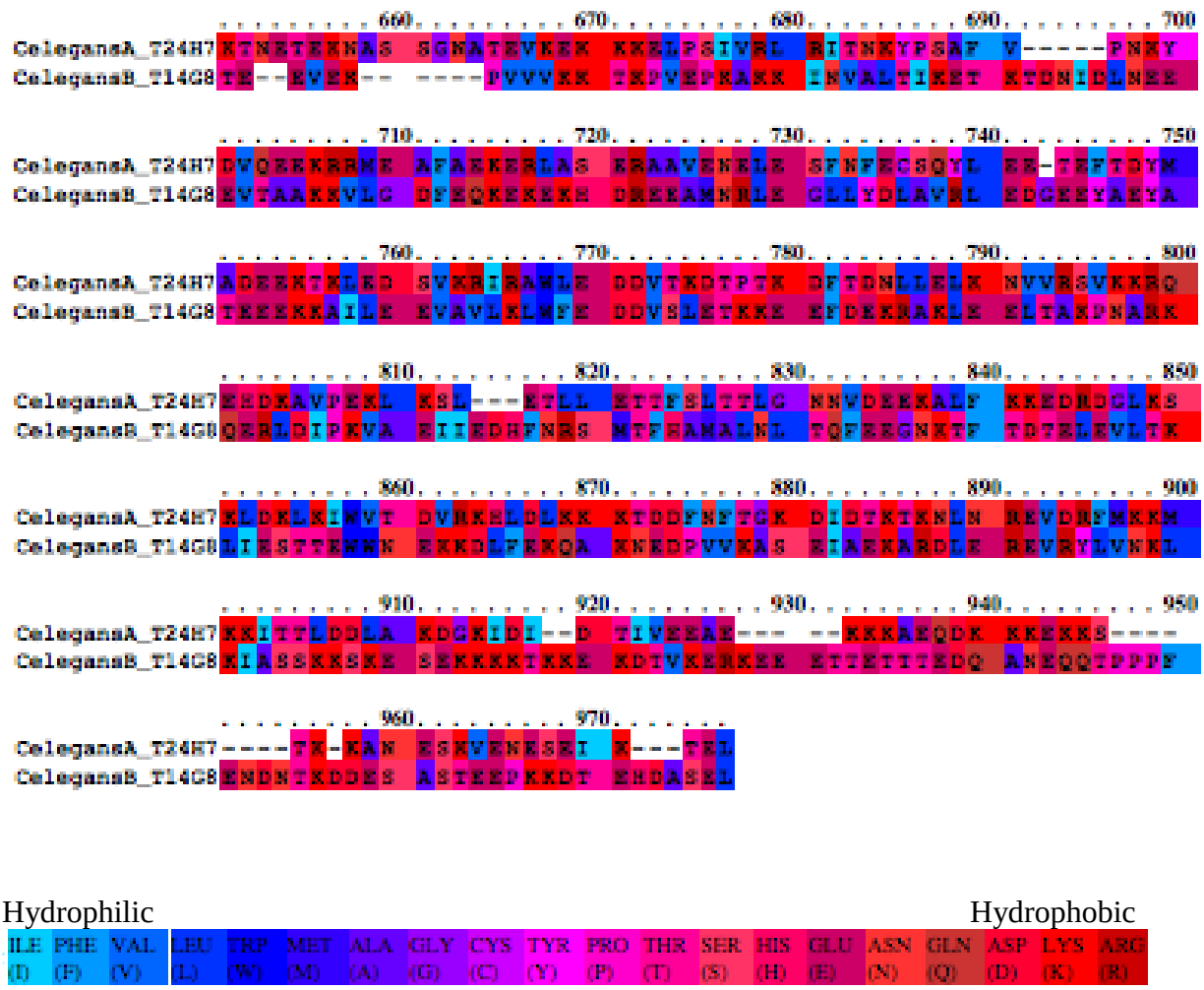


Figure 6: Distribution of Hydrophilic Amino Acids within *C. elegans* GRP170 isoforms. The *C. elegans* genes GRP170a (T24H7) and GRP170b were alignment using the PRALINE algorithm and the relative hydrophobicity of amino acids indicated by color with light blue being the most hydrophilic and red being the most hydrophobic. The key to color coding of amino acids is included.

Works Cited

Andreasson C, Rampelt H, Fiaux J, Druffel-Augustin S, Bukau B. 2010. The endoplasmic reticulum GRP170 acts as a nucleotide exchange factor of HSP70 via a mechanism similar to that of the cytosolic HSP110. J Biol Chem. 285(16):12445-12453.

