GLUCOSE REGULATED PROTEIN AND HEAT SHOCK PROTEIN EXPRESSION IN HIBERNATING MAMMALS

By

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Abstract

During mammalian hibernation, most physiological activities are dramatically suppressed, but selected genes are up-regulated to provide protein products that protect cells and organs for long term survival in the hypometabolic, hypothermic state. In this study, the roles of chaperone proteins including glucose regulated proteins and heat shock proteins were assessed in two species: thirteen-lined ground squirrels, *Spermophilus tridecemlineatus,* and little brown bats, *Myotis lucifugus*. RT-PCR and Western blot techniques were used to examine gene and protein expression. Compared with euthermic control squirrels and bats, glucose regulated protein 75 (Grp75), Grp94 and Grp170 were elevated in some tissues at the mRNA and/or protein levels with organ-specific patterns of response by *grp* transcripts and GRP protein. The up-regulation of *grp* mRNA may be important for rapidly elevating the protein content of these chaperones during hibernation and arousal; elevated GRP protein would then aid in the folding of other proteins that are newly synthesized during hibernation and/or in the renaturation of proteins that become misfolded at low body temperatures. Heat shock proteins (Hsps) including Hsp40, Hsp72, Hsp73 and Hsp90 were elevated in some tissues of hibernating ground squirrels and bats. Analysis of partial amino acid sequences of Grps and Hsps showed very high identities (88-100%) compared with human or mouse sequences which indicates similar structures and functions of Grps and Hsps among mammalian species. The data support the idea that Grps and Hsps are up-regulated during hibernation to function as chaperones to bind non-native polypeptides and suppress protein aggregations caused by low temperature during hibernation.

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List of Abbreviations

2D-PAGE	Two-dimensional polyacrylamide gel electrophoresis
ATF	activating transcription factor
ATP	adenosine triphosphate
BAT	brown adipose tissue
bHLH	basic helix-loop-helix
BLAST	Basic local alignment search tool
BZIP	basic leucine zipper
cDNA	complementary DNA
CMRS	Canadian Molecular Research Services
DEPC	diethylpyrocarbonate
DjA4	another type of Hsp40
DnaJ	*Escherichia coli* Hsp40 protein
DnaK	*Escherichia coli* Hsp70 homologue
ECL	enhanced chemiluminescence
EDTA	ethylenediamine tetraacetic acid
eEF2	eukaryotic elongation factor 2
EGTA	ethylenebis(oxyethylenenitrilo)tetraacetic acid
eIF2	eukaryotic initiation factor 2
ER	endoplasmic reticulum
ERSE	ER stress-response element
EtBr	ethidium bromide
GD	glucose deprivation

gp96	grp94
GR	glucocorticoid receptor
GRP94	94 kDa glucose-regulated protein
GTP	guanosine triphosphate
HEPES	N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid)
HIF-1	hypoxia-inducible transcription factor 1
Hop	Hsp organizing protein
HPD motif	canonical tripeptide motif His-Pro-Asp
HRE	hypoxia response elements
hsc70	heat-shock cognate protein 70
HSE	heat shock element
HSF	heat shock factor
HSP	heat shock protein
kb	kilobase
$MLC1_v$	myosin light chain 1
MOPS	2-[N-morpholine] proanesulfonic acid
mRNA	messenger RNA
mthsp70	mitochondrial hsp70
NLS	nuclear localization signal
OGD	oxygen-glucose deprivation
ORF	open reading frame
ORP	oxygen regulated protein
PCR	polymerase chain reaction

PDK	pyruvate dehydrogenase kinase
pI	isoelectric point
PMSF	phenylmethanesulfonyl fluoride
PVDF	polyvinylidene difluoride
RNP	ribonucleoprotein
RT	room temperature
RT-PCR	reverse transcription-polymerase chain reaction
SDS	sodium dodecyl sulfate
TAE	Tris-acetate-EDTA buffer
TEMED	N,N,N',N'-tetramethylethylenediamine
TPR	tetratricopeptide repeat
UPR	unfolded protein response
UPRE	unfolded protein response element
WAT	white adipose tissue
Ydj1	one member of Hsp40 family
ZBD	zinc-binding domains
ZFLR	zinc finger-like region

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CHAPTER 1

GENERAL INTRODUCTION

1.1. Hibernation

During the winter, many small mammals adopt hibernation to protect themselves from the cold temperatures and the scarcity of food. Hibernation is a state in which physiological and metabolic activities are greatly depressed (Storey, 2002). There are three major characteristics of torpor including a profound reduction of metabolic rate, greatly reduced physiological functions (including heart rate, breathing, etc.) and extremely low body temperature (Tb) (Wang and Lee, 2000). Hibernators drop their Tb to near ambient temperature and can achieve energy savings of as much as 90% compared with the energy that they would otherwise spend to remain euthermic (Tb ~ 37°) over the winter (Wang and Lee, 1996). Some species such as the Arctic ground squirrel can even maintain Tb below 0°C for up to 3 weeks (Barnes, 1989). In the case of hibernating ground squirrels, the heart rate drops to only 5-10 beat per minute compared with the euthermic value of 350-400 beats per minute (Storey, 2003). Breathing is also greatly reduced from ~40 to about 1 breath per minute (Milsom, 1992). Metabolic rate in torpor can drop to just 1-5% of the corresponding resting rate in euthermia (Storey, 2003).

Thirteen-lined ground squirrels (*Spermophilus tridecemlineatus*) have become one of the favoured model organisms for studies of the molecular basis of mammalian hibernation. In nature, thirteen-lined ground squirrels exhibit a strict rhythm of reproduction, fattening, and hibernation each year (Fig.1) (Kenagy et al., 1989). The cycle includes mating, gestation, and birth in the spring. Then the young babies gain sufficient body mass in the summer to prepare themselves for the severe cold winter. All animals must put on enough fat to supply their fuel needs throughout the winter, usually

doubling their body weight by the end of summer. In the fall, the animals disappear into their burrows to hibernate through the whole winter until the next spring. Over the winter months, these mammals spend most of their time in deep torpor with their Tb near ambient. They don't eat or drink. Periodically they arouse from torpor, re-warming themselves to core Tb values near 37°C. The interbout arousal period usually lasts from 20 minutes to 2 hours before the animals begin to sink into torpor again, with their Tb stabilizing at a low value after about 1 day. The arousals are very energy-expensive and, indeed, they can account for ~90% of the total energy consumption over the hibernation season (Wang, 2000). Lyman *et al.* (1982) postulated that periodic arousals must be essential for both hibernation and survival because otherwise continuous torpor would save much more energy. However, to date, the true purpose of periodic arousals from hibernation is not known.

The little brown bat (*Myotis lucifugus*) is a small insectivorous species. Their body weight is only 7-11 g and they are found across most of North America (Fenton and Barclay, 1980). In winter, this species finds caves and mines in which to hibernate in large numbers. In New England, bats begin to arrive at swarming sites in July and enter hibernation in early to mid-October (Kunz and Anthony, 1996). During the prehibernation period between leaving the maternity roosts and entering hibernation, bats feed nightly and enter torpor during the day in order to fatten for the hibernation (Kronfeld-Schor *et al.*, 2000). *M. lucifugus* often hibernates for 8-9 months of the year.

Hibernators keep their blood circulation and respiration well maintained during hibernation, although at much lower rates than normal (Wang *et al.*, 2002). Non-hibernators, such as humans, will die when core Tb falls to low values because some very

important physiological activities are halted; for instance, the shivering response stops at a Tb of 30-32°C, the heart fibrillates at 27-29°C, and ventilation stops at 23-27°C (Hirvonen, 1979; Ivanov, 2000). However, hibernators do not experience these problems. They not only survive prolonged cold exposures without damage but also regulate the transitions between warm and cold Tb values (Breukelen *et al.,* 2000). The mechanisms involved are not fully understood yet. Many studies on hibernation have shown evidence of adaptation in hibernators by means of differential gene expression, altering both the mRNA transcripts and the protein product levels of a variety of genes. A variety of examples of hibernation-responsive gene activation have been reported. Expression of the gene encoding α_2-macroglobulin, a broad-spectrum protease inhibitor which has a very important role in controlling blood clotting, increased at both mRNA and protein levels during the winter (Srere *et al.*, 1992). Some "intermediate-early" gene mRNAs such as *c-fos, c-jun*, and *junB* also increased in the brain across the hibernation cycle (O'Hara *et al.*, 1999). Pyruvate dehydrogenase kinase (PDK) isozyme-4 mRNA was elevated in the heart of hibernating ground squirrels; production of this enzyme which inactivates pyruvate dehydrogenase is important for the regulated suppression of carbohydrate catabolism during torpor (Andrews *et al.*, 1998; Brooks and Storey, 1992). Studies in our lab showed that genes coding for the ventricular isoform of myosin light chain 1 ($MLC1_v$) and NADH ubiquinone-oxidoreductase subunit 2 (ND2) were also up-regulated in heart and skeletal muscle of another ground squirrel, *S. lateralis* (Fahlman and Storey 2000). Fatty acid binding proteins are also up-regulated in both ground squirrel and bat organs during hibernation (Hittel and Storey, 2001; Eddy and Storey, 2004) to provide intracellular transport of lipid fuels to the mitochondria. Protein levels also change during

hibernation. One particular example is insulin-like growth factor which was down-regulated during hibernation along with the plasma binding protein (IGFBP-3) that carries it. However, despite many recent advances, the full range of protein changes that are needed to support hibernation is still far from completely understood.

1.2. Mammalian stress response

The mammalian stress response is an evolutionarily conserved mechanism that allows cells to response to adverse environmental or metabolic conditions (Lee, 2001). Two sets of cellular protein families with protective functions are often over-produced when cells are under stress. These are the heat shock proteins and the glucose-regulated proteins. These proteins are called molecular chaperones and they play essential roles in protein folding, assembling, transportation and disposal by degradation (Hartl, 1996). By binding to denatured proteins, they prevent their aggregation and aid in their refolding into the native state.

1.3. Heat shock proteins

Heat shock proteins are so-called because they were first discovered in salivary glands and other tissues of *Drosophila melanogaster* that were given transient sublethal heat shock (Tb elevated by ~5°C). To date, in eukaryotic cells, six families of HSPs have been identified based on their molecular weight including HSP100, HSP90, HSP70, HSP60, HSP40, and the small HSPs (Katschinski, 2004). HSP families have both constitutive and stress-inducible members whose primary functions are to interact with naïve and denatured proteins to prevent the aggregation of unfolded proteins, facilitate

the folding of naïve proteins and the re-folding of misfolded proteins, and aid intracellular protein trafficking (Gething *et al.*, 1992; Becker and Craig, 1994). These functions make contributions to the maintenance of cellular homeostasis and promote cell survival in response to stressful conditions. HSP sequences are strongly conserved across phylogeny; for example, the *Drosophila hsp70*-gene and the prokaryotic *Escherichia coli dnaK*-gene show 72% and 50% identity, respectively, with the human *hsp70*-gene (Luc *et al.*, 2001). The transcription of *hsp* genes is regulated by heat shock factors (HSFs), which are constitutively synthesized cellular transcription factors. In human tissues, three HSFs have been discovered (HSF1, HSF2, HSF4) but only HSF1 is involved primarily in the stress response. The HSF is negatively regulated since HSF can bind DNA only under stress conditions.

In eukaryotes, the Hsp proteins are found in the cytoplasm, nucleus and mitochondria. In addition to heat shock, other several stressful events such as hypoxia, ischemia, inflammation, and exposure to toxins including heavy metals, endotoxins, and reactive oxygen species also induce HSPs. The physiological roles and the protective potential of HSPs under pathophysiological circumstances have been identified clearly in animal models. However, there is relatively little known to date about HSP roles in mammalian hibernation. Hibernators naturally experience very low body temperatures, hypoxia, and ischemia – all conditions that could trigger HSP production in nonhibernating species. Hence, an exploration of the role that HSPs could play in hibernation is warranted.

Hsp40 (also known as HDJ1) is one member of the HSP40-family. It is found in both the cytoplasm and the nucleus. Hsp40 is constitutive and can bind to unfolded

proteins and couple them to Hsc70 or bind directly to the already formed Hsc70-unfolded protein complex. Hsp40 binds through its J-domain to regulate the function of Hsp70 by stimulating its adenosine triphosphatase activity (Freeman *et al.*, 1995; Borges *et al.*, 2005).

Hsp72 is the major inducible member of the HSP70 family. It is located in both the cytoplasm and nucleus and contains a peptide-binding site and an enzymatic catalytic site. It has a function in guiding protein synthesis and binding proteins under stress conditions. Under unstressed conditions Hsp72 protein can stabilize unfolded nascent precursor peptides (Beckmann *et al.*, 1990; Hartl and Martin, 1992). Hsp72 has been found to associate with cytoskeletal proteins (Tsang, 1993). Under stress conditions, Hsp72 and Hsp73 translocate into the cellular nucleus, particularly to the nucleolus (Welch and Feramisco, 1984). Expression of Hsp72 can significantly reduce nuclear protein aggregation and accelerate refolding of luciferase after heat shock (Nollen *et al.*, 1999; Stege *et al.*, 1994). Hsp72 has a protective role in the cardiovascular system and has received intensive studies in human cardiovascular pathophysiology. Luss *et al.* (2002) found that Hsp72 was elevated in stunned human myocardium, which suggests a cardioprotective action. Hsp72 was up-regulated when myocardial hibernation of rat heart was induced, which could be viewed as a stereotypical adaptational reaction of the cardiac cell to stress conditions (Ferrari *et al.*, 1996).

Hsp73 (also known as Hsc70 due to its 73 kDa molecular weight) is another member of the HSP70 family. Hsp73 also contains a peptide-binding site and enzymatic catalytic site like Hsp72. Unlike Hsp72, Hsp73 is constitutively expressed, but it is also induced under stress. It is located in the cytoplasm, peroxisomes, and nucleus and acts to

guide protein synthesis and import for protein degradation. Under normal conditions, Hsp73 can act as a cellular chaperone by forming a complex with an unfolded protein (Freeman *et al.*, 1995) and can fold and release the newly formed protein in an ATP hydrolysis-dependent process. Under heat stress conditions, Hsp73 was found to associate with topoisomerase I and refolded this nuclear protein. During recovery after heat shock, both Hsp70 and Hsp73 return to the cytoplasm (Welch and Feramisco, 1984).

Hsp90 is one of the most abundant cytosolic Hsps; it is located in cytoplasm and nucleus. Hsp90 is constitutive but up-regulated and phosphorylated under stress. It can bind steroid receptors, protein kinases, intermediate filaments, microtubules and actin microfilaments in a very specific manner (Koyasu *et al.*, 1986). Hsp90 is an essential component of the glucocorticoid receptor (Pratt, 1993); it can bind the receptor at the chaperone site and bind cofactors at other sites to translocate the receptor to the nucleus (Pratt *et al.*, 2004). Data from Nadeau *et al.* (1993) revealed that Hsp90 possesses ATPase activity and binds heat shock transcription factors. Hsp90 has two isoforms: the more inducible Hsp90α and the less inducible and more constitutively expressed Hsp90β.

1.4. Glucose regulated proteins

Another set of stress proteins were first identified in 1977 as responses to glucose starvation of cells and are called glucose regulated proteins (GRPs) (Lee, 1992; Little *et al.*, 1992; Lee, 2001). Two proteins were discovered with molecular weights of 78 and 94 kDa that were strongly induced when glucose was absent in the culture medium of growing embryo fibroblasts (Shiu *et al.*, 1977). This stress resulted in the accumulation of misfolded proteins in the ER, and the elevation of *Grp* genes has been become known as

a marker for the unfolded protein response (UPR). The principal Grps now known in mammals are Grp75, Grp94, Grp78, and Grp170. Hypoxia exposure as well as other perturbations of ER function, such as agents that affect calcium stores or inhibit glycosylation, also result in *Grp* synthesis. These proteins have been shown to bind to newly synthesized, unfolded, and/or incompletely glycosylated proteins in the lumen of the ER, the mitochondria, and other compartments of cells.

Stress induction of mammalian *Grp* genes is mainly regulated at the transcription level. In mammalian cells, the *Grp* promoters contain multiple endoplasmic reticulum stress elements (ERSEs). Transcription factors including the nuclear form of ATF6, a strong activator of mammalian *Grp* genes, and other transcription factors such as NF-Y (CCAAT-binding factor) (Roy and Lee, 1999; Marcus and Green, 1997; Yoshida *et al.,* 2001), YY1 (Ying Yang 1) binding to the CCACG motif (Li *et al.,* 1997) and TFII-I (known to facilitate protein-protein interactions) (Parker *et al.,* 2001) can bind and activate the ERSE. The promoter region of some *Grps*, for example *Grp78,* also contain another element called the unfolded protein response element (UPRE) to which yeast Hac1 can bind and activate transcription.

Grp75 (also known as Mortalin-2 or mthsp70), a member of the hsp70 family of proteins, has been localized to mitochondria, endoplasmic reticulum, plasma membrane (Shin *et al.,* 2003), and cytoplasmic vesicles (Domanico *et al.*, 1993; Singh *et al.*, 1997; Ran *et al.,* 2000). Mizzen *et al.* (1991) showed that Grp75 interacts with and facilitates the folding and assembly of proteins as they enter into the mitochondria. It can also bind other proteins such GRP94 and p53 (Takano *et al.*, 2001; Wadhwa *et al.,* 2002). The level of GRP75 protein can be enhanced by exposure to low levels of ionizing radiation,

glucose deprivation (Merrick *et al.*, 1997), and calcium ionophore (Massa *et al.*, 1995). In humans, Grp75 is up-regulated in tumours and transformed and tumour cell lines (Kaul *et al.*, 1998; Taknao *et al.*, 1997; Bini *et al.*, 1997). The level of Grp75 was also reported to be elevated in bat brain during arousal from hibernation (Lee *et al.*, 2002) and was consistently higher in intestinal mucosa of 13-lined ground squirrels at five stages of the hibernation cycle (entrance, short-bout torpid, long-bout torpid, arousal, and interbout euthermia) as compared with summer-active squirrels (Carey *et al.*, 2000).

GRP94 (also known as gp96), a member of the heat shock protein 90 family of proteins, is another stress-inducible protein and also one of the most abundant and well-characterized ER molecular chaperone. GRP94 has been studied more extensively because it's involved in antigen processing and has potential use in immune therapy (Nicchitta *et al.*, 2004; Gidalevitz *et al.*, 2004; Argon and Simen, 1999). As a molecular chaperone, GRP94 can bind to malfolded proteins and unassembled complexes. They are induced in response to stress and are posttranscriptionally modified into biologically inactive forms after removing the stress. The promoter of Grp94 is well conserved and can interact with several transcription factors to induce grp94 expression (Little *et al.*, 1994; Yoshida *et al.*, 2001; Parker *et al.*, 2001). Paris *et al.* (2005) found that there are three hypoxia response elements (HRE) in the human GRP94 promoter and GRP94 was up-regulated in endothelial cells in response to hypoxia. Jeon *et al.* (2004) evaluated GRP94 expression level after giving an intracerebroventricular injection of kainic acid to adult mice. They found an elevated GRP expression compared with control, by which they think that GRP94 may stabilize the astroglial cytoskeleton and participate in astroglial antioxidant mechanisms.

Grp170 (also known as oxygen-regulated protein, Orp150) is a novel endoplasmic-reticulum-associated chaperone induced by hypoxia/ischemia. Induction of this protein under ischemic stress protected cells from death (Ozawa *et al.*, 1999). Yoshitane *et al.* (1998) found that Grp170 was up-regulated in human breast tumors compared with normal breast tissue. In addition to a protective role for cells under stress conditions, Grp170 was also described as making a contribution to the import of proteins and peptides. Dierks *et al.* (1996) found that Grp170 was an efficient ATP-binding protein. Grp170 was also involved with peptide transport into the ER via the transporter associated with antigen processing, suggesting that Grp170 may be involved in the antigen presentation pathway.

1.5. Hypothesis and proposed studies

Adaptations that support hibernation should predictably include changes to the protein make-up of cells to deal with a number of stresses and potential problems. These could include protein changes that allow cells to maintain coordinated functions under very low Tb values in torpor or that may deal with potential ischemia/hypoxia stresses, especially associated with the arousal process when metabolic demand by warming tissues may rise very quickly Furthermore, because protein synthesis is strongly suppressed during torpor and because animals remain torpid for many weeks at a time, mechanisms may be needed to help stabilize cellular proteins over the long term. These actions may be jobs for HSPs and GRPs in hibernating mammals. Indeed, as reported above, Grp75 is already known to be elevated in bat brain during arousal from hibernation (Lee *et al.*, 2002) and in intestinal mucosa of 13-lined ground squirrels in

winter as compared with summer active animals (Carey *et al.*, 2000). This suggests that Grp75 may have a wide role to play in all tissues. In addition, other Grp and Hsp family members may also have important roles in hibernation.

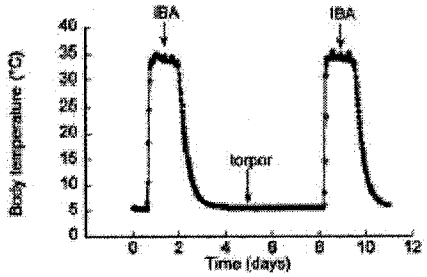
I hypothesize that multiple HSP and GRP family members are up-regulated during hibernation in the organs of two mammalian species, ground squirrels and bats. To test this hypothesis, relative quantitative RT-PCR was employed to examine *Hsp* and *Grp* gene expression and Western blotting was used to evaluate HSP and GRP protein levels in the organs of ground squirrels and bats, comparing euthermic and hibernating states. Partial cDNA sequences were also obtained from squirrel and/or bat for each gene studied and were compared with the corresponding sequences from other mammals to highlight similarities and differences between hibernator and non-hibernator proteins.

In Chapter 3, the responses of several *Grps* are quantified at the mRNA level and/or protein level in squirrel and bat tissues. Levels of *Grp75* expression in both squirrel and bat tissues were analyzed for changes in both mRNA and protein levels. *Grp94* expression was also analyzed at both mRNA and protein levels in squirrel tissues whereas *Grp170* transcript levels were quantified in squirrel and bat tissues.

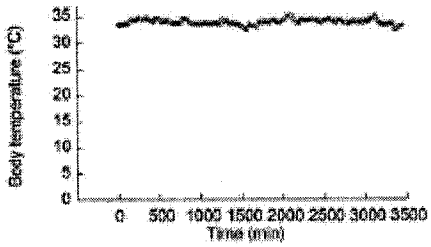
In Chapter 4, the effect of hibernation on HSP protein expression is quantified in squirrel and in bat tissues. Western blots were used to analyze HSP70, HSP73, and HSP40 levels in both squirrel and bat organs and HSP90 protein expression was also evaluated in squirrel tissues.

Figure 1.1 Life history of a hibernator.

Hibernating rodents routinely transition between extremely low body temperatures and euthermy. Graphs plot body temperature against time, showing the dynamic cycling between longer periods of torpor and interbout arousals (IBA) during hibernation (*top*) vs. the relative constancy in summer (*bottom*).



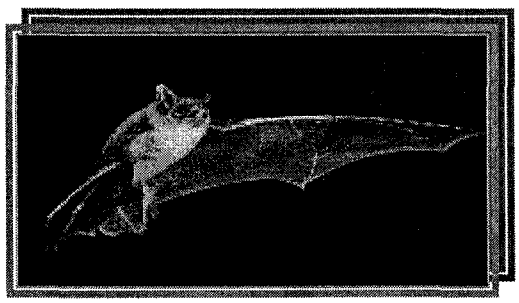
Winter



Summer

Fig. 1.2. Thirteen-lined ground squirrel, *Spermophilus tridecemlineatus* (A) and little brown bat, *Myotis lucifugus* (B).

A



B

CHAPTER 2

MATERIALS AND METHODS

Animals

Thirteen-lined ground squirrels, *Spermophilus tridecemlineatus* (130-180 g), were obtained in September 2000 and transported to an animal housing facility on the National Institutes of Health campus (Bethesda, MD) where they were held at the Animal Hibernation Facility. Hibernation experiments were carried out by the laboratory of Dr. J.M. Hallenbeck (National Institute of Neurological Disorders and Stroke). Animals were held at 21°C on a 12/12-h light/dark cycle and fed *ad libitum* until they entered and finished the pre-hibernation phase of hyperphagia that maximized the body lipid reserves prior to hibernation. When animals had exhibited a rapid increase in body weight to 220-240 g, they were placed in constant darkness in chambers at 5-6°C and 60% humidity to induce hibernation; noise within the chamber was kept to a minimum. These environmental conditions approximate those to which the animals are normally exposed in their burrows over the winter season. After hibernation for 2-6 days (as indicated by a constant low body temperature of ~5°C), animals were sacrificed by decapitation. At the same time, animals that were kept as euthermic controls at 21°C were sacrificed. Brain, liver, kidney, heart, lung, brown adipose tissue (B.A.T.), white adipose tissue (W.A.T.) and skeletal muscle were quickly excised, flash frozen in liquid nitrogen, and then stored at –80°C. The tissues were packed in dry ice and air-freighted to Carleton University where they were again stored at –80 °C until use.

Little brown bats, *Myotis lucifugus* (7-8 g body mass), were collected by Dr. Don Thomas (Université de Sherbrooke) in November 1999 from a disaffected slate mine near Sherbrooke, Quebec where they had been hibernating since October (cave air temperature was ~5°C). They aroused during transportation to the Université de Sherbrooke. Some

were kept at euthermic conditions at 23-24°C for 48 hours and then euthanized by cervical dislocation while others were placed at 5°C and allowed to re-enter hibernation. They were sacrificed after 36 hours of continuous torpor with body temperature lower than 6°C. The tissues from both euthermic and hibernating bats were collected as described above.

General procedures for molecular biology methods

All materials and solutions used for RNA isolation were treated with 0.1% v/v diethylpyrocarbonate (DEPC) and subsequently autoclaved. Lab tools and equipment were routinely sterilized in a UV hood (VWR Canada). Lab benches were washed down with ethanol and gloves were worn for all procedures and changed frequently to prevent DNA contamination.

Total RNA extraction

Total RNA was extracted from the tissues obtained above. Trizol reagent (Gibco-BRL) was used for the extraction and purification of total RNA following the manufacturer's protocol and as reported previously (McMullen, 2004). Briefly, about 50-100mg of tissue was weighed, placed in a 2 ml microcentrifuge tube and homogenized in 1ml of Trizol reagent. A 200 µl aliquot of chloroform was added (0.2 ml/ml of Trizol) and the sample was incubated at room temperature (RT) for 2-10 min. The sample was then centrifuged at 10,000 rpm for 15 min at 4°C to separate the sample into an aqueous phase and an organic phase. After centrifugation, the upper aqueous phase containing RNA was removed to a fresh 1.5 ml tube containing 500 µl isopropanol and RNA was

precipitated over 10 min at RT followed by centrifugation at 12,000 rpm for 10 min. The supernatant was discarded and the pellet was washed with 70% ethanol and centrifuged again at 12,000 rpm for 5 min. The total RNA pellet was resuspended in 50-200 µl of RNAase-free distilled water depending on the amount of total RNA.

The quality of total RNA was assessed by separation on a denaturing formaldehyde agarose gel; visual inspection showing the presence of sharp and distinct 28S and 18S rRNA bands indicated good quality. Briefly, a 1.2% gel was prepared by melting 0.75 g of agarose (electrophoresis grade, GIBCO BRL) in 52.75 ml DEPC-treated water, then allowed to cool to ~60°C with addition of 6.25 ml 10× MOPS buffer [0.2 M 3-N-morpholino propanesulfonic acid (MOPS), 50 mM sodium acetate, 10 mM ethylenediamine tetraacetate (EDTA), pH 7.0] and 3.5 ml of 37% formaldehyde (v/v). To prepare RNA samples, 10-15 µg of total RNA from each sample (in a volume of 6-10 µl of sterile DEPC-treated water) and an equal volume of RNA denaturing buffer (made of 12.5 µl formamide, 2.5 µl 10× MOPS buffer and 4 µl 37% formaldehyde) were mixed and heated at 65°C for 5 min, then chilled on ice, and then 2.5 µl of 10× RNA loading buffer [0.9 ml of 50% glycerol, 2 µl of 0.5 M EDTA (pH 8.0), 50 µl of 0.25% bromophenol blue, 50 µl of 0.25% xylene cyanol FF] was added to the mixture. After a quick spin, the RNA sample was loaded onto an agarose gel which had been pre-run at 90 V (4.5 V/cm) with 1× MOPS running buffer for 10-15 min. A 7µg aliquot of RNA ladder (1µg/µl, 0.24-9.5 kb, GIBCO BRL) was applied as a size marker in one lane. The quantity of the total RNA was measured using spectrophotometry at 260 nm with an OD_{260} of 1 corresponding to 40 µg of RNA per ml. RNA was stored at -80°C until required for further use.

First strand synthesis

Briefly, 15 μg of total RNA in 10 μl of DEPC-treated water was placed in a 0.5ml tube to which 1 μl 200 ng/μl of Oligo dT primer (5'-TTTTTTTTTTTTTTTTTTTV-3'; V=A or G or C) (Sigma Genosys) was added, and the tube was put in a 65°C water bath for 5 min. Then the following components were added: 2 μl DTT, 4 μl of 5X sample buffer, 1 μl of 10 μM dNTPs, and 1 μl reverse transcriptase (Superscript, GIBCO). The mixture was incubated at 40°C for 40 min to reverse transcript the mRNA to cDNA. Then a series of cDNA dilutions of 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, and 10^{-5} were made and stored at 4°C.

Degenerate primer design

Degenerate primers were designed using DNAMAN and Primer Designer software. Several mammalian sequences for the gene of interest were retrieved from Genbank and then compared using DNAMAN. Highly conserved regions were identified in the sequences and these were used to design primers using Primer Designer based on the variables including primer length, melting temperature, G/C content and 3'-end sequence. The designed primers were then synthesized by Sigma Genosys.

RT-PCR

The reverse transcription-polymerase chain reaction (RT-PCR) was used to retrieve the segments of genes and to access the levels of mRNA transcripts in the tissues of control and hibernating ground squirrels and bats. To retrieve the segments of genes, gradient PCR was performed with a 10^{-2} cDNA dilution and the designed primers,

essentially as described by Morin and Storey (2005). PCR tubes contained the following: ddH_2O 15 µl, cDNA 5µl, 10X PCR buffer 2.5 µl, 50 µM $MgCl_2$ 1.25 µl, 10 µM dNTPs 0.5µl, Taq 0.125µl and 30 nmol/ml of forward and reverse primer mixture (1:1) 1.25 µl. The program for i-Cycler PCR machine was set as follows: denaturation at 95°C for 30 sec, primer annealing for 30 sec, and primer extension at 72°C for 30 sec. These cycles were carried out 35 times.

The resulting PCR products were separated on 1% agarose gels together with a DNA ladder. The optimum temperature for the PCR reaction was determined to be the one at which the PCR products showed the brightest band. The PCR products were sequenced by either Canadian Molecular Research Services (CMRS) Inc. (Ottawa, Ontario) or by CORTEC (Kingston, ON) using an automated DNA sequencing procedure.

For the analysis of DNA sequencing data, the segments were analyzed using computer-assisted programs. Nucleotide sequences and deduced polypeptide sequences were analyzed with the Blast Program on the World Wide Web (WWW) to search for homologous sequences in Genbank. The DNAMAN program (Lynnon BioSoft, Vaudreuil, Quebec) was used for sequence comparison and alignment.

For analyzing the levels of gene expression, relative RT-PCR was employed. First, primers (Forward AAGGAAGATGCTGCCAATAA, Reverse GGTCACATTTCACCATCTG, Sigma Genosys) for a housekeeping gene, α-tubulin, were subjected to PCR using the series of cDNA dilutions made from control and hibernating tissues; α-tubulin expression was used to normalize the amount of cDNA present in control versus hibernated samples. Then RT-PCR was carried out using the

primers for the gene of interest and the same dilution series. A tube with reaction mix and alpha-tubulin primers but with no cDNA was also used as a negative control. A tube with reaction mix and alpha-tubulin primers but with total RNA was used as control to avoid genomic contamination. PCR products were separated on a 1% agarose/ethidium bromide gel. DNA bands were visualized under UV light in ChemiGenius Bio Imaging System (SynGene) and quantified using SynGene tools. The lowest dilutions for alpha-tubulin and the gene of interest which had visible bands were chosen for quantification. The mRNA band density of the gene of interest was normalized against the corresponding alpha-tubulin band density for the same sample and then normalized values for euthermic and hibernating samples (n=4 for each) were tested for significant differences using the Student's t-test with $P<0.05$ accepted as a significant difference. Next, the values for hibernator samples were expressed relative to euthermic controls and plotted as histograms. Overall, the ratios shown in histograms represent the following:

(hibernating gene / hibernating tubulin) / (euthermic gene / euthermic tubulin).

Protein isolation and Western blotting

The technique of Western blotting was employed to assess the protein expression of the genes in tissues from hibernating versus euthermic animals. Methodology was essentially as described in Eddy et al. (2005) and Morin and Storey (2005). Frozen tissue samples (~50 mg) from euthermic and hibernating ground squirrels and bats were homogenized in 0.5ml buffer (20 mM Tris, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% v/v Triton X-100, 10 mM ß-glycerol phosphate, 1 mM Na_3VO_4, 1 mM PMSF, pH7.8). Homogenates were centrifuged at 1,1000r/min for 13 min at 4°C, and

supernatants were collected. Soluble protein concentration was measured with the Coomassie blue dye-binding method and the Bio-Rad commercial kit. Volumes containing equal amounts of protein from euthermic and hibernating samples were mixed with 1:1 v:v with 2X SDS –PAGE loading buffer (100 mM Tris-HCl, pH 6.8, 20% v/v glycerol, 4% w/v SDS, 0.2 % w/v 2-mercaptoethanol, 0.2% w/v bromophenol blue) and boiled for 5 min. The samples were then loaded to 8-12% acrylamide gels and run at 180 V for 45 minutes using the BioRad Mini-PROTEAN 3 System. Proteins were transferred to polyvinylidene difluoride (PVDF) membranes by electroblotting using wet transfer with pre-chilled solution containing 25 mM Tris (pH 8.5), 192 mM glycine, and 20% v/v methanol at 4°C 70 V for 2 hours.

Membranes were then blocked with 2.5% milk in TBST buffer (10 mM Tris, pH 7.5, 150 mM NaCl, 0.05% v/v Tween-20) at RT for 25-35 minutes and then probed with primary antibodies at 4°C for overnight. For Grp94, a negative control was performed using the Grp94 peptide (Cal. Sc-1794p, Santa Cruz Biotechnology) which was used to raise the antibodies against Grp94. The membranes were washed with TBST for 3 x 5 min and then incubated in secondary antibody at RT for 2 hours. After a further three washes, immunoreactive bands were detected by use of enhanced chemiluminescence (ECL) reagents (PIERCE) in Syngene. Subsequently, the membrane was stained by Coomassie blue. The relative amount of the target protein as well as Coomassie stained bands were quantified using Syngene tools. Data were collected from n=4 trials for both hibernating and euthermic samples and significant differences between the two groups were tested using the Student t-test. Histograms were constructed showing the ratio hibernating:euthermic values with error bars that are the sum of SEM values for

euthermic and hibernating trials.

In selected cases, the specificity of the antibody reaction was tested by using the immunizing peptide to block the immunoreaction with the band of interest on the blot. Duplicate blots were prepared. One was reacted as normally with primary antibodies. The second blot was incubated with primary antibody that had previously been incubated with 10 ug of GRP94 immunizing peptide in TBST for 2 hours at room temperature. Both blots were then incubated with secondary antibody and developed as normally.

2-Dimentional Polyacrylamide Gel Electrophoresis (2-D PAGE)

To verify that the antibody used in Western blotting was specific to its protein or peptide target, 2-D PAGE was employed using the Mini-PROTEAN II system, essentially as described by Eddy et al. (2005). The first dimension gel was prepared in a capillary tube containing monomer solution [9.2 M urea, 4% acrylamide, 1.6% pH 5-8 ampholytes (Sigma), 0.4% pH 3.5-10 ampholytes (Sigma), 0.01% ammonium persulfate, 0.1% TEMED]. After polymerization, the capillary gels were pre-electrophoresed for 10 min at 200V, 15 min at 300V and 15 min at 400V with 100 mM NaOH as the upper chamber running buffer and 10 mM H_3PO_4 as the lower chamber running buffer.

Tissue samples from ground squirrels and bats (~100mg) were homogenized with a Polytron homogenizer in 1ml (1:10 w/v) of buffer (25 mM HEPES, 25 mM KCl, 250 mM sucrose, 1 mM EGTA, 1 mM EDTA, pH 7.4). The protease inhibitor phenylmethylsulphonyl fluoride (PMSF) was added just prior to homogenization. The samples were then centrifuged at 5,000 rpm for 5 min at 4°C in a Biofuge 15 (Baxter Canlab). The supernatant was removed and total protein content was quantified using the

BioRad protein assay. Protein samples were mixed with equal volume of 2x SDS-PAGE sample buffer (9.5 M urea, 2% v/v Triton X-100, 5% v/v β-mercaptoethanol, 1.6% ampholines pH 4-6 and 0.4% ampholines pH 3.5-10), and then boiled for 5 min. Samples were loaded into pre-electrophoresed first dimensional gel capillary sample reservoirs and overlaid with the first dimension sample overlay buffer (9 M urea, 0.8% pH 5-8 ampholytes, 0.2% pH 3.5-10 ampholytes, 0.005% w/v bromophenol blue). Separation generally occurred over 10 min at 500 V and then 3 h at 750 V. Following isoelectric focusing, the gels were removed from the capillary tubes and incubated for 10 min in SDS equilibration buffer containing 62.5 mM Tris-HCl, pH 6.8, 2.3% w/v SDS, 5.0% β-mercaptoethanol, 10% w/v glycerol and 0.00125% w/v bromophenol blue. The gels were then loaded onto an SDS polyacrylamide gel (12%) for second dimension separation by molecular mass and probed with primary and second antibodies with the same procedures as used for Western blotting.