Behnke J, Hendershot LM. 2013. The large HSP70 GRP170 binds to unfolded protein substrates in vivo with a regulation distinct from conventional HSP70s. J Biol Chem. 289(5):2899-2907.

Behnke J, Feige MJ, Hendershot LM. 2015. BiP and it's nucleotide exchange factors GRP170 and SIL1: Mechanisms of action and biological functions. J Mol Biol. 427(7):1589-1608.

Bhaskaran S, Butler JA, Becerra S, Fassio V, Girotti M, Rea SL. 2011. Breaking *C. elegans* the easy way using the Balch Homogenizer: An old tool for a new application. Anal Biochem. 413(2):123-132.

Bracher A, Verghese J. 2015. The nucleotide exchange factors of Hsp70 molecular chaperones. Front Mol Biosci. 2(1):10.

Bradford M. 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem. 72(1):248-254.

Brignull HR, Morley JF, Morimoto RI. 2007. Molecular aspects of the stress response: chaperones, membranes and networks. New York (NY): Springer Science.

Budde MW, Roth MB. 2009. Hydrogen sulfide increases hypoxia-inducible factor 1 activity independently of the Von Hippel-Lindau tumor suppressor 1 in *C. elegans*. Mol Biol Cell. 21(1):212-217

Bukau B, Horwich AL. 1998. The HSP70 and HSP60 chaperone machines. Cell. 92(3):351-366.

Chen G. 2002. Discordant protein and MRNA expression in lunch adenocarcinomas. Mol Cell Proteomics. 1(4):304-313.

Drupaz S. 2016. Why is my 150kDa protein showing up at around 70kDa in SDS-PAGE, western blot? [Msg 7]. Message posted at https://www.researchgate.net/post/Why_is_my_150kDa_protein_showing_up_at_around_70kDa_in_SDS-PAGE_Western_Blot

Easton DP, Kaneko Y, Subjeck JR. 2000. The HSP110 and GRP170 stress proteins: Newly recognized relatives of the HSP70s. Cell Stress and Chaperones. 5(4):276.

Emerson, JJ. 2004. Extensive gene traffic on the mammalian X chromosome. Science 303(5657):537-540.

Geysen, HM, Barteling SJ, Meloen. 1985. Small peptides induce antibodies with a sequence and structural requirement for binding antigen comparable to antibodies raised against the native protein. Proc Nat Acad Sci. 82(1):178-182.

Guo Y, Xiao P, Lei S, Deng F, Xiao GG, Liu Y, Chen X, Li L, Wu S, Chen Y, Jiang H, Tan L, Xie J, Zhu X, Liang S, Deng H. 2008. How is MRNA expression predictive for protein expression? A correlation study on human circulating monocytes. J Biochem Biophys Sci. 40(5):426-436.

Gupta A, Dong A, Lowe AW. 2011. AGR2 gene function requires a unique endoplasmic reticulum localization motif. J Biol Chem. 287(7):4773-4782.

Hancock DC, O'Reilly NJ. 2005. Synthetic peptides as antigens for antibody production. Immunochem Protocols. 13-25.

Hancock JM, Bishop MJ. EMBOSS, the European molecular biology open software suite. Dict Bioinform Comp Biol. 2014.

Hanly WC, Artwohl JE, BT Bennett. 1995. Review of polyclonal antibody production procedures in mammals and poultry. ILAR J. 37(3):93-118.

Harris TW, Antoshechkin I, Bieri T. 2009. WormBase: a comprehensive resource for nematode research. Nuc Acid Res. 38(1):463-467.

Hopp TP, Woods KR. 1981. Prediction of protein antigenic determinants from amino acid sequences. Proc Nat Acad Sci. 78(6):3824-3828.

Ikeda J, Kaneda S, Kuwabara K. 1997. Cloning and expression of cDNA encoding the human 150 kDa oxygen-regulated protein, ORP150. Biochem Biophys Res Comm. 230(1):94-99.

Ko, Rim H, Chang YS. 2016. Opposing roles of the two isoforms of ERBB3 binding protein 1 in human cancer cells. Int J Cancer. 139(6):1202-1208.

Kolaskar AS, Tongaonkar PC. A semi-empirical method for prediction of antigenic determinants on protein antigens. FEBS Letters. 276(1-2)172-174.

Lee AS. 2001. The glucose-related proteins: Stress induction and clinical applications. Trend Biochem Sci. 26(8):504-510.

Li, Xumei, Zhang K, Li Z. 2011. Unfolded protein response in cancer: The physician's perspective. J Hematol Oncol. 4(1):8.

Li L, Ernsting BR, Wishart MJ, Lohse DL, Dixon JE. 1997. A family of putative tumor suppressors is structurally and functionally conserved in humans and yeast. J Biol Chem. 272(47):29403-29406.