Comparison of gene expression in nuclear and cytoplasmic fractions

Differential centrifugation was used to separate nuclear and cytoplasmic fractions as described by Morin and Storey (2005). Briefly, frozen tissue samples (0.5 g) were weighed and added to 0.5 ml of homogenization buffer (1:1 w:v) containing 10 mM HEPES, pH 7.9, 10 mM KCl, 10 mM EDTA, 10 mM DTT, and 10 µl of protease inhibitor mixture (1 mM PMSF, 0.015% w/v aprotimin, 10 µM leupeptin, 1 µM pepstatin, 1 mM NaF). Tissue was disrupted in a Dounce homogenizer and then centrifuged at 10,000 x g for 10 min at 4°C. The supernatant representing the cytoplasmic extract was removed into a sterile microcentrifuge tube and stored at -80°C. The pellet

was resuspended in extraction buffer containing 20 mM HEPES, pH 7.9, 0.4 M NaCl, 1 mM EDTA, 10% v/v glycerol, 0.1 mM DTT and 1.5 µl of protease inhibitor cocktail and then incubated on ice with shaking for 1 h. After centrifugation at 10,000 x g for 10 min at 4°C, the supernatant representing the nuclear extract was removed and stored at -80°C. SDS-PAGE and Western blotting were carried to check the protein localization and expression level.

The integrity and purity of the nuclei isolated by the above procedure was checked by running the following tests on the nuclear versus cytoplasmic fractions. Firstly, DNA content was measured spectrophotometrically at 260 vs 280 nm in the two fractions; compared with the cytoplasmic fractions, the nuclear fractions that were isolated were found to contain virtually all of the DNA. Secondly, the distribution of the transcription factor ATF4 was assessed by western blotting. ATF4 is typically found in the cytoplasm under control conditions and moves to the nucleus under stress conditions; nuclei from euthermic animals showed ATF4 in the cytoplasm whereas this shifted to the nuclear fraction during hibernation. Thirdly, the distribution of the phosphorylated form of the transcription factor CREB was assessed; CREB is not phosphorylated in its inactive state in the cytoplasm and when activated moves to the nucleus and is phosphorylated CREB. Phospho-CREB was detected only in the nuclear fraction and only in hibernating animals.3

CHAPTER 3

UP-REGULATION OF GLUCOSE REGULATED PROTEINS IN HIBERNATION

Results

3.1 GRP75

3.1.1. RT-PCR retrieval of squirrel and bat grp75

RT-PCR was carried out to retrieve cDNA for *grp75* from liver of both ground squirrels and bats. Degenerate primers were designed based on the consensus sequences of *grp75* from several other mammal species: Forward 5'-GGAATGGCCTTAGTCATGAG-3' and Reverse 5'-CCTGTC(T/G)CTG(C/T)GAGTCATTG-3'. The optimum annealing temperature was 54°C. Fig. 3.1 shows the cDNA segments that were amplified for ground squirrel (A) and bat (B) liver and the corresponding deduced amino acid sequences. Analysis using the BLAST program at the NCBI website and DNAMAN software confirmed that the sequences encoded *grp75*. The primers amplified the same 482 nucleotide segment of cDNA from both species. These partial sequences showed 94% identity, respectively, with the corresponding segment of the human *grp75* cDNA sequence (Fig. 3.2). The amplified nucleotide sequences encoded 160 amino acids, about 25% of the full protein (659 amino acids in humans and mice), from a region near the N terminal (beginning at amino acid 37 of the human/mouse sequence). Amino acid sequence identity was very high; squirrel and bat GRP75 shared 99% and 97% identity, respectively, with human GRP75 (Fig.3.3), which shows that GRP75 is very conserved among mammals and furthermore confirmed that the GRP75 partial sequences obtained in this experiment for squirrel and bat were reliable.

3.1.2. Expression of grp75 mRNA in tissues from hibernating squirrels and bats

The technique of RT-PCR was employed to analyze *grp75* mRNA expression levels in tissues of euthermic (control) and hibernating squirrels and bats. For this, the degenerate *grp75* primers (listed above) were used; the very high nucleotide sequence similarity between the mammalian cDNAs made it unnecessary to redesign species-specific primers for these studies. Fig.3.4 shows a typical pattern of PCR product bands after amplification of normalized serial dilutions of squirrel liver cDNA using primers for α-tubulin or *grp75*. α-tubulin, used as a control, showed no difference in band intensities between control and hibernating states at any of the dilutions of liver cDNA tested. The same result was found for alpha-tubulin mRNA levels in other squirrel tissues and also in bat tissues (data not shown). As the figure shows, the intensity of the bands decreased with the increasing dilution. The bands chosen for quantification (10^{-5} for tubulin, 10^{-3} for *grp75*) were clearly nonsaturating in intensity, indicating that they fell within the linear portion of the [cDNA] versus band intensity relationship. The intensity of the bands in higher dilutions was measured according to the method described in Chapter 2 and the formula listed in Fig.3.4. Following the calculation using Syngene software, the mean values from multiple runs were statistically analyzed.

Fig.3.5A shows the relative expression level of *grp75* mRNA in eight organs of ground squirrels. Expression in BAT (brown adipose tissue) tissue from hibernating animals was significantly higher (2.9-fold higher) than in euthermic controls. Transcripts of *grp75* were also moderately elevated in kidney by 1.2-fold. By contrast, *grp75* expression was reduced in WAT (white adipose tissue) of hibernating squirrels to 51% of

the euthermic value. Transcript levels were unaltered during hibernation in liver, muscle, lung, heart and brain.

The pattern seen in bat tissues was quite different (Fig. 3.5B). Transcripts of *grp75* were moderately elevated in muscle, lung, and brain of hibernating animals by 1.22-, 1.2-and 1.3-fold, respectively, as compared with euthermic controls. By contrast, expression in BAT of hibernators was reduced to 74% of the control value. Expression levels were unaltered in bat liver and kidney and *grp75* mRNA was not detected in bat WAT.

3.1.3 Expression of GRP75 protein in hibernated squirrel and bat tissues

The technique of Western blotting was used to determine the protein levels of GRP75 in hibernator tissues. The antisera used was anti-GRP75 goat polyclonal IgG antibody (1:200) (sc-1058, Santa Cruz Biotech.). Horseradish peroxidase-conjugated bovine anti-goat IgG (sc-2350, Santa Cruz) was used as secondary antibody (1:1000). Band intensities on Western blots were calculated using Syngene software and the data were analyzed statistically in Excel. Fig. 3.6A shows representative immunoblots for GRP75 in squirrel and bat tissues from hibernating versus euthermic control animals. Histograms in Fig. 3.6B show mean ratios (hibernating:euthermic) for multiple independent trials. In ground squirrels, GRP75 protein levels increased significantly during hibernation in two tissues, brain and liver, by 1.68- and 1.23-fold, respectively (Fig.3.6B). By contrast, GRP75 levels fell in squirrel muscle and heart to 61% of the euthermic values, respectively, while there was no change in BAT, kidney and lung. In bats, GRP75 protein levels increased significantly in muscle and brain during hibernation

by 1.32- and 1.14-fold, respectively (Fig.3.6C). However, protein levels decreased to 84% and 70% in liver and lung, respectively, while there was no change in BAT and kidney.

3.1.4. Anti-GRP75 antibody specificity confirmed by 2D electrophoresis

To confirm that the antibodies used were specific for a single protein, 2-D gel electrophoresis was employed. After running the first dimension isoelectrofocusing gel to separate the proteins on the basis of charge, the proteins were separated in the second dimension by molecular mass using SDS-PAGE. The resolved proteins were then probed with primary and secondary antibodies, as described above in section 3.1.3. Fig.3.7 shows the protein spots that reacted with GRP75 antibodies in squirrel liver (A) and bat liver (B). In both cases strong immunoreactive spots were seen at a position corresponding to a molecular weight of ~70 kDa and pI value of ~5.4, which is consistent with the characteristics of human GRP75 (NP_004125) which has a molecular weight of 73 kDa and theoretical pI of 5.87 as calculated from the Compute pI/Mw tool at http://www.expasy.org/tools/ website. Some minor crossreacting spots on the squirrel liver gel were at a much lower molecular weight; these would not interfere with the detection and quantification of the GRP75 band on the one-dimensional western blot. Hence, the results demonstrate that the antibody used for Western blotting is specific to GRP75 protein and the protein levels examined in different tissues were reliable.

3.1.5 Localization of GRP75 protein in squirrel muscle

Subcellular fraction and immunoblotting studies using anti-GRP75 antibody (the same as used in Western blotting) showed that GRP75 protein was present in both the cytoplasm and the nucleus of ground squirrel skeletal muscle. Fig.3.8 shows that the relative amounts of GRP75 were lower in both subcellular fractions in muscle from hibernating squirrels, in agreement with the decrease in total GRP75 in hibernating muscle seen in Fig. 3.6. GRP75 protein in the cytoplasmic fraction from hibernating animals was only 45% of the amount in euthermic cytoplasm whereas in nuclei, the amount in hibernators was reduced to 70% of the euthermic value.

3.2 GRP94

3.2.1 Similarity analysis of squirrel GRP94 in both DNA and protein level

RT-PCR was used to retrieve a cDNA for *grp94* from ground squirrel brain. Degenerate primers were designed based on the consensus sequences of *grp94* from other mammalian species: Forward 5'-TGCTG(C/T)GTCCTGCTGACCTT-3' and Reverse 5'-GC(T/G/C)ACAAGGAAGGC(G/T)GAATA-3'. The optimum annealing temperature was 63°C. A 550 bp segment was amplified from squirrel brain. Fig. 3.9 shows the squirrel *grp94* cDNA segment and the corresponding deduced amino acid sequence. Analysis using the BLAST program at the NCBI website and DNAMAN software confirmed that the sequence encoded *grp94.* The partial cDNA sequence shared 94% and 90% identities, respectively, with human and mouse *grp94* (Fig. 3.10). The amplified nucleotide sequence encoded 183 amino acids, about 23% of the full protein (803 and 802 amino acids in human and mouse, respectively), from a region near the N-terminal

(beginning at amino acid 17 of the human/mouse sequence). Amino acid identity was high; the squirrel GRP94 protein segment shared 98% identity, respectively, with the human, mouse and cow sequences (Fig. 3.11). These data show that *grp94* is well conserved among mammals and that the partial nucleotide cDNA sequence obtained in this experiment is reliable.

3.2.2 grp94 mRNA expression in squirrel tissues

To study the effects of hibernation on the mRNA expression of *grp94* in squirrel tissues, the technique of RT-PCR was employed. Samples were normalized by amplification of tubulin primers (the same as in section 3.1.2), and then amplification of cDNA serial dilutions were conducted with the *grp94* degenerate primers (listed above). The PCR products were separated on agarose gels and the DNA bands were stained with ethidium bromide and visualized under UV light with the Syngene. The intensities of the bands in the higher dilution samples were measured and the histogram in Fig. 3.4 shows data derived from the amplification of the cDNA dilutions according to the method described in Chapter 2 and the formula listed in Fig. 3.4. Fig.3.12 shows the relative expression level of *grp94* transcripts in seven tissues. In squirrel BAT, brain and lung expression was significantly higher in hibernating animals compared with euthermic controls; levels were 1.17-fold, 1.31-fold and 1.24-fold higher than controls, respectively. By contrast, *grp94* expression did not change in heart, kidney, liver and muscle and *grp94* was not detected in WAT.

3.2.3 GRP94 protein expression in squirrel tissues

To check the GRP94 protein expression in squirrel tissues, tissue extracts of soluble proteins were prepared, separated on SDS-PAGE gels, transferred to PVC membrane and then immuno-reacted with anti-GRP94 goat polyclonal IgG antibody (sc-1794, Santa Cruz) (1:200 v:v). Following washing, the membrane was probed with secondary antibody, bovine anti-goat IgG HRP (sc-2350, Santa Cruz) (1:1000 v:v), and then bands were developed with ECL and the intensities of bands was quantified using Syngene software. Fig. 3.13A shows representative Western blots of GRP94 protein levels in seven squirrel tissues and the histogram (Fig. 3.13B) shows the relative GRP94 protein expression in tissues from hibernating versus euthermic animals. In brain, liver, lung and muscle, GRP94 protein was significantly higher by 1.17-, 1.15-, 1.3- and 1.2-fold, respectively, in hibernating animals versus controls. GRP94 protein levels did not change during hibernation in BAT, heart or kidney.

3.2.4 *Anti-GRP94 antibody specificity confirmed by negative control test*

The specificity of anti-GRP94 antibody was assessed by a negative control test. Samples of squirrel control and hibernating BAT and liver proteins were separated by SDS-PAGE on three gels and transferred to PVC membranes (Fig. 3.14). One blot was immunoblotted with primary and secondary antibodies (as in 3.2.3) and immunoreacting protein bands were visualized at ~94 kDa (Fig. 3.14A). The second blot was also immunoreacted with primary and secondary antibodies, but the primary antibody had been previously incubated with 10 ug of GRP94 immunizing peptide in TBST for 2 hours

at room temperature to allow the peptide to bind to the antibody. When developed, this blot showed no cross-reacting bands because the GRP94 primary antibody cross-reacted with the GRP94 peptide, leaving no free antibodies left to bind with GRP94 proteins on the membrane. This result demonstrated that the GRP94 antibody used in this experiment was specific for GRP94 protein and crossreacted with squirrel GRP94. The immunoblot shown in Fig. 3.14C shows a comparable Western blot for GRP94 in squirrel liver and BAT but with protein molecular weight markers run in the far right lane; this shows that the immunoreacting band is of the expected molecular weight.

3.2.5 *Localization of GRP94 protein in squirrel muscle*

Subcellular fractionation and immunoblotting was used to study the distribution of GRP94 protein in the cytoplasm versus nucleus of ground squirrel skeletal muscle. Fig. 3.15 (A) shows that levels of GRP94 were significantly elevated in both compartments during hibernation; GRP94 protein content was 1.23-fold higher in cytoplasmic fractions of hibernating versus control animals whereas GRP94 was 1.37-fold higher in nuclear fractions of hibernators (Fig.3.15 B).

3.3 GRP170

3.3.1 *Similarity analysis of squirrel and bat GRP170 at mRNA and protein level*

RT-PCR was employed to retrieve cDNA for *grp170* from bat brain. Degenerate primers were designed based on the consensus sequences of *grp170* from several other

mammal species. These were: Forward 5'-GACCTGTTGGCACTGAGTGA-3' and Reverse 5'-CGGAAGACACCATAGCTGAG-3'. The optimum annealing temperature was 66°C. The primers for squirrel *grp170* were subsequently designed based on a consensus sequence derived from bat *grp170* cDNA as well as other mammalian *grp170* sequences: Forward 5'-CCATGAAGGTGGCCAT(C/T)GTC-3' and Reverse 5'-C(G/A/T)GTGGC(A/G)GTGTTGTCATTG-3'. The optimum annealing temperature was 66°C. Fig. 3.16 shows the cDNA segments that were amplified for squirrel kidney (A) and bat brain (B) and the corresponding deduced amino acid sequences. Analysis using the BLAST program at the NCBI website and DNAMAN software confirmed that the sequences encoded *grp170*. The primers amplified 460- and 538-nucleotide segments (primers excluded from both sequences) of cDNA from both species. These partial sequences showed 88% and 90% identity, respectively, with the corresponding segment of the human *grp170* cDNA sequence (Fig. 3.17). The amplified squirrel *grp170* nucleotide sequence encoded 153 amino acids or about 15% of the full protein (999 amino acids in mouse and hamster), from a region near the N terminal (beginning at amino acid 51 of the mouse/hamster sequence). The amplified bat *grp170* nucleotide sequence encoded 179 amino acids about 18% of the full protein, again from a region near the N terminal (beginning at amino acid 32 of the mouse/hamster sequence). Squirrel and bat GRP170 amino acid sequences shared 94% identity with human GRP170 (Fig. 3.18).

3.3.2 *Grp170 mRNA expression in squirrel and bat tissues*

RT-PCR was carried out to check the mRNA expression level in tissues from hibernating versus euthermic squirrels and bats. Since the cDNA bands were not bright

using the degenerate primers for squirrel and bat, specific primers for squirrel and bat *grp170* were redesigned from the 500 bp of squirrel *grp170* partial nucleotide sequence and bat 580 bp of bat partial *grp170* nucleotide sequence, respectively. The specific primers for squirrels were: Forward 5'-TGGAGTGCCCATGGAGAT-3' and Reverse 5'-GCTCAGCCTGGTTGAAGA-3'; the optimum temperature is 66°C. For bat, the specific primers were: Forward 5'-TCCATGAAGGTGGCCATCGT-3' and Reverse 5'-CGGTGGCAGTGTTGTCATTG-3'; the optimum temperature is 64°C. After normalization of hibernating and control samples using amplification of tubulin primers (as in section 3.1.2), RT-PCR was carried out on the serial dilutions of each cDNA sample using the *grp170* specific primers. The PCR products were separated on an agarose gel and DNA bands were stained with ethidium bromide and band intensities were quantified under UV light in Syngene. The intensity of the bands at the higher dilutions was measured according to the method described in Chapter 2 and the formula listed in Fig.3.4. Following the calculation using Syngene software, the mean values from multiple runs were statistically analyzed.

In ground squirrels, levels of *grp170* transcripts were significantly increased in heart of hibernating animals by 1.8-fold over control values (Fig. 3.19a). By comparison, *grp170* transcripts in lung and kidney of hibernating animals were reduced to levels that were 76 and 84% of the euthermic values, respectively. Transcripts were stable in brain and liver and *grp170* transcripts were not detected in muscle, BAT and WAT. In bats, levels of *grp170* transcript did not change in brain and liver during hibernation but they decreased to 62- and 52 % of the euthermic value in kidney and lung, respectively (Fig.3.19b). The message was not detected in heart, muscle, BAT and WAT of bats.

Unlike *grp75* and *grp94* that were widely expressed with abundant transcript detected, *grp170* mRNA expression levels were very low in all tissues where they were detected and undetectable in most tissues.

Fig. 3.1 Partial nucleotide sequences of (A) thirteen-lined ground squirrel (*Spermophilus tridecemlineatus*) and (B) little brown bat (*Myotis lucifugus*) *grp75* and their deduced amino acid sequences. The partial cDNA sequences were amplified from squirrel and bat liver by RT-PCR using degenerate primers. A single open reading frame was predicted from the 482 bp nucleotide sequence encoding peptides of 160 amino acids.

A)

```
1       GCTTTTAGGTTTGTTTCAAGGAGAGATTATGCATCAGAAGCAATCAAGGGCGCAGTTGTT
1        A  F  R  F  V  S  R  R  D  Y  A  S  E  A  I  K  G  A  V  V

61      GGTATTGATTTGGGTACTACCAACTCCTGTGTGGCAGTTATGGAGGGTAAACAAGCAAAG
21       G  I  D  L  G  T  T  N  S  C  V  A  V  M  E  G  K  Q  A  K

121     GTGCTGGAGAATGCTGAAGGTGCCAGAACCACCCCTTCCGTTGTTGCCTTTACAGCAGAT
41       V  L  E  N  A  E  G  A  R  T  T  P  S  V  V  A  F  T  A  D

181     GGTGAACGACTTGTTGGGATGCCAGCGAAGCGACAGGCTGTCACCAACCCAAACAATACA
61       G  E  R  L  V  G  M  P  A  K  R  Q  A  V  T  N  P  N  N  T

241     TTCTATGCCACCAAGCGTCTCATTGGCCGGCGATACGATGACCCTGAAGTACAGAAAGAC
81       F  Y  A  T  K  R  L  I  G  R  R  Y  D  D  P  E  V  Q  K  D

301     ATTAAAAATGTTCCCTTCAAAATTGTTCGTGCCTCCAATGGTGATGCTTGGGTTGAGGCT
101      I  K  N  V  P  F  K  I  V  R  A  S  N  G  D  A  W  V  E  A

361     CACGGAAAACTGTATTCTCCAAGTCAAATTGGAGCATTTGTGTTGATGAAGATGAAAGAA
121      H  G  K  L  Y  S  P  S  Q  I  G  A  F  V  L  M  K  M  K  E

421     ACTGCAGAAAATTACTTGGGTCACACAGCAAAAAATGCTGTAATCACAGTCCCTGCTTAT
141      T  A  E  N  Y  L  G  H  T  A  K  N  A  V  I  T  V  P  A  Y

481     TT
```

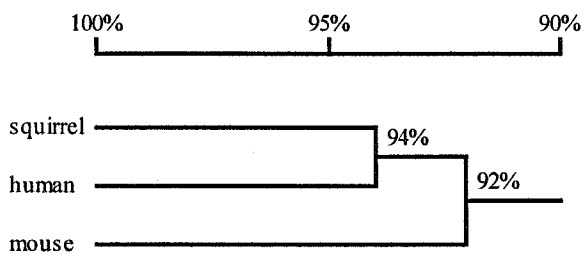
B)

```
1       ACTTTTAGAATTGTCTCAAAGCGGGACTATGCATCAGAAGCAATCAAGGGAGCAGTTGTT
1        T  F  R  I  V  S  K  R  D  Y  A  S  E  A  I  K  G  A  V  V

61      GGTATTGATTTGGGTACTACCAATTCCTGTGTGGCCGTTATGGAAGGTAAACAAGCAAAG
21       G  I  D  L  G  T  T  N  S  C  V  A  V  M  E  G  K  Q  A  K

121     GTGCTGGAGAATGCTGAAGGTGCCAGAACCACCCCTTCAGTTGTAGCCTTTACAACAGAT
41       V  L  E  N  A  E  G  A  R  T  T  P  S  V  V  A  F  T  T  D

181     GGTGAGCGACTTGTTGGCATGCCTGCCAAGCGACAGGCTGTCACCAACCCAAACAACACA
61       G  E  R  L  V  G  M  P  A  K  R  Q  A  V  T  N  P  N  N  T

241     CTCTATGCTACCAAGCGTCTTATTGGCCGGCGATATGACGACCCTGAAGTCCAGAAAGAC
81       L  Y  A  T  K  R  L  I  G  R  R  Y  D  D  P  E  V  Q  K  D

301     ATTAAAAATGTTCCCTTTAAAATTGTCCGTGCCTCCAATGGTGATGCCTGGGTTGAAGCT
101      I  K  N  V  P  F  K  I  V  R  A  S  N  G  D  A  W  V  E  A

361     CATGGAAAACTCTATTCTCCAAGTCAGATTGGAGCGTTTGTGTTGATGAAGATGAAAGAG
121      H  G  K  L  Y  S  P  S  Q  I  G  A  F  V  L  M  K  M  K  E

421     ACTGCAGAAAATTACCTGGGGCATACAGCAAAAAATGCTGTGATCACAGTCCCAGCTTAT
141      T  A  E  N  Y  L  G  H  T  A  K  N  A  V  I  T  V  P  A  Y

481     TT
```

Fig. 3.2 Comparison of *grp75* partial nucleotide sequences from (A) ground squirrel (*Spermophilus tridecemlineatus*) and (B) bat (*Myotis lucifugus*) with the human and mouse sequences and homology tree showing the percent identity between the sequences. Genbank accession numbers are: human (*Homo sapiens*, NM_004134) and mouse (*Mus musculus*, D17556). The nucleotide sequences were aligned using DNAMAN software and the homology tree generated by DNAMAN shows the percent identities between squirrel or bat *grp75* nucleotide sequences and the human and mouse sequences. For the human and mouse sequences dashes (-) replace those nucleotides that are identical with either the squirrel or bat sequences.

A)

```
squirrel   GCTTTTAGGTTTGTTTCAAGGAGAGATTATGCATCAGAAGCAATCAAGGGCGCAGTTGTT    60
human      --------ac----------c-g-------------------------a---------    60
mouse      --------a----------a-----------------------------t-----g---    60

squirrel   GGTATTGATTTGGGTACTACCAACTCCTGTGTGGCAGTTATGGAGGGTAAACAAGCAAAG   120
human      ----------------------------c--------------a---------------   120
mouse      --------------------t--------------t-----------c------------   120

squirrel   GTGCTGGAGAATGCTGAAGGTGCCAGAACCACCCCTTCCGTTGTTGCCTTTACAGCAGAT   180
human      --------------c-----------------------a-----g---------------   180
mouse      --c---------------------------t--------t--g-----------------   180

squirrel   GGTGAACGACTTGTTGGGATGCCAGCGAAGCGACAGGCTGTCACCAACCCAAACAATACA   240
human      -----g----------a-----g--c---------------------------------   240
mouse      --a--------------t--------a--a--g--a-----------t-----------c   240

squirrel   TTCTATGCCACCAAGCGTCTCATTGGCCGGCGATACGATGACCCTGAAGTACAGAAAGAC   300
human      --t-----t-------------------------t-----t------------------   300
mouse      --------t--t--------t-----a--a-----t-------------------------   300

squirrel   ATTAAAAATGTTCCCTTCAAAATTGTTCGTGCCTCCAATGGTGATGCTTGGGTTGAGGCT   360
human      ----------------t--------c--------------------c-------------   360
mouse      -c---g--------t--t--------c---------------------------------   360

squirrel   CACGGAAAACTGTATTCTCCAAGTCAAATTGGAGCATTTGTGTTGATGAAGATGAAAGAA   420
human      --t--g---t----------g-----g---------------------------------g   420
mouse      --t--------c--------------g---------------------------------g   420

squirrel   ACTGCAGAAAATTACTTGGGTCACACAGCAAAAAATGCTGTAATCACAGTCCCTGCTTAT   480
human      --------------------g-------------------g-----------a------   480
mouse      --------------------c-------------------g-----------------   480

squirrel   TT                                                           482
human      --                                                           482
mouse      --                                                           482
```



B)

```
bat       ACTTTTAGAATTGTCTCAAAGCGGGACTATGCATCAGAAGCAATCAAGGGAGCAGTTGTT   60
human     g--------c----t----g------t-------------------------------   60
mouse     g--------t----t----gaa-a--t----------------------t-----g---   60

bat       GGTATTGATTTGGGTACTACCAATTCCTGTGTGGCCGTTATGGAAGGTAAACAAGCAAAG  120
human     ----------------------c-----c-----a------------------------  120
mouse     --------------------t--c-----------t--------g--c------------  120

bat       GTGCTGGAGAATGCTGAAGGTGCCAGAACCACCCCTTCAGTTGTAGCCTTTACAACAGAT  180
human     --------------c-------------------------------g---------g-----  180
mouse     --c---------------------------t--------t--g--t---------g-----  180

bat       GGTGAGCGACTTGTTGGCATGCCTGCCAAGCGACAGGCTGTCACCAACCCAAACAACACA  240
human     -----------------a-----g-----------------------------------t---  240
mouse     --a--a-----------t-----a--a--a--g--a----------t--------t--c    240

bat       CTCTATGCTACCAAGCGTCTTATTGGCCGGCGATATGACGACCCTGAAGTCCAGAAAGAC  300
human     t-t-----------------c-----------------t--t--------a---------  300
mouse     t----------t--------------a--a--------t----------a---------  300

bat       ATTAAAAATGTTCCCTTTAAAATTGTCCGTGCCTCCAATGGTGATGCCTGGGTTGAAGCT  360
human     ------------------------------------------------------------g---  360
mouse     -c---g--------t-----------------------------------t--------g---  360

bat       CATGGAAAACTCTATTCTCCAAGTCAGATTGGAGCGTTTGTGTTGATGAAGATGAAAGAG  420
human     -----g---t-g--------g--------------a------------------------  420
mouse     ------------------------------------a------------------------  420

bat       ACTGCAGAAAATTACCTGGGGCATACAGCAAAAAATGCTGTGATCACAGTCCCAGCTTAT  480
human     ---------------t-------c-------------------------------------  480
mouse     ---------------t----c--c-----------------------------t------  480

bat       TT                                                            482
human     --                                                            482
mouse     --                                                            482
```

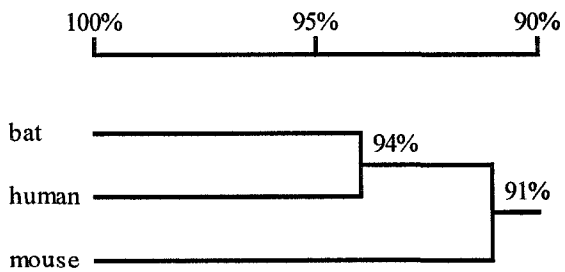


Fig. 3.3 Comparison of GRP75 partial amino acid sequences from ground squirrel (*Spermophilus tridecemlineatus*), bat (*Myotis lucifugus*), human, mouse and rat. Genbank accession numbers are: human (*Homo sapiens*, NM_004134), rat (*Rattus sp.*, S78556) and mouse (*Mus musculus*, D17556). DNAMAN software was used to translate the nucleotide sequences, generate the alignment and compute the percent identities. Dashes (-) replace those amino acids that are identical the squirrel.

A) Alignment of the five GRP75 partial amino acid sequences

B) Homology tree for GRP75 partial amino acid sequences

A)

```
squirrel   AFRFVSRRDYASEAIKGAVVGIDLGTTNSCVAVMEGKQAKVLENAEGARTTPSVVAFTAD     60
bat        t--i--k-----------------------------------------------------t-     60
human      ---l--------------------------------------------------------     60
mouse      --------------------------------------------------------------     60
rat        -----------------------------------------------------------p-     60

squirrel   GERLVGMPAKRQAVTNPNNTFYATKRLIGRRYDDPEVQKDIKNVPFKIVRASNGDAWVEA    120
bat        --------------------l-----------------------------------------    120
human      --------------------------------------------------------------    120
mouse      ----------------------------------------t---------------------    120
rat        ----------------------------------------t---------------------    120

squirrel   HGKLYSPSQIGAFVLMKMKETAENYLGHTAKNAVITVPAY                        160
bat        -----------------------------------------                        160
human      -----------------------------------------                        160
mouse      -----------------------------------------                        160
rat        -----------------------------------------                        160
```

B)

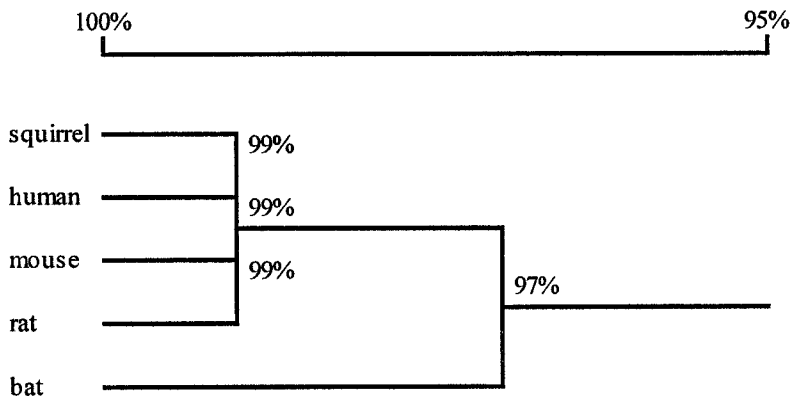
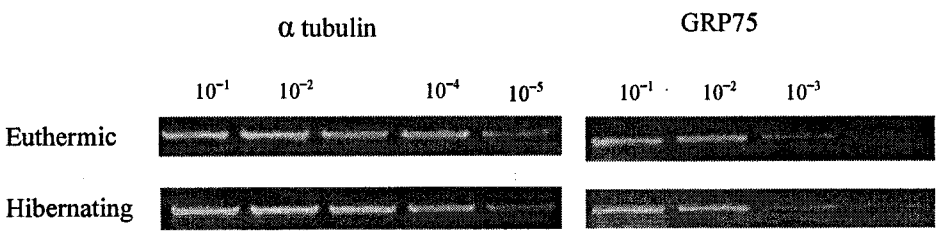


Fig. 3.4 Representative pictures showing RT-PCR product levels after amplification of serial dilutions of α-tubulin or *grp75* cDNA from squirrel liver extracts cDNA and the formula used for the calculation of mRNA expression level. Band intensities from higher dilutions, (10^{-5}) for α-tubulin and (10^{-3}) *grp75*, were used to calculate the relative mRNA expression levels in euthermic versus hibernating situations.



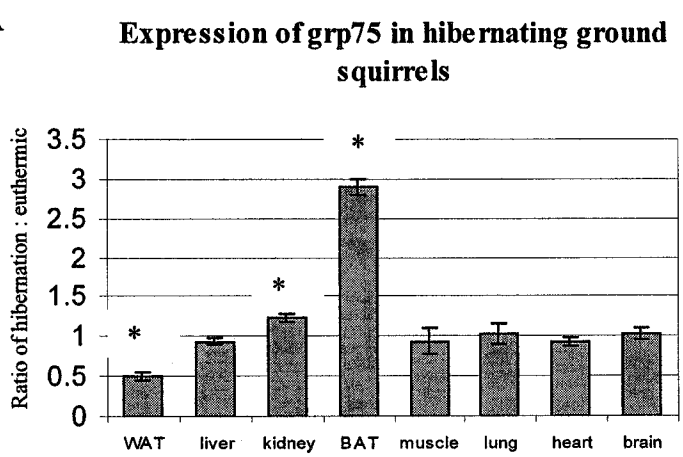
$$\text{Relative mRNA level} = \frac{\text{Hibernating GRP75 } 10^{-3} \text{ / hibernating tubulin } 10^{-5}}{\text{Euthermic GRP75 } 10^{-3} \text{ / euthermic tubulin } 10^{-5}}$$

Fig. 3.5 The effects of hibernation on (A) ground squirrel and (B) bat *grp75* mRNA expression in different tissues as determined from RT-PCR. Histograms show the ratio of *grp75* mRNA levels in hibernating versus euthermic tissues. Data are means ± SEM for n = 3 independent experiments for both euthermic and hibernating samples. * - Mean value for hibernating sample is significantly different from the corresponding euthermic value, $P < 0.05$.

A) Squirrel *grp75* relative mRNA expression level.

B) Bat *grp75* relative mRNA expression level.

A



B

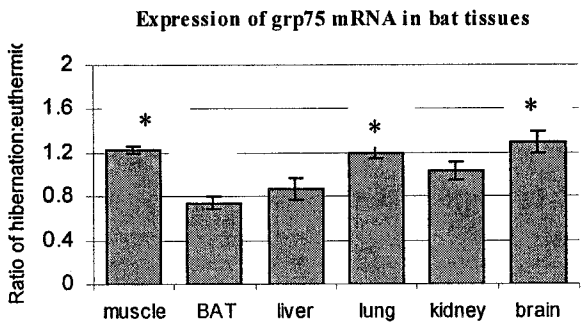


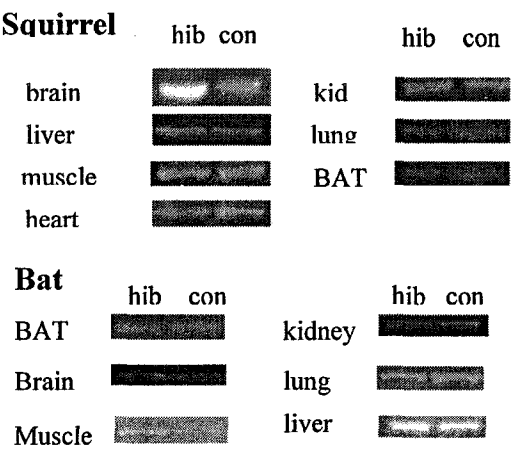
Fig. 3.6. The effects of hibernation on ground squirrel and bat GRP75 protein expression in different tissues.

A) Representative Western blots showing GRP75 protein levels in seven tissues of euthermic (con) and hibernating (hib) ground squirrels and bats.

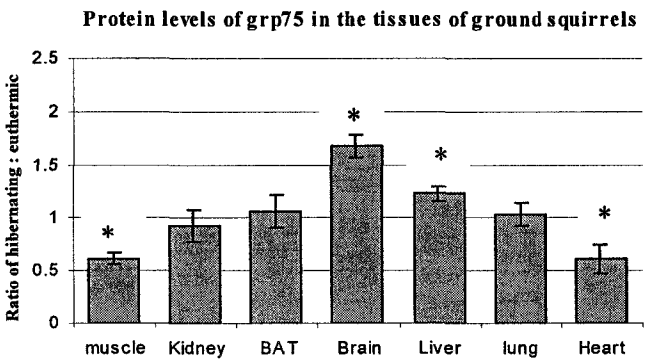
B) Histograms show the ratio of GRP75 protein levels in hibernating versus euthermic ground squirrels. Mean values (± SEM) were calculated from n = 3 trials. * - Mean value for hibernating sample is significantly different from the corresponding euthermic value, $P < 0.05$.

C) Histograms show the ratio of GRP75 protein levels in hibernating versus euthermic bats. Mean values (± SEM) were calculated from n = 3 trials. * - Mean value for hibernating sample is significantly different from the corresponding euthermic value, $P < 0.05$.

A)



B)



C)

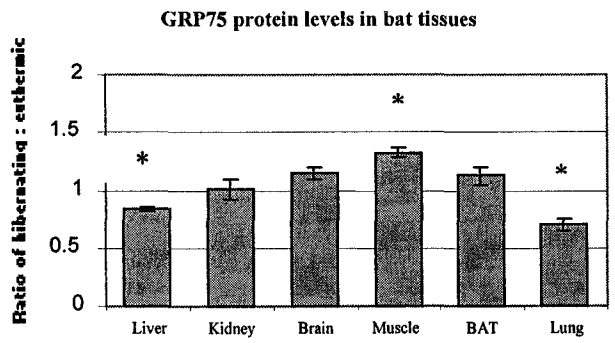
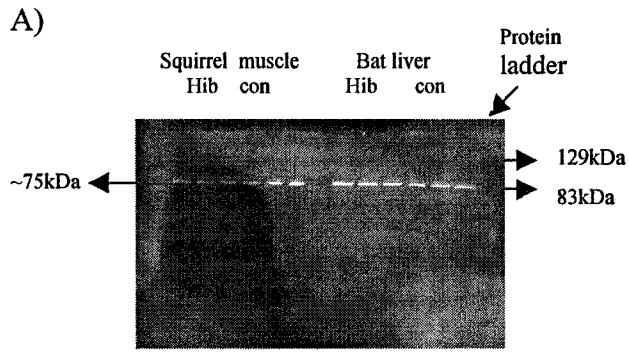
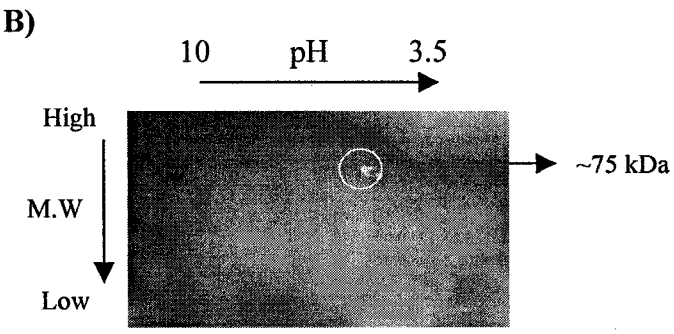


Fig. 3.7 Representative Western blots and 2D-PAGE Western blots showing GRP75 protein bands in ground squirrel muscle (A) and spots in liver (B) and bat liver (C), respectively.