Li Y. 2015. Distinct physiological roles for the two isoforms of the ER chaperone GRP170 in *Caenorhabditis elegans* [thesis]. State University of New York College at Buffalo.

Lipman NS, Jackson LR, Trudel LJ, Weis-Garcia F. 2005. Monoclonal versus polyclonal antibodies: Distinguishing characteristics, applications, and information resources. ILAR J. 46(3):258-268.

Lopez R, Cowley A, Li W, McWilliam H. 2014. Using EMBL-EBI services via web interface and programmatically via web services. Curr Protocol Bioinfo. 48(3):12-15.

Van Regenmortel MHHV, Muller S. 1999. Synthetic peptides as antigens. Amsterdam (NL): Elsevier.

Michaud GA, Salcius M, Zhou F, Bangham R, Bonin J, Guo H, Snyder M, Predki PF, Schweitzer BI. 2003. 21(12):1509-1512.

Moresco JJ, Carvalho PC, Yates JR. 2010. Identifying components of protein complexes in *C. elegans* using co-immunoprecipitation and mass spectrometry. J Proteom. 73(11):2198-2204.

Nikolaidis N, Nei M. 2003. Concerted and nonconcerted evolution of the HSP70 gene superfamily in two sibling species of nematodes. Mol Biol Evol. 21(3):498-505.

Park J, Easton DP, Chen X, MacDonald IJ, Wang XY, Subjeck JR. 2003. The chaperoning properties of mouse GRP170, a member of the third family of HSP70 related proteins. Biochem. 42(50):14893-14902.

Pearson WR. 2013. Selecting the right similarity scoring matrix. Curr Prot Bioinfo.

Rockwell A. 2015. Differential regulation of the two grp170 paralogues of *Caenorhabditis elegans* [thesis]. State University of New York College at Buffalo.

Ruan L, Huang HS, Jin WX, Chen HM, Li XJ, Gong QJ. 2013. Tetrandrine attenuated cerebral ischemia/reperfusion injury and induced differential proteomic changes in a MCAO mice model using 2-D DIGE. Neurochem Res. 38(9):1871-1879.

Rutkowski, Thomas D, Kaufman R. 2004. A trip to the ER: Coping with stress. Trends Cell Bio. 14(1):20-28.

Scherer PE, Tang Z, Chun M, Sargiacomo M, Lodish HF, Lisanti MP. 1995. Caveolin isoforms differ in their N-terminal protein sequence and subcellular distribution. Identification and epitope mapping of an isoform-specific monoclonal antibody probe. J Biol Chem. 270(27):16395-16401.

Shaner L, Morano KA. 2007. All in the family: Atypical HSP170 chaperones are conserved modulators of HSP70 activity. Cell Stress and Chaperones. 12(1):1.

Simossis VA, Heringa J. 2005. PRALINE: A multiple sequence alignment toolbox that integrates homology-extended and secondary structure information. Nuc Acids Res. 33(1):289-294.

Stein LD, Bao Z, Blasiar D. 2003. The genome sequence of *Caenorhabditis briggsae*: A platform for comparative genomics. PLoS Biol. 1(2).

Stiernagle T. 2006. Maintenance of *C. elegans* [Internet]. Minneapolis (MN): University of Minnesota [cited 2017 Aug 6]. Available from https://www.ncbi.nlm.nih.gov/books/NBK19649

Stormo GD. 2009. An introduction to sequence similarity (homology) searching. Curr Protocols in Bioinfo.

Taniguchi Y, Choi PJ, Li GW, Chen H, Babu M, Hearn J, Emili A, Xie XS. 2010. Science. 329(5991):533-538.

Tyson J, Stirling C. 1997. A novel subfamily of HSP70s in the endoplasmic reticulum. Trends in Cell Biol. 7(7):277-282.

UniProt: A hub for protein information. Nuc Acids Res. 43(1).

Wang XY, Arnouk H, Chen X, Kazim L. 2006. Extracellular targeting of endoplasmic reticulum chaperone glucose-regulated protein 170 enhances tumor immunity to a poorly immunogenic melanoma. J Immunol. 177(3):1543-1551.

Whalen RG, Butler-Browne GS, Gros F. 1976. Protein synthesis and actin heterogeneity in calf muscle cells in culture. PNAS. 73(6):2018-2022.

Williams EJB, Pal C, Hurst LD. 2000. The molecular evolution of signal peptides. Gene. 253(2):313-322.

Wolfe PB, Zwizinski C, Wickner W. 1983. Purification and characterization of leader peptidase from Escherichia coli. Met Ezymol. 40-46.