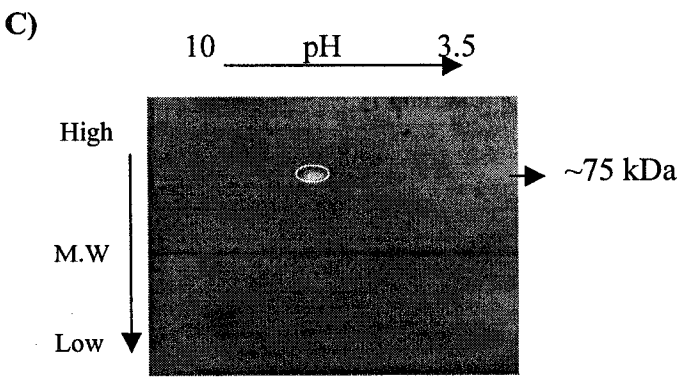


Fig.3.8 GRP75 protein distribution in nuclear versus cytoplasmic fractions as assessed after subcellular fractionation and Western blotting.

A) Representative Western blots show GRP75 levels in cytoplasmic and nuclear fractions of ground squirrel skeletal muscle from euthermic control (con) versus hibernating (hib) animals.

B) Histogram shows the relative expression levels of GRP75 (hibernating versus control) in cytoplasmic and nuclear fractions of squirrel muscle. Mean values (± SEM) were calculated from n = 3 independent trials. * - Mean value for hibernating sample was significantly different from the corresponding euthermic value, $P < 0.05$.

A)

B)

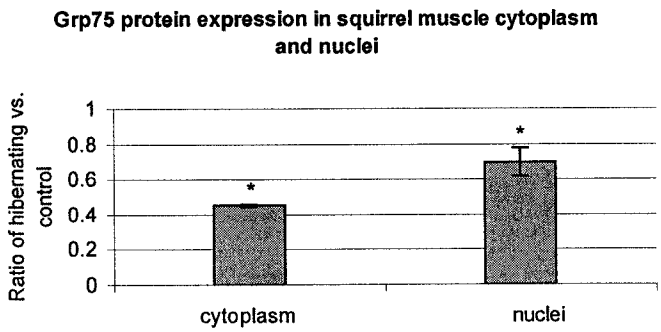


Fig. 3.9 Partial nucleotide sequence of ground squirrel (*Spermophilus tridecemlineatus*) *grp94* and its deduced amino acid sequence. The partial cDNA sequence was amplified from squirrel brain by RT-PCR using degenerate primers. A single open reading frame was predicted from the 550 bp of nucleotide sequence which encoded a peptide with 183 amino acid residues.

```
1          CGGGTCAGTTAGAGCTGACGATGAAGTCGATGTGGATGGTACAGTGGAAGAGGACCTGGG

1            G  S  V  R  A  D  D  E  V  D  V  D  G  T  V  E  E  D  L  G

61         CAAAAGCAGAGAAGGCTCAAGGACAGATGATGAAGTAGTACAGAGAGAGGAAGAAGCTAT
21           K  S  R  E  G  S  R  T  D  D  E  V  V  Q  R  E  E  E  A  I

121        ACAGTTGGATGGATTAAATGCATCACAAATACGAGAACTTAGAGAGAAATCTGAAAAGTT
41           Q  L  D  G  L  N  A  S  Q  I  R  E  L  R  E  K  S  E  K  F

181        TGCCTTCCAAGCTGAAGTTAACAGGATGATGAAACTTATCATCAATTCATTATATAAAAA
61           A  F  Q  A  E  V  N  R  M  M  K  L  I  I  N  S  L  Y  K  N

241        TAAAGAGATTTTCCTGAGAGAACTGATTTCCAATGCTTCTGATGCTTTAGATAAGATAAG
81           K  E  I  F  L  R  E  L  I  S  N  A  S  D  A  L  D  K  I  R

301        GCTAATATCACTAACTGATGAAAATGCGCTTTCTGGAAATGAGGAATTAACTGTCAAAAT
101          L  I  S  L  T  D  E  N  A  L  S  G  N  E  E  L  T  V  K  I

361        TAAGTGTGACAAGGAGAAAAACCTACTACATGTCACAGACACTGGTGTAGGAATGACCAG
121          K  C  D  K  E  K  N  L  L  H  V  T  D  T  G  V  G  M  T  R

421        AGAAGAGTTGGTTAAAAACCTTGGTACCATAGCCAAATCTGGGACAAGTGAATTTTTAAA
141          E  E  L  V  K  N  L  G  T  I  A  K  S  G  T  S  E  F  L  K

481        AAAAATGMCTGAAGCACAAGAAGATAGCCAGTCAACTTCTGAATTGATTGGCCAGTTTGG
161          K  M  X  E  A  Q  E  D  S  Q  S  T  S  E  L  I  G  Q  F  G

541        TGTTGGTTTC
181          V  G  F
```

Fig. 3.10 Comparison of squirrel *grp94* partial nucleotide sequence with (*Homo sapiens*, NM_003299), mouse (*Mus musculus*, NM_011631), and cow (*Bos taurus*, NM_174700) *grp94* partial sequences and the corresponding homology tree. The Genbank accession numbers are provided in brackets. Nucleotide sequences are aligned and the homology tree was generated using DNAMAN software. The ground squirrel *grp*94 nucleotides are displayed, whereas for the other sequences dashes (-) are used to replace bases that are the same as in the ground squirrel sequence.

```
squirrel   CGGGTCAGTTAGAGCTGACGATGAAGTCGATGTGGATGGTACAGTGGAAGAGGACCTGGG    60
human      ------g--c----------------t----------------a--------t-----    60
mouse      -----tc--c--------t-----------c--------c-------------------    60
cow        ---------gc-g--------c-----g----------g----------a--t-----    60

squirrel   CAAAAGCAGAGAAGGCTCAAGGACAGATGATGAAGTAGTACAGAGAGAGGAAGAAGCTAT   120
human      t-----t--------a--------g-----------------------------------   120
mouse      t------c-----------------------------t--g-------------------   120
cow        t-----t-----------------------------------------------------   120

squirrel   ACAGTTGGATGGATTAAATGCATCACAAATACGAGAACTTAGAGAGAAATCTGAAAAGTT   180
human      t-------------------------------a----------------g--g--------   180
mouse      t----------g-----c--------g---a-------------a--------------   180
cow        t----------------------c------a-------------------a--------   180

squirrel   TGCCTTCCAAGCTGAAGTTAACAGGATGATGAAACTTATCATCAATTCATTATATAAAAA   240
human      ------------c----------a-----------------------g--------   240
mouse      c----------------g-----------------------------t--g--------   240
cow        ----------------------a----------c--------------g--------   240

squirrel   TAAAGAGATTTTCCTGAGAGAACTGATTTCCAATGCTTCTGATGCTTTAGATAAGATAAG   300
human      ------------------------------a-----------------------------   300
mouse      ------------------------------a--------------------c--------   300
cow        ----------------c-------------a-----------------------------   300

squirrel   GCTAATATCACTAACTGATGAAAATGCGCTTTCTGGAAATGAGGAATTAACTGTCAAAAT   360
human      ----------g--------------t-------------------c----a--------   360
mouse      ---c--c--c-----------------a--cg------------g-----g-----g--   360
cow        a----------g--------------t---g------------g--g--g--t-----   360

squirrel   TAAGTGTGACAAGGAGAAAAACCTACTACATGTCACAGACACTGGTGTAGGAATGACCAG   420
human      ---------t--------g-----g--g--------------c----------------   420
mouse      ------------a-----------g--g--------------g--------------t--   420
cow        ------------------g-----g--g-------------------------------c-   420

squirrel   AGAAGAGTTGGTTAAAAACCTTGGTACCATAGCCAAATCTGGGACAAGTGAATTTTTAAA   480
human      ------------------------------------------------c--g--------   480
mouse      ---g--------------t--c--c-----------------a-----c--g--------   480
cow        g--g--------g----------c----------g-----a-----c--g--------   480

squirrel   AAAAATGMCTGAAGCACAAGAAGATAGCCAGTCAACTTCTGAATTGATTGGCCAGTTTGG   540
human      c------a----------g------g-------------------------------   540
mouse      c------a-a-----t---------g-t--------c------c----------------   540
cow        c------a----g-----------g----------a--g---c----c--t--------   540

squirrel   TGTTGGTTTC                                                   550
human      ---c------                                                   550
mouse      ---c-----t                                                   550
cow        ----------                                                   550
```

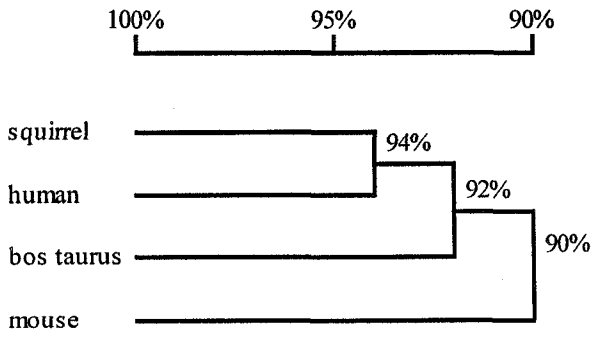


Fig. 3.11. Comparison of GRP94 partial amino acid sequences. Alignment of partial GRP94 amino acid sequences from ground squirrel (*Spermophilus tridecemlineatus*), human (*Homo sapiens*, NM_003299), mouse (*Mus musculus*, NM_011631), and cow (*Bos taurus*, NM_174700) using DNAMAN software. The partial amino acid sequences were the translation products of the nucleotides using DNAMAN software. The Genbank accession numbers for the nucleotide sequences are provided in brackets. The ground squirrel GRP94 sequence is displayed, whereas for the other sequences dashes (-) are used to replace amino acids that are the same as in the ground squirrel sequence.

```
squirrel   GSVRADDEVDVDGTVEEDLGKSREGSRTDDEVVQREEEAIQLDGLNASQIRELREKSEKF   60
human      ------------------------------------------------------------   60
mouse      -f----------------------------------------------------------   60
cow        ------------------------------------------------------------   60

squirrel   AFQAEVNRMMKLIINSLYKNKEIFLRELISNASDALDKIRLISLTDENALSGNEELTVKI  120
human      ------------------------------------------------------------  120
mouse      ----------------------------------------------------a-------  120
cow        ----------------------------------------------------a-------  120

squirrel   KCDKEKNLLHVTDTGVGMTREELVKNLGTIAKSGTSEFLKKMXEAQEDSQSTSELIGQFG  180
human      ----------------------------------------n--t-----g-----------  180
mouse      ----------------------------------------n--t-----g-----------  180
cow        ----------------------------------------n--t-----g-----------  180

squirrel   VGF                                                           183
human      ---                                                           183
mouse      ---                                                           183
cow        ---                                                           183
```

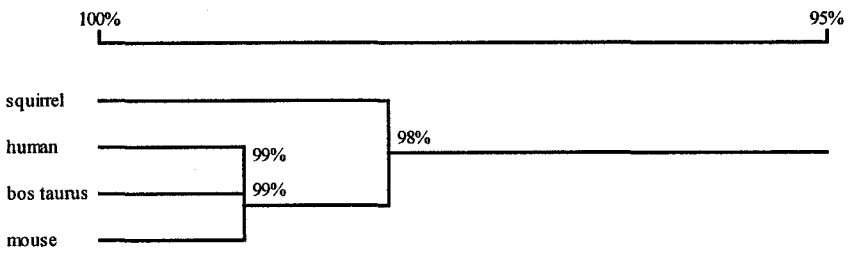


Fig. 3.12 The effects of hibernation on ground squirrel *grp94* mRNA expression in different tissues. Histograms show the ratio of *grp94* mRNA levels in tissues of hibernating versus euthermic animals. Mean values (± SEM) for band intensities were calculated from n = 3 separate isolations of mRNA from both euthermic and hibernating tissues. *- Mean value for hibernating sample was significantly different from the corresponding euthermic value, $P < 0.05$.

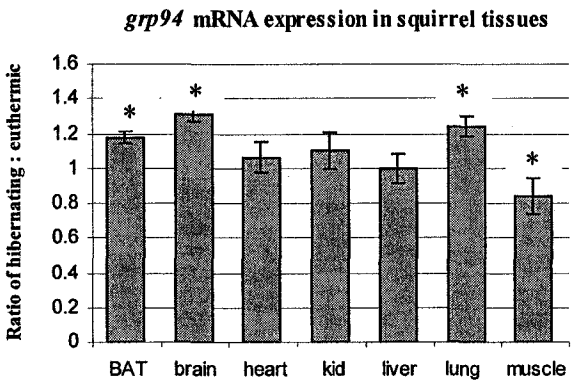
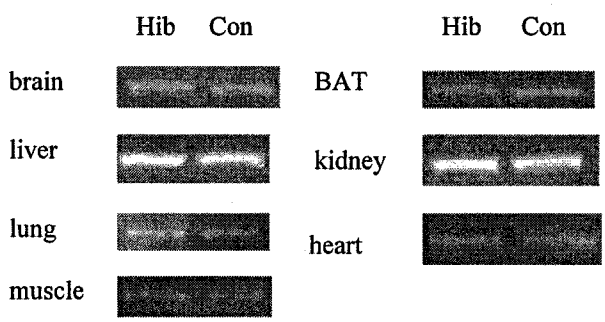


Fig. 3.13 The effects of hibernation on ground squirrel GRP94 protein expression in different tissues.

A) Representative western blots showing GRP94 protein levels in seven tissues of control euthermic (con) and hibernating (hib) ground squirrels.

B) Histograms show the ratio of GRP94 protein levels in hibernating versus euthermic ground squirrels. Mean values (± SEM) were calculated from n = 3 independent trials. * - Mean value for hibernating samples was significantly different from the corresponding euthermic value, $P < 0.05$.

A)



B)

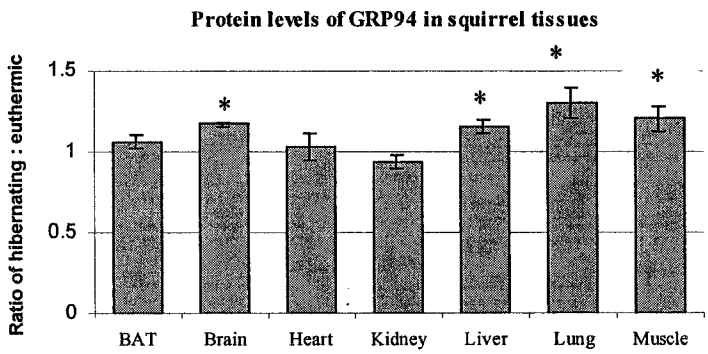
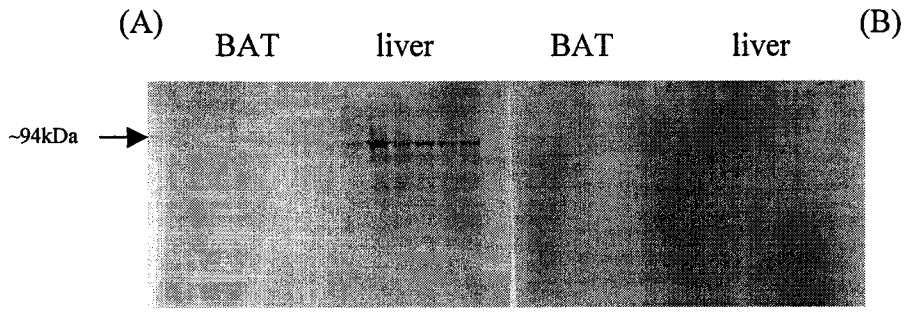


Fig. 3.14 Representative Western blots run without (A, left) and with (B, right) the GRP94 blocking peptide using extracts from ground squirrel BAT and liver. (C) The 94 kDa band of GRP94 in western blot bands of liver is shown together with the protein standard ladder.



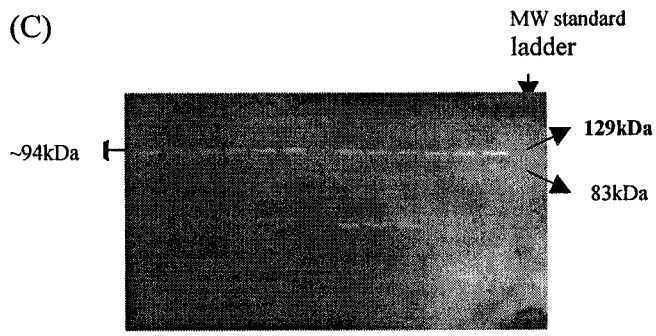


Fig. 3.15 GRP94 protein distribution between nuclear and cytoplasmic fractions as assessed after subcellular fractionation and Western blotting.

A) Representative Western blots show GRP94 present in both cytoplasmic and nuclear fractions of skeletal muscle from control euthermic (con) and hibernating (hib) squirrels.

B) Histogram shows GRP94 relative expression levels in squirrel muscle cytoplasmic and nuclear fractions in hibernating versus euthermic control animals. Mean values (± SEM) were calculated from n = 3 trials. *- Mean value for hibernating sample was significantly different from the corresponding euthermic value, $P < 0.05$.

A)

B)

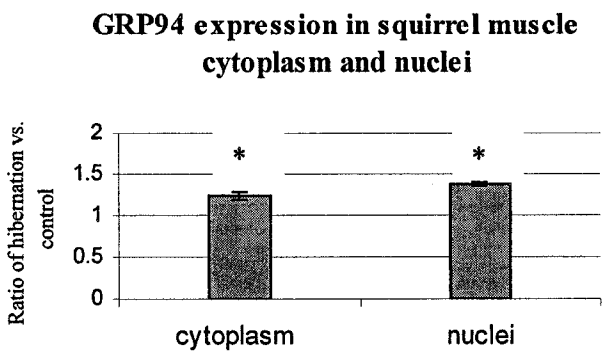


Fig. 3.16 Partial nucleotide sequences of *grp170* from (A) thirteen-lined ground squirrel (*Spermophilus tridecemlineatus*) and (B) little brown bat (*Myotis lucifugus*) with their corresponding deduced amino acid sequences. The partial cDNA sequences were amplified from squirrel kidney and bat brain by RT-PCR using degenerate primers. A single open reading frame was predicted from the 460 bp squirrel and 538 bp bat nucleotide sequences which encoded peptides of 153 and 179 amino acids, respectively.

A)

```
1          AAACCTGGAGTGCCCATGGAGATTGTCTTGAACAAGGAATCTCGGAGGAAAACCCCAGTA
1           K  P  G  V  P  M  E  I  V  L  N  K  E  S  R  R  K  T  P  V

61         ACCGTGACCCTAAAAGAAAATGAAAGATTCTTTGGAGACACTGCAGCAGGCATGGCCATC
21          T  V  T  L  K  E  N  E  R  F  F  G  D  T  A  A  G  M  A  I

121        AAGAACCCAAAGGCTACGTTACGTTATTTCCAGCACCTTCTAGGGAAACAGGCAGATAAC
41          K  N  P  K  A  T  L  R  Y  F  Q  H  L  L  G  K  Q  A  D  N

181        CCCCATGTGGCCCTGTATCGGGCCCGTTTTCCAGAGCATGAGCTAAGCTTTGACCCACAG
61          P  H  V  A  L  Y  R  A  R  F  P  E  H  E  L  S  F  D  P  Q

241        AGGCAGACTGTGTACTTCCAGATCAGCCCGCAGCTTCAGTTCTCACCTGAGGAGGTTTTA
81          R  Q  T  V  Y  F  Q  I  S  P  Q  L  Q  F  S  P  E  E  V  L

301        GGCATGGTTCTCAATTACTCCCGGTCCCTGGCTGAAGATTTCGCAGAGCAGCCCATCAAG
101         G  M  V  L  N  Y  S  R  S  L  A  E  D  F  A  E  Q  P  I  K

361        GATGCTGTCATCACCGTGCCAGCCTTCTTCAACCAGGCTGAGCGTAGAGCTGTGCTTCAA
121         D  A  V  I  T  V  P  A  F  F  N  Q  A  E  R  R  A  V  L  Q

421        GCTGCCCGAATGGCTGGCCTTAAAGTACTGCAGCTCATCA
141         A  A  R  M  A  G  L  K  V  L  Q  L  I
```

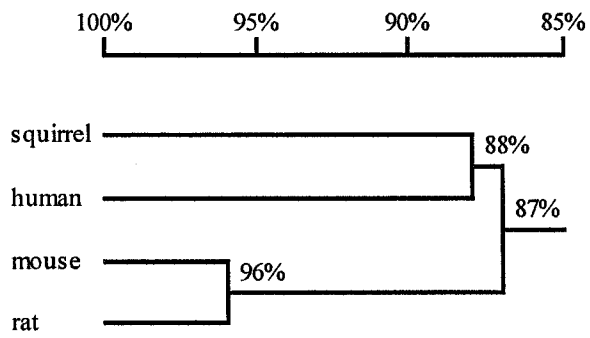
B)

```
1          CATGCTGGCAGTGATGTCAGTGGACCTGGGCAGCGAGTCCATGAAGGTGGCCATCGTCAA
1            M  L  A  V  M  S  V  D  L  G  S  E  S  M  K  V  A  I  V  K

61         ACCTGGAGTTCCCATGGAAATTGTCCTGAACAAAGAATCCCGGAGGAAAACCCCAGTGAC
21           P  G  V  P  M  E  I  V  L  N  K  E  S  R  R  K  T  P  V  T

121        TGTGACCCTGAAGGAAAATGAAAGATTCTTTGGAGACAGTGCAGCAAGCATGGCCATCAA
41           V  T  L  K  E  N  E  R  F  F  G  D  S  A  A  S  M  A  I  K

181        GAATCCAAAGGCTACGCTGCGTTACTTCCAGCAGCTCCTGGGGAAGCAGGAGGATAACCC
61           N  P  K  A  T  L  R  Y  F  Q  Q  L  L  G  K  Q  E  D  N  P

241        CCATGTGGCCCTTTACAGAGAGCGGTTCCCAGAGCATGAGCTGGGCTTCGACCCGCAGAG
81           H  V  A  L  Y  R  E  R  F  P  E  H  E  L  G  F  D  P  Q  R

301        GCAGACTGTGCGCTTCCAAATCAGCCCGCAGCTGCAGTTCTCACCTGAGGAGGTACTGGG
101          Q  T  V  R  F  Q  I  S  P  Q  L  Q  F  S  P  E  E  V  L  G

361        CATGGTTCTCAATTACTCCCGTTCCCTGGCTGAAGACTTTGCAGAGCAGCCCATCAAGGA
121          M  V  L  N  Y  S  R  S  L  A  E  D  F  A  E  Q  P  I  K  D

421        TGCAGTGATCACCGTGCCAGCCTTCTTCAATCAGGCCGAGCGCCGAGCTGTGCTGCAAGC
141          A  V  I  T  V  P  A  F  F  N  Q  A  E  R  R  A  V  L  Q  A

481        TGCTCGCATGGCTGGCCTCAAAGTGCTGCAGCTCATCAATGACAACACTGCCACCGCC
161          A  R  M  A  G  L  K  V  L  Q  L  I  N  D  N  T  A  T  A
```

Fig. 3.17 Comparison of *grp170* partial nucleotide sequences from (A) ground squirrel (*Spermophilus tridecemlineatus*) and (B) bat (*Myotis lucifugus*) with the human, mouse and rat sequences and a homology tree showing the percent identities between the sequences. Genbank accession numbers are: human (*Homo sapiens*, U65785), mouse (*Mus musculus*, AF228709) and rat (*Rattus norvegicus*, U41853). The nucleotide sequences were aligned and the homology tree was generated using DNAMAN software. For the human, mouse and rat sequences dashes (-) replace those nucleotides that are identical with either the squirrel or bat sequence.

A)

```
squirrel   AAACCTGGAGTGCCCATGGAGATTGTCTTGAACAAGGAATCTCGGAGGAAAACCCCAGTA    60
human      --------------------a----------t--------------------a--g--g    60
mouse      --g----------------------a-------------------------t-----g    60
rat        --g----------------------a-------------------------t--g--g    60

squirrel   ACCGTGACCCTAAAAGAAAATGAAAGATTCTTTGGAGACACTGCAGCAGGCATGGCCATC   120
human      -t---------g-----------------------------g-------a-------g--t   120
mouse      --t------t-g--------------g--t--a--t--t-g------c------------   120
rat        --t------t-g--g-----c-----g--tc-a--t----g------t------------   120

squirrel   AAGAACCCAAAGGCTACGTTACGTTATTTCCAGCACCTTCTAGGGAAACAGGCAGATAAC   180
human      -----t------------c-------c-----------c--g-----g------------   180
mouse      ------------------c-c-----------------c--t--a--------g------   180
rat        ------------------c-c-----------------c--t--a--g------------   180

squirrel   CCCCATGTGGCCCTGTATCGGGCCCGTTTTCCAGAGCATGAGCTAAGCTTTGACCCACAG   240
human      --------a--t--t--c-a------c--c--g-----c-----g-ct--c---------   240
mouse      --t-----------t--c---t-------c-----a----------ttg-----------   240
rat        --t---------t--t--c---t-------c-----a--------c-atg-----------   240

squirrel   AGGCAGACTGTGTACTTCCAGATCAGCCCGCAGCTTCAGTTCTCACCTGAGGAGGTTTTA   300
human      ------------c----t---------t-------g----------------a--g--g   300
mouse      ------------cg----------t--------g--------t--c--------ac-g   300
rat        ------------cg----------t--------g--------t--c--------gc-g   300

squirrel   GGCATGGTTCTCAATTACTCCCGGTCCCTGGCTGAAGATTTCGCAGAGCAGCCCATCAAG   360
human      -----------------t--t--t--t--a----------t-----------------   360
mouse      -----------g--c--------t---t------------t--t--a--a-----t---   360
rat        --------------c--------t----------------t-----a--a--t--t---   360

squirrel   GATGCTGTCATCACCGTGCCAGCCTTCTTCAACCAGGCTGAGCGTAGAGCTGTGCTTCAA   420
human      -----a--g-------------t---------------c-----cc----------g--g   420
mouse      -----a--g-----------------t-----------------cc----------g--g   420
rat        -----a--g-----------------t----------c-----cc----------g--g   420

squirrel   GCTGCCCGAATGGCTGGCCTTAAAGTACTGCAGCTCATCA                      460
human      -----t--t-----------c-----g-------------                      460
mouse      -----t--g-----------c--g--g-------------                      460
rat        -----t--t-----------c--g--g-------------                      460
```



B)

```
bat       CATGCTGGCAGTGATGTCAGTGGACCTGGGCAGCGAGTCCATGAAGGTGGCCATCGTCAA   60
human     t-ca----------t----------t----------t-----                     60
mouse     --cat----t--------t--a----------t--a----------t-----           60
rat       --ca-----t--------t----------t--a----------t-----              60

bat       ACCTGGAGTTCCCATGGAAATTGTCCTGAACAAAGAATCCCGGAGGAAAACCCCAGTGAC  120
human     ---------g----------t----t--g-----t----------a--g----t         120
mouse     g--------g--------g-----at-------g-----t----------t--------    120
rat       g--------g--------g-----at-------g-----t----------t--g-----    120

bat       TGTGACCCTGAAGGAAAATGAAAGATTCTTTGGAGACAGTGCAGCAAGCATGGCCATCAA  180
human     c----------a-------------------------------------------g--t--  180
mouse     -------t----a----------g--t--a--t--t--------cg----------      180
rat       -------t----------c-----g--tc-a--t----------tg----------      180

bat       GAATCCAAAGGCTACGCTGCGTTACTTCCAGCAGCTCCTGGGGAAGCAGGAGGATAACCC  240
human     ----------------a----------c----------ca--------              240
mouse     ---c----------c-----t--------c-----t--a--a----c---------      240
rat       ---c----------c-----t--------c-----t--a-------ca--------      240

bat       CCATGTGGCCCTTTACAGAGAGCGGTTCCCAGAGCATGAGCTGGGCTTCGACCCGCAGAG  300
human     ------a--t------cag-cc--c-----g-----c------act--------a-----  300
mouse     t---------------c-gtcc--t--------a--------aattg-t-----a-----  300
rat       t--------t------c-gtcc--t--------a--------caatg-t-----a-----  300

bat       GCAGACTGTGCGCTTCCAAATCAGCCCGCAGCTGCAGTTCTCACCTGAGGAGGTACTGGG  360
human     ----------a---t--g------t----------a--gt----                  360
mouse     ----------------g-----t----------t--c----------              360
rat       ----------------g-----t----------t--c--------g-----           360

bat       CATGGTTCTCAATTACTCCCGTTCCCTGGCTGAAGACTTTGCAGAGCAGCCCATCAAGGA  420
human     ---------------t--t-----t--a--------t----------               420
mouse     ---------g--c----------t----------t-----t--a--a-----t-----    420
rat       ----------c----------t--------a--a--t--t-----                 420

bat       TGCAGTGATCACCGTGCCAGCCTTCTTCAATCAGGCCGAGCGCCGAGCTGTGCTGCAAGC  480
human     ----------t---------c----------g--                            480
mouse     ----------t-----c-----t----------g--                          480
rat       ----------t-----c----------g--                                480

bat       TGCTCGCATGGCTGGCCTCAAAGTGCTGCAGCTCATCAATGACAACACTGCCACCGCC    538
human     ------t----------c-----t---                                   538
mouse     ------g----------g----------a---                              538
rat       ------t----------g----------a---                              538
```

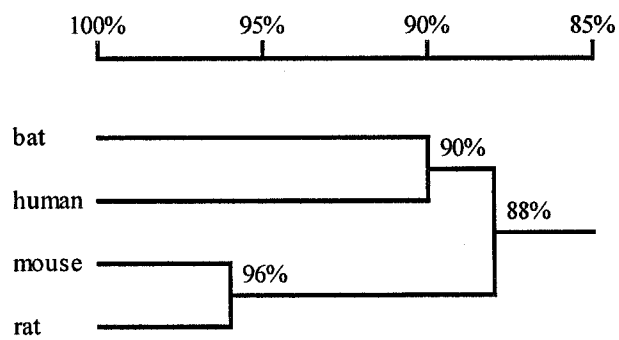
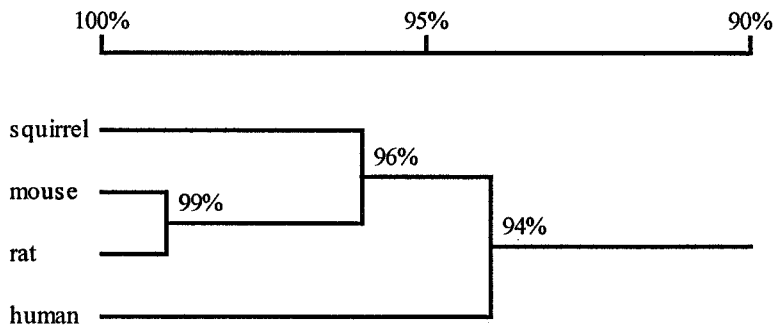


Fig.3.18 Comparison of squirrel (A) or bat (B) GRP170 partial amino acid sequence with corresponding human, mouse and rat amino acid sequences; all the five amino acid sequences were compared as well (C). Genbank accession numbers are: human (*Homo sapiens*, U65785), mouse (*Mus musculus*, AF228709) and rat (*Rattus sp.*, U41853). DNAMAN software was used to translate the nucleotide sequences, generate the alignment and compute the percent identities. Dashes (-) replace those amino acids that are identical with the squirrel or bat sequences. Dots (.) represent the absence of the amino acids.

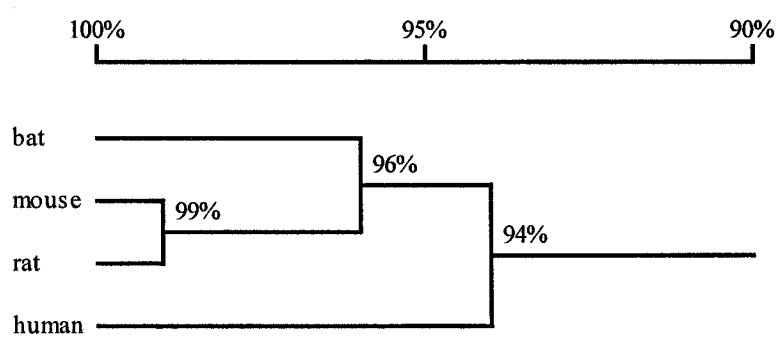
A)

```
squirrel   MEIVLNKESRRKTPVTVTLKENERFFGDTAAGMAIKNPKATLRYFQHLLGKQADNPHVAL   60
human      ---------------i-----------s--s-----------------------------   60
mouse      ------------------------l--s-----------------------------   60
rat        ------------------------l--s-----------------------------   60

squirrel   YRARFPEHELSFDPQRQTVYFQISPQLQFSPEEVLGMVLNYSRSLAEDFAEQPIKDAVIT   120
human      -q--------t--------h----s-----------------------------------   120
mouse      --s-------iv-------r-------------------------------------   120
rat        --s-------nv-------r-------------------------------------   120

squirrel   VPAFFNQAERRAVLQAARMAGLKVLQLI                                148
human      --v-------------------------                                148
mouse      ----------------------------                                148
rat        ----------------------------                                148
```



B)

```
bat        MLAVMSVDLGSESMKVAIVKPGVPMEIVLNKESRRKTPVTVTLKENERFFGDSAASMAIK   60
human      t-------------------------------------i--------------------   60
mouse      t-----------------------------------------------l-----g----   60
rat        t-----------------------------------------------l-----g----   60

bat        NPKATLRYFQQLLGKQEDNPHVALYRERFPEHELGFDPQRQTVRFQISPQLQFSPEEVLG   120
human      ----------h-----a--------qa-------t--------h----s-----------   120
mouse      ----------h-----a---------s-------iv-----------------------   120
rat        ----------h-----a---------s-------nv-----------------------   120

bat        MVLNYSRSLAEDFAEQPIKDAVITVPAFFNQAERRAVLQAARMAGLKVLQLINDNTATA   179
human      -------------------------v-------------------------------   179
mouse      -----------------------------------------------------------   179
rat        -----------------------------------------------------------   179
```



C)

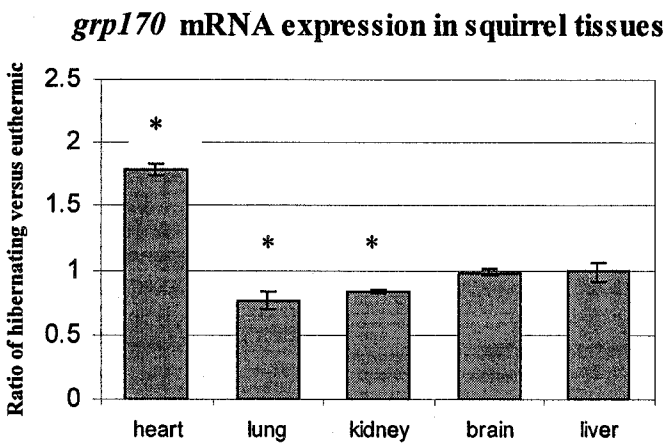
```
bat        ............................MLAVMSVDLGSESMKVAIVKPGVPMEIVL     29
squirrel   ..................................................----------     10
human      madkvrrqrprrrvcwalvavlladllalsdt----------------------------     60
mouse      maatvrrqrprrllcwalvavlladllalsdt----------------------------     60
rat        maatvrrqrprrllcwalvavlladllalsdt----------------------------     60

bat        NKESRRKTPVTVTLKENERFFGDSAASMAIKNPKATLRYFQQLLGKQEDNPHVALYRERF     89
squirrel   ----------------------t--g--------------h-----a---------a--     70
human      ----------i-----------------------------h-----a---------qa--    120
mouse      --------------------l-----g--------------h-----a---------s--    120
rat        --------------------l-----g--------------h-----a---------s--    120

bat        PEHELGFDPQRQTVRFQISPQLQFSPEEVLGMVLNYSRSLAEDFAEQPIKDAVITVPAFF    149
squirrel   -----s--------y-----------------------------------------------    130
human      -----t--------h----s-------------------------------------v--    180
mouse      -----iv--------------------------------------------------------    180
rat        -----nv--------------------------------------------------------    180

bat        NQAERRAVLQAARMAGLKVLQLINDNTATA.............................    179
squirrel   ----------------------.....................................    153
human      ------------------------------lsygvfrrkdinttaqnimfydmgsgstvc    240
mouse      ------------------------------lsygvfrrkdinstaqnvmfydmgsgstvc    240
rat        ------------------------------lsygvfrrkdinstaqnimfydmgsgstvc    240
```

Fig. 3.19 The effects of hibernation on (A) ground squirrel and (B) bat *grp170* mRNA expression in different tissues as determined from RT-PCR. Histograms show the ratio of *grp170* mRNA levels in tissues from hibernating versus euthermic animals. Data are means ± SEM for n = 3 independent experiments for both euthermic and hibernating samples. * - Mean value for hibernating sample is significantly different from the corresponding euthermic value, $P < 0.05$.

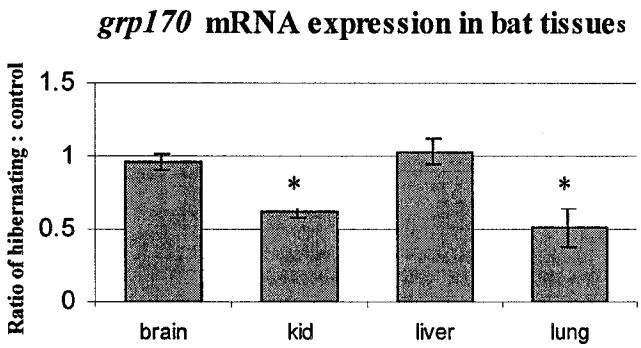
A) Squirrel *grp170* relative mRNA expression level.

B) Bat *grp170* relative mRNA expression level

A



B



CHAPTER 4

UP-REGULATION OF HEAT SHOCK PROTEINS DURING HIBERNATION

Results

4.1 HSP72

4.1.1. RT-PCR retrieval of squirrel and bat hsp72

RT-PCR was carried out to retrieve the cDNA segments for *hsp72* from squirrel and bat. Degenerate primers were designed based on the consensus sequences of *hsp72* from several other mammalian species: Forward 5'-CACGGCAAGGTGGAGATCAT-3' and Reverse 5'-CGCTTGTTCTGGCTGATGTC-3'. The optimum annealing temperature for the primers was 56°C and 64°C, respectively, for the tissue sources used: squirrel kidney and bat brain. Fig. 4.1 shows the *hsp72* cDNA segments that were amplified for squirrel (A) and bat (B) and the corresponding translated amino acid sequences. Analysis using the BLAST program at the NCBI website and DNAMAN software confirmed that the sequences encoded *hsp72*. DNA alignment results showed that the 660 bp of the squirrel *hsp72* segment shared 95% identity with cow and human sequences, respectively, whereas the 660 bp of bat *hsp72* segment shared 97% and 96% identities with human and cow, respectively (Fig. 4.2). The squirrel and bat nucleotide segments encoded peptides with 219 amino acids, about 34% of the full protein (641 amino acids in humans) from a region near N terminal (beginning at amino acid 37 of the human sequence). Amino acid sequence identity was high; squirrel and bat HSP72 shared 99% and 96% identity, respectively, with the human protein after amino acid alignment (Fig. 4.3).

4.1.2 HSP72 protein expression in squirrel and bat tissues

To assess HSP72 protein expression levels, Western blotting was employed. The primary antibody was rabbit anti-Hsp70 (Hsp72) polyclonal antibody (#SPA-812, Santa Cruz, 1:30,000), and the second antibody was goat anti-rabbit IgG-HRP (#sc-2004, Santa Cruz, 1:1000). Band intensities on Western blots were quantified using Syngene software and the data were analyzed statistically in Excel. Fig. 4.4A shows representative immunoblots for HSP72 in squirrel and bat tissues from hibernating versus euthermic control animals. Histograms in Fig. 4.4B show mean ratios (hibernating:euthermic) for multiple independent trials. In ground squirrels, HSP72 protein levels increased significantly during hibernation in skeletal muscle by 1.42-fold. By contrast, HSP72 levels fell in squirrel BAT, brain, heart and lung to 87%, 64%, 64% 45% of the corresponding euthermic values, respectively. There were no changes in HSP72 levels in squirrel kidney and liver. In bats, HSP72 protein levels increased slightly in brain and liver during hibernation by 1.27- and 1.29-fold, respectively (Fig. 4.4C). However, protein levels decreased to 63%, 61% and 69% in BAT, lung and muscle, respectively, whereas there was no change in kidney.

4.1.3 Anti-HSP72 antibody specificity confirmation by 2-D gel electrophoresis

To confirm that the antibodies used were specific for a single protein, 2-D gel electrophoresis was employed. After running the first dimension isoelectrofocusing gel to separate the proteins on the basis of charge, the proteins were separated in the second dimension by molecular mass using SDS-PAGE. The resolved proteins were then probed with primary and secondary antibodies, as described above in section 4.1.2. Fig. 4.5A shows the protein bands on a one-dimensional gel that reacted with HSP72 antibodies in

squirrel muscle and bat liver. There was only one band in each lane at a molecular weight of ~72 kDa, which indicates that the HSP72 antibody used was specific to the HSP72 protein. The two-dimensional gels in Fig. 4.5B and C confirm this by showing only a single strong immunoreactive spot in squirrel liver (B) and bat liver (C). In both cases, strong immunoreactive spots were seen at a position corresponding to a molecular weight of ~70 kDa and pI value of ~5.4, which is consistent with the characteristics of human HSP72 (NP_005336) which has a molecular weight of 70 kDa and theoretical pI of 5.48 as calculated from the Compute pI/Mw tool website (http://www.expasy.org/tools/). One very small additional crossreacting spot was seen on the squirrel muscle gel at a much higher pI value, which would not interfere with the detection and quantification of the HSP72 band on the one-dimensional western blot. Hence, the results demonstrate that the antibody used for Western blotting is specific to HSP72 protein and the protein levels examined in different tissues were reliable.

4.1.4 HS72 subcellular localization in squirrel muscle

Subcellular fractionation and immunoblotting studies using anti-HSP72 antibody (the same as used in Western blotting) showed that HSP72 protein was present in both the cytoplasm and the nucleus of ground squirrel skeletal muscle. Fig. 4.6 shows that the relative amount of HSP72 was higher in the cytoplasmic fraction of muscle from hibernating squirrels (by 1.58-fold) versus euthermic controls, where the relative HSP72 expression level did not change between the two states in the nuclear fraction. The overall effect of hibernation on HSP72 expression in skeletal muscle seen here is in agreement with the data in Fig. 4.4B.

4.2 HSP73

4.2.1. RT-PCR retrieval of squirrel and bat hsp73

RT-PCR was carried out to retrieve the cDNA segments for *hsp73* from squirrel and bat. Degenerate primers were designed based on the consensus sequences of *hsp73* from several other mammalian species: Forward 5'-TTGC(T/A/C)TATGGTGCAGCTGTC-3' and Reverse 5'-ATTCTTCCTTCTC(G/T)GCAGTC-3'. The optimum annealing temperature for the primers was 58°C for both species. Fig. 4.7 shows the *hsp73* cDNA segments that were amplified from squirrel BAT (A) and bat lung (B) and their corresponding amino acid sequences. Analysis using the BLAST program at the NCBI website and DNAMAN software confirmed that the sequences encoded *hsp73*. DNA alignment results showed that the 632 bp of squirrel *hsp73* segment shared 93% and 89% identities, respectively, with the human and mouse sequences whereas the 632 bp of the bat *hsp73* segment shared 91% and 89% identities, respectively, with human and mouse (Fig. 4.8). The squirrel and bat cDNA segments encoded peptides of 210 amino acids, about 32% of the full protein (646 amino acids in humans) from a region near the C terminal of the protein (beginning at amino acid 382 of the human sequence). Amino acid sequence identity was high; squirrel and bat HSP73 shared 99% identity and identity was 98% with nonhibernating mammals (Fig. 4.9).

4.2.2 HSP73 protein expression in squirrel and bat tissues

To assess the effect of hibernation on the expression level of HSP73 protein, Western blotting was employed. The primary antibody was rat anti-Hsc70 monoclonal antibody (SPA 815, Stressgen, 1:10,000) and the secondary antibody was rabbit anti-rat IgG horseradish peroxidase conjugate (SAB-200, Stresssgen, 1:2000). Fig. 4.10A shows representative immunoblots for HSP73 in squirrel and bat tissues from hibernating versus euthermic control animals and histograms in Fig. 4.10B and C show mean ratios (hibernating:euthermic) for multiple independent trials. In ground squirrels, HSP73 protein levels increased during hibernation in heart, kidney and lung by 1.49-, 1.32- and 1.41-fold, respectively, whereas levels were unchanged in BAT, liver, brain, and muscle (Fig.4.10 B). In bats, HSP73 protein levels increased slightly in liver during hibernation (by 1.18-fold). However, protein levels deceased in lung, kidney and muscle to 24, 57, or 55 % of euthermic values (Fig.4.10 B). HSP73 levels were unchanged in BAT and brain of bats.

4.2.3 Anti-HSP73 antibody specificity confirmed by 2-D electrophoresis

To confirm that the antibodies used were specific for a single protein, 2-D gel electrophoresis was again employed. Fig. 4.11A shows the protein bands on a one-dimensional gel that reacted with HSP73 antibodies in squirrel BAT and muscle. Only one band was detected in each lane at a molecular weight of ~73 kDa, which suggests that the HSP73 antibody used was specific to the HSP73 protein. Fig 4.11B and C support this interpretation. Two-dimensional separations of squirrel and bat liver extracts showed strong immunoreactive spots at a position corresponding to a molecular weight of

~70 kDa and pI value of ~5.4, which is consistent with the characteristics of human HSP73 (P11142) which has a molecular weight of 70 kDa and theoretical pI of 5.37 as calculated from the Compute pI/Mw tool website. Some minor spots on the membrane would not interfere with the detection and quantification of the HSP73 band on the one-dimensional western blot. Hence, the results demonstrate that the antibody used for Western blotting is specific to HSP73 protein and the protein levels examined in different tissues were reliable.

4.2.4 HSP73 subcellular localization in squirrel muscle

Subcellular fractionation and immunoblotting studies using anti-HSP73 antibody (the same as used in Western blotting) showed that HSP73 protein was present in both the cytoplasm and the nucleus of ground squirrel skeletal muscle. Fig. 4.12 shows that the relative amount of HSP73 did not change in either subcellular fraction during hibernation, in agreement with the relative expression level of total HSP72 in hibernating muscle seen in Fig. 4.10B.

4.3 HSP40

4.3.1. RT-PCR retrieval of squirrel and bat hsp40

RT-PCR was employed to retrieve the cDNA segments for *hsp40* from ground squirrel and bat. Degenerate primers were designed based on the consensus sequences of *hsp40* from several other mammalian species. The primers, Forward 5'-CGAGGAGYTGTTCAGGAAGA-3' and Reverse 5'-GAGGTCSGAGTGGATGTCTG-3' were used to amplify the squirrel *hsp40* nucleotide segment, and the primers, Forward 5'-CAGCTGGCAGAAGCYTATGA-3' and Reverse 5'-

CTCGGCRTAGCTCAGGATCA-3', were used to retrieve the bat *hsp40* partial nucleotide sequence. The optimum annealing temperature for the primers was 66°C and 62°C, respectively, for the tissue sources used: squirrel heart and bat liver. Fig. 4.13 shows the *hsp40* cDNA segments that were amplified for squirrel (A) and bat (B) and the corresponding translated amino acid sequences. Analysis using the BLAST program at the NCBI website and DNAMAN software confirmed that the sequences encoded *hsp40*. DNA alignment showed that the 452 bp of the squirrel sequence and 649 bp of bat *hsp40* shared 90% and 86% identity, respectively, with the human sequence (Fig. 4.14 A and B). The squirrel nucleotide segment encoded a peptide with 150 amino acids, about 32% of the full protein (460 amino acids in mouse) from a region near the middle of the protein (beginning at amino acid 196 of the mouse sequence). The 649 bp nucleotide segment of bat *hsp40* encoded a peptide with 216 amino acids, about 47% of the full protein, again from a region near the middle of the protein (beginning at amino acid 150 of the mouse sequence). Fig. 4.15A shows that the 150 amino acid segment of squirrel HSP40 shared 96% and 94% identity with the mouse and human amino acid sequences, respectively, whereas the 216 amino acids of the bat sequence shared 88% identity with the human and mouse proteins after amino acid alignment (Fig. 4.15B). Fig. 4.15C shows that the 150 amino acid sequence that was common to the squirrel and bat proteins shared 90% identity.

4.3.2 HSP40 protein expression in squirrel and bat tissues

HSP40 protein expression was assessed by Western blotting; the primary antibody was rabbit anti-Hsp40 polyclonal antibody (#SPA-400, StressGen, 1:10,000), and secondary antibody was goat anti-Rabbit IgG-HRP (sc-2004, Santa Cruz, 1:1000). Fig.

4.16A shows representative immunoblots for HSP40 in squirrel and bat tissues from hibernating versus euthermic control animals. Histograms in Fig. 4.16 B and C show mean ratios (hibernating:euthermic) for multiple independent trials. In ground squirrels, HSP40 protein levels increased significantly during hibernation in BAT, heart, kidney and liver by 2.62-, 2.14-, 1.23- and 1.64-fold, respectively. By contrast, HSP40 levels fell during hibernation to 61% of the euthermic value in lung and muscle. There was no change in HSP40 in squirrel brain. In bats, HSP40 protein levels increased significantly in liver during hibernation by 3.35-fold and increased slightly by 1.26-fold and 1.21-fold in kidney and lung, respectively. However, HSP40 protein in muscle decreased during hibernation to 67 % of the euthermic value, whereas levels were unchanged change in BAT and brain.

4.3.3 Anti-HSP40 antibody specificity confirmation by 2-D gel electrophoresis

Two dimensional electrophoresis was again used to confirm the specificity of the antibody. Fig. 4.17 A and B show that there was only one strong immunoreactive spot in squirrel liver and bat liver, respectively. This corresponded to a molecular weight of ~40 kDa and pI value of ~9.0, which is consistent with the characteristics of mouse HSP40 (BC027240) which has a molecular weight of 49 kDa and theoretical pI of 9.41 as calculated by the Compute pI/Mw tool website. A few other spots showed up on the membranes, but since they were either trace amounts or their molecular weight was substantially different from 40 kDa, these spots would not interfere with the detection and quantification of the HSP40 band on the one-dimensional Western blot. Only the spots with the same molecular weight might interfere with detection, but since they were too tiny compared with the large HSP40 spot, these would cause only minor interference.

Hence, the results demonstrate that the antibody used for Western blotting is specific for HSP72 protein and the protein levels examined in different tissues were reliable.

4.3.4 HSP40 subcellular localization in squirrel muscle

Subcellular fractionation and immunoblotting studies using anti-HSP40 antibody showed that HSP40 protein was present in both the cytoplasm and the nucleus of ground squirrel skeletal muscle. Fig. 4.18 shows that the relative amount of HSP40 decreased in both the cytoplasmic fraction and nuclear fractions of muscle from hibernating squirrels to 57% and 53%, respectively, of the euthermic control values. The overall effect of hibernation on HSP40 expression in skeletal muscle seen here is in agreement with the data in Fig. 4.16B.

4.4 HSP90

4.4.1. RT-PCR retrieval of ground squirrel hsp90α and hsp90β

RT-PCR was carried out to retrieve the cDNA segments for *hsp90α* and *hsp90β* from squirrel kidney. Degenerate primers were designed based on the consensus sequences of *hsp90α* and *hsp90β* from several other mammalian species. The primers, Forward 5'-TTGC(T/A/C)TATGGTGCAGCTGTC-3' and Reverse 5'-ATTCTTCCTTCTC(G/T)GCAGTC-3', were used for *hsp90α* retrieval and the primers, Forward 5'-CAGGCTGGTGCAGACATCTC-3' and Reverse 5'-AAGTGCTTGACTGCCAAGTG-3', were for *hsp90β* retrieval. The optimum annealing temperature for the primers was 61°C for both genes. Fig. 4.19 shows the *hsp90α* (A) and *hsp90β* (B) cDNA segments that were amplified from squirrel kidney and their corresponding amino acid sequences. Analysis using the BLAST program at the NCBI

website and DNAMAN software confirmed that the sequences encoded *hsp90α* and *hsp90β*. DNA alignment results showed the 620 bp of squirrel *hsp90α* segment shared 92% and 84% identity, respectively, with the human and mouse nucleotide sequences (Fig. 4.20A) whereas the 560 bp of the squirrel *hsp90β* segment shared 90% and 87% identity with human and mouse, respectively (Fig. 4.20B). The 620 bp of *hsp90α* encoded a peptide with 206 amino acids, about 28% of the full protein (732 amino acids in humans) from a region near the C terminal of the protein (beginning at amino acid 511 of the human sequence). The 560 bp of HSP90*β* encoded a peptide with 186 amino acids, about 26% of the full protein (724 amino acids in humans) from a region near the N terminal of the protein (beginning at amino acid 131 of the human sequence). Amino acid sequence identity was high; squirrel HSP90α and HSP90β shared 100% and 97% identity, respectively, with the corresponding human amino acid sequences (Fig. 21).

4.4.2 HSP90α/β protein expression in squirrel tissues

Expression levels of HSP90α/β protein were analyzed in ground squirrel organs by Western blotting; the primary antibody was anti-HSP90α/β goat polyclonal IgG (sc-1055, Santa Cruz, 1:200) which crossreacts with both isoforms of Hsp90 and the secondary antibody was bovine anti-goat IgG HRP (sc-2350, Santa Cruz, 1:1000). Fig. 4.22A shows representative immunoblots for HSP90α/β in tissues from hibernating versus euthermic control squirrels and histograms in Fig. 4.22B shows mean ratios (hibernating:euthermic) for three independent trials. HSP90α/β protein increased during hibernation in kidney by 1.76-fold, whereas levels decreased in lung and BAT to 52- and

64 %, respectively, of the control value. HSP90 levels remained unchanged during hibernation in lung, liver and WAT and the protein was not detected in skeletal muscle.

4.4.3 Anti-HSP90α/β antibody specificity confirmed by 2-D electrophoresis

To confirm that the antibodies used were specific for a single protein, 2-D gel electrophoresis was again employed. Fig. 4.23 shows a strong immunoreactive spot on the 2-D gel at a position corresponding to a molecular weight of 90 kDa and pI value of 5.0, which is consistent with the characteristics of human HSP90α (NM_005348) and HSP90β (NM_007355) which have molecular weights of 89 and 87 kDa and theoretical pI values of 4.73 and 4.76, respectively. Some minor crossreacting spots were seen but these would not interfere with the detection and quantification of the HSP90α/β band on one-dimensional Western blots. Hence, the results demonstrate that the antibody used for Western blotting is specific to HSP90α/β protein and the protein levels examined in different tissues were reliable.

4.4.4 HSP90 subcellular localization in squirrel liver

Subcellular fractionation and immunoblotting studies using anti-HSP90α/β antibody showed that HSP90α/β protein was present in both the cytoplasm and the nucleus of ground squirrel liver. Fig. 4.24 shows that the relative amount of HSP90α/β significantly increased in the nuclear fraction of squirrel liver during hibernation by 3.07-fold. By contrast, HSP90α/β content decreased in the cytoplasm during hibernation to 72 % of the control value. This shows an increase in HSP90α/β translocation to the nucleus during hibernation.

Fig. 4.1 Partial nucleotide sequences of *hsp72* from (A) thirteen-lined ground squirrel (*Spermophilus tridecemlineatus*) and (B) little brown bat (*Myotis lucifugus*) and their deduced amino acid sequences. The partial cDNA sequences were amplified from squirrel kidney and bat brain by RT-PCR using degenerate primers. A single open reading frame was predicted from the 660 bp of squirrel *hsp72* nucleotide sequence encoding a peptide of 219 amino acids and from the 660 bp of bat *hsp72* nucleotide sequence encoding a peptide of 219 amino acids, respectively. "N" represents an unknown nucleotide, and "X" represents an unknown amino acid.

A)

```
1          GCACCACCCCCAGCTACGTGGCCTTCACGGACACCGAGCGGCTCATCGGCGATGCCGCC
1            T  T  P  S  Y  V  A  F  T  D  T  E  R  L  I  G  D  A  A

60         AAGAACCAGGTGGCGCTGAACCCGCAGAACACGGTGTTCGACGCGAAGCGGCTGATCGGC
20          K  N  Q  V  A  L  N  P  Q  N  T  V  F  D  A  K  R  L  I  G

120        CGCAAGTTCGGCGACCCGGTGGTGCAGTCGGACATGAAGCACTGGCCCTTCCAGGTGATC
40          R  K  F  G  D  P  V  V  Q  S  D  M  K  H  W  P  F  Q  V  I

180        AACGACGGCGACAAGCCCAAGGTGCAGGTGAGCTACAAGGGCGAGAGCAAAGCGTTCTAC
60          N  D  G  D  K  P  K  V  Q  V  S  Y  K  G  E  S  K  A  F  Y

240        CCGGAGGAGATCTCTTCCATGGTGCTGACCAAGATGAAGGAGATCGCCGAGGCGTACCTG
80          P  E  E  I  S  S  M  V  L  T  K  M  K  E  I  A  E  A  Y  L

300        GGCTACCCGGTGACCAACGCGGTGATCACCGTGCCGGCCTACTTCAACGACTCGCAGCGC
100         G  Y  P  V  T  N  A  V  I  T  V  P  A  Y  F  N  D  S  Q  R

360        CAGGCCACCAAGGACGCGGGCGTGATCGCCGGGCTCAACGTGCTGAGGATCATCAACGAG
120         Q  A  T  K  D  A  G  V  I  A  G  L  N  V  L  R  I  I  N  E

420        CCCACGGCGGCCGCCATCGCCTACGGCCTGGACCGCACNGGCAAGGGGGAGCGCAACGTG
140         P  T  A  A  A  I  A  Y  G  L  D  R  T  G  K  G  E  R  N  V

480        CTCATCTTTGACCTGGGCGGGGGCACCTTCGACGTGTCCATCCTGACGATCGACGACGGC
160         L  I  F  D  L  G  G  G  T  F  D  V  S  I  L  T  I  D  D  G

540        ATCTTCGAGGTGAAGGCCACTGCCGGCGACACGCACCTGGGCGGGGAGGACTTCGACAAC
180         I  F  E  V  K  A  T  A  G  D  T  H  L  G  G  E  D  F  D  N

600        CGGCTGGTGAACCACTTCGTGGAGGAGTTCAAGAGGAAACACAAGAAGGACATCAGCCCAA
200         R  L  V  N  H  F  V  E  E  F  K  R  K  H  K  K  D  I  S  P
```

B)

```
1          GCACCACCCCCAGCTACGTGGCCTTCACCGACACCGAGCGGCTCATCGGGGACGCGGCCA
1            T  T  P  S  Y  V  A  F  T  D  T  E  R  L  I  G  D  A  A

61         AGAACCAGGTGGCGCTGAACCCGCAGAACACCGTGTTCGACGCCAAGCGGCTGATCGGCC
20          K  N  Q  V  A  L  N  P  Q  N  T  V  F  D  A  K  R  L  I  G

121        GCAAGTTCGGCGACGCGGTGGTGCAGTCGGACATGAAGCACTGGCCTTTCCAGGTGATCA
40          R  K  F  G  D  A  V  V  Q  S  D  M  K  H  W  P  F  Q  V  I

181        GCGACGGGGACAAGCCCAAGGTGCAGGTGAGCTACAAAGGGGAGACCAAGGCGTTCTTCC
60          S  D  G  D  K  P  K  V  Q  V  S  Y  K  G  E  T  K  A  F  F

241        CCGAGGAGATCTCGTCCATGGTGCTGACCAAGATGAAGGAGATCGCCGAGGCGTACCTGG
80          P  E  E  I  S  S  M  V  L  T  K  M  K  E  I  A  E  A  Y  L

301        GCTACGCGGTGACCAACGCGGTGATCACCGTGCCGGCCTACTTCAACGACTCGCAGCGCC
100         G  Y  A  V  T  N  A  V  I  T  V  P  A  Y  F  N  D  S  Q  R

361        AGGCCACCAAGGACGCGGGGGTGATCGCGGGGCTGAACGTGCTGAGGATCATCAACGAGC
120         Q  A  T  K  D  A  G  V  I  A  G  L  N  V  L  R  I  I  N  E

421        CCACGGCCGCCGNCATCGNCTACGGCCTGGACAGGACGGGCAAGGGGGAGCGCAACGTGC
140         P  T  A  A  X  I  X  Y  G  L  D  R  T  G  K  G  E  R  N  V

481        TCATCTTCGACCTGGGCGGGGGCACCTTCGACGTGTCCATCCTGACGATCGACGACGGCA
160         L  I  F  D  L  G  G  G  T  F  D  V  S  I  L  T  I  D  D  G

541        TCTTCGAGGTGAAGGCCACGGCCGGGGACACCCACCTGGGAGGGGAGGACTTTGACAACA
180         I  F  E  V  K  A  T  A  G  D  T  H  L  G  G  E  D  F  D  N

601        GGCTGGTGAACCACTTCNTGGAGGAGTTCAAGCGCAAGCACAAGAAGGACATCAGCCAGA
200         R  L  V  N  H  F  X  E  E  F  K  R  K  H  K  K  D  I  S  Q
```

Fig. 4.2 Comparison of *hsp72* partial nucleotide sequences from ground squirrel (*Spermophilus tridecemlineatus*), bat (*Myotis lucifugus*), human, dog and cow (A) and homology tree (B) showing the percent identity between the sequences. Genbank accession numbers are: human (*Homo sapiens*, BC009322), dog (*Canis familiaris*, AB114672) and cow (*Bos taurus,* NM_203322). The nucleotide sequences were aligned and the homology tree was generated using DNAMAN software. For the bat, human, dog and cow sequences dashes (-) replace those nucleotides that are identical with the squirrel sequence. "n" represents an unknown nucleotide.

A) Alignment of squirrel, bat, human, dog and cow Hsp72 nucleotide partial sequences

B) Homology tree of the five hsp72 partial nucleotide sequences

A)

```
squirrel   GCACCACCCCCAGCTACGTGGCCTTCACGGACACCGAGCGGCTCATCGGCGATGCCGCCA    60
bat        ----------------------------c-------------------g--c--g----    60
human      ----------------------------------------------------g-----g----    60
dog        ----------------------------c-------------------g-----g----    60
cow        ----------------------------c--t-----------------a-----g----    60

squirrel   AGAACCAGGTGGCGCTGAACCCGCAGAACACGGTGTTCGACGCGAAGCGGCTGATCGGCC   120
bat        --------------------------------c-----------c----------------   120
human      --------------------------------c-----t--------------------   120
dog        --------------------------------c--------------------c-------   120
cow        ------------------------------------------------------------   120

squirrel   GCAAGTTCGGCGACCCGGTGGTGCAGTCGGACATGAAGCACTGGCCCTTCCAGGTGATCA   180
bat        --------------g-------------------------------t-------------   180
human      ------------------------------------------------t-------------   180
dog        -------t---------------------------------------t---------g---   180
cow        ----------a-----------------------------------t----gc--c----   180

squirrel   ACGACGGCGACAAGCCCAAGGTGCAGGTGAGCTACAAGGGCGAGAGCAAAGCGTTCTACC   240
bat        g------g-------------------------------a--g----c---g-------t--   240
human      -------a-----------------------------------g----c---g--a-------   240
dog        -------g-----------------------------------g----c---g----------   240
cow        -------a--------t-----------------------g----c---g----------   240

squirrel   CGGAGGAGATCTCTTCCATGGTGCTGACCAAGATGAAGGAGATCGCCGAGGCGTACCTGG   300
bat        -c-----------g-----------------------------------------------   300
human      -c-----------g-----------------------------------------------   300
dog        -c-----------g-------------------------------------------c-------   300
cow        -------------g--g-----------------------------------------------   300

squirrel   GCTACCCGGTGACCAACGCGGTGATCACCGTGCCGGCCTACTTCAACGACTCGCAGCGCC   360
bat        -----g-------------------------------------------------------   360
human      ------------------------------------------------------------   360
dog        ------------------------------------------------------------   360
cow        --c-----------------------------------------------------------g-   360

squirrel   AGGCCACCAAGGACGCGGGCGTGATCGCCGGGCTCAACGTGCTGAGGATCATCAACGAGC   420
bat        -------------------g--------g-----g------------------------   420
human      -------------t-----t--------g---------------c---------------   420
dog        -------------------g--------g-------------------------------   420
cow        -------------------g--------g-----g------------------------   420

squirrel   CCACGGCGGCCGCCATCGCCTACGGCCTGGACCGCACNGGCAAGGGGGAGCGCAACGTGC   480
bat        -------c----n-----n-------------a-g--g----------------------   480
human      -------c-------------------------a-a--g----------------------   480
dog        ----t--c-------------------------a-g--c----------------------   480
cow        -------c-------------------------a-g--g----------------------   480

squirrel   TCATCTTTGACCTGGGCGGGGGCACCTTCGACGTGTCCATCCTGACGATCGACGACGGCA   540
bat        -------c-----------------------------------------------------   540
human      ------------------------------------------------------------   540
dog        ------------------------------------------------------------   540
cow        ----------t-----a--------g-----------------------------------   540

squirrel   TCTTCGAGGTGAAGGCCACTGCCGGCGACACGCACCTGGGCGGGGAGGACTTCGACAACC   600
bat        --------------------g-----g-----c---------a-----------t------a   600
human      --------------------g-----g-----c---------t-----------t------a   600
dog        --------------------g-----g-----------------------------------a   600
cow        --------------------g-----g--------------g-----------------a   600

squirrel   GGCTGGTGAACCACTTCGTGGAGGAGTTCAAGAGGAAACACAAGAAGGACATCAGCCCAA   660
bat        -----------------n--------------c-c--g-------------------ag-   660
human      ---------------------------------a---------------------ag-   660
dog        ---------------------------------c----g-----------------ag-   660
cow        ------------------------------------g-----------------ag-   660
```

B)

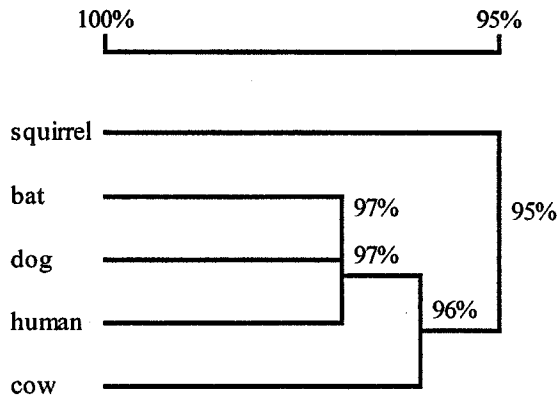


Fig. 4.3 Comparison of HSP72 partial amino acid sequences from ground squirrel (*Spermophilus tridecemlineatus*), bat (*Myotis lucifugus*), human, dog and cow (A) and homology tree (B) showing the percent identity between the sequences. Genbank accession numbers are: human (*Homo sapiens*, BC009322), dog (*Canis familiaris*, AB114672) and cow (*Bos taurus,* NM_203322). DNAMAN software was used to translate the nucleotide sequences, generate the alignment and compute the percent identities. Dashes (-) replace those amino acids that are identical to the squirrel sequence. "X" represents an unknown amino acid.

A)

```
squirrel   TTPSYVAFTDTERLIGDAAKNQVALNPQNTVFDAKRLIGRKFGDPVVQSDMKHWPFQVIN   60
bat        ------------------------------------------a--------------s   60
human      ------------------------------------------------------------   60
dog        ------------------------------------------------------------v-   60
cow        ------------------------------------------------------------r---   60

squirrel   DGDKPKVQVSYKGESKAFYPEEISSMVLTKMKEIAEAYLGYPVTNAVITVPAYFNDSQRQ   120
bat        --------------t---f----------------------a------------------   120
human      --------------t-------------------------------------------   120
dog        --------------t-------------------------------------------   120
cow        --------------t------------------------h-------------------   120

squirrel   ATKDAGVIAGLNVLRIINEPTAAAIAYGLDRTGKGERNVLIFDLGGGTFDVSILTIDDGI   180
bat        ----------------------x-x---------------------------------   180
human      ------------------------------------------------------------   180
dog        ------------------------------------------------------------   180
cow        ------------------------------------------------------------   180

squirrel   FEVKATAGDTHLGGEDFDNRLVNHFVEEFKRKHKKDISP                       219
bat        -------------------------x------------q                       219
human      ------------------------------------q                       219
dog        ------------------------------------q                       219
cow        ------------------------------------q                       219
```

B)

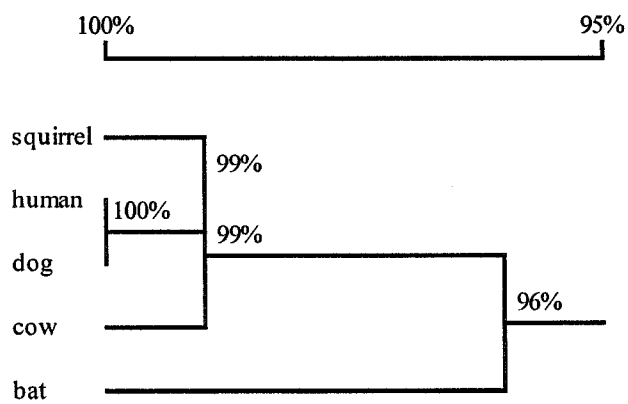
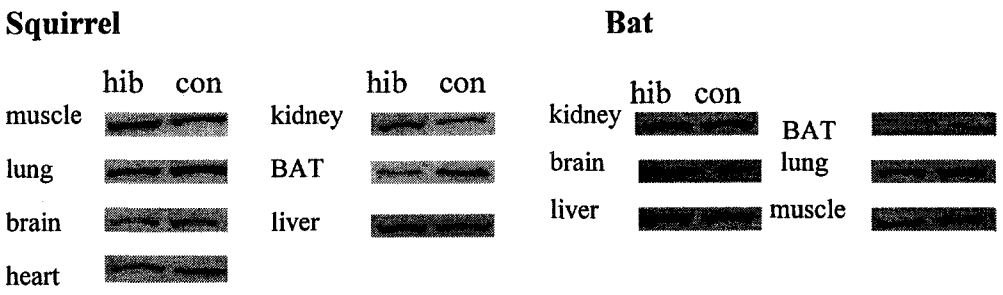


Fig. 4.4 The effects of hibernation on ground squirrel and bat HSP72 protein expression in different tissues.

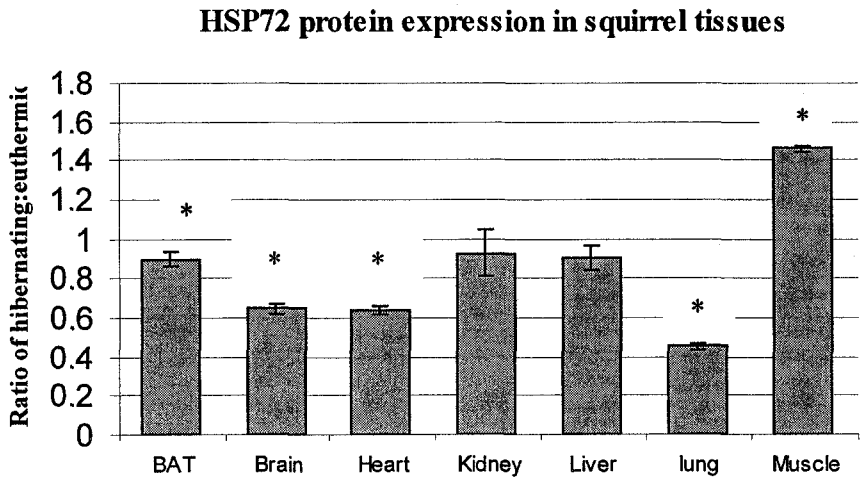
A) Representative Western blots showing HSP72 protein levels in seven tissues of euthermic (con) and hibernating (hib) ground squirrels and bats.

B) Histograms show the ratio of HSP72 protein levels in hibernating versus euthermic ground squirrels. Mean values (± SEM) were calculated from n = 3 trials on independent extracts of tissue. * - Mean value for hibernating sample is significantly different from the corresponding euthermic value, $P < 0.05$.

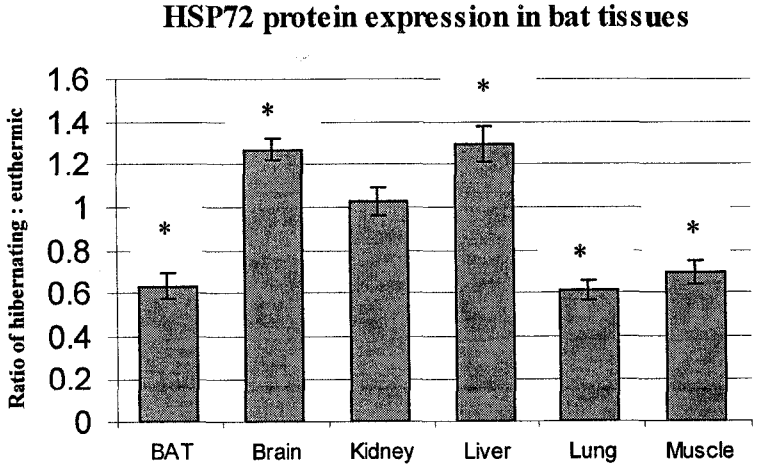
C) Histograms show the ratio of HSP72 protein levels in hibernating versus euthermic bats. Mean values (± SEM) were calculated from n = 3 trials on independent extracts of tissue. * - Mean value for hibernating sample is significantly different from the corresponding euthermic value, $P < 0.05$.



A)



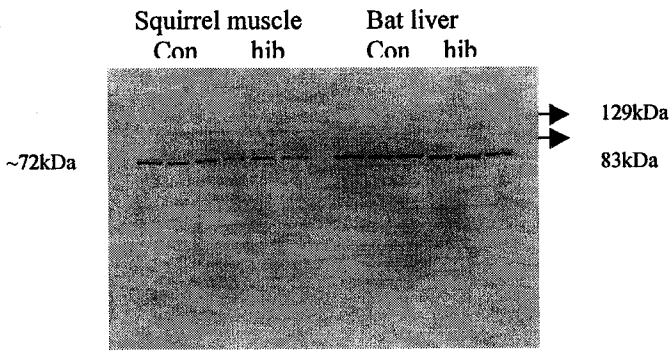
B)



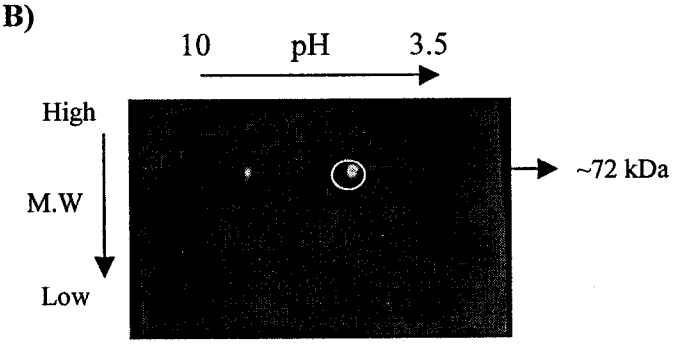
C)

Fig. 4.5 Representative Western blots of HSP72 expression: (A) one dimensional blots of ground squirrel skeletal muscle and bat liver (B) 2D-PAGE blots of ground squirrel liver, and (C) 2D-PAGE blots of bat liver.

A)



B)



C)

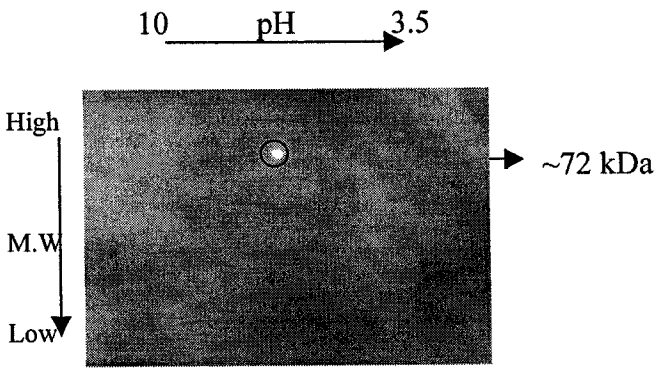
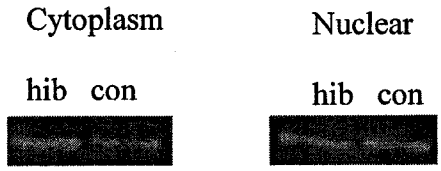


Fig. 4.6 HSP72 protein distribution in nuclear versus cytoplasmic fractions as assessed after subcellular fractionation and Western blotting.

A) Representative Western blots show HSP72 levels in cytoplasmic and nuclear fractions of ground squirrel skeletal muscle from euthermic control (con) versus hibernating (hib) animals.

B) Histogram shows the relative expression levels of HSP72 (hibernating versus control) in cytoplasmic and nuclear fractions of squirrel muscle. Mean values (± SEM) were calculated from n = 3 independent trials. * - Mean value for hibernating sample was significantly different from the corresponding euthermic value, $P < 0.05$.

A)



B)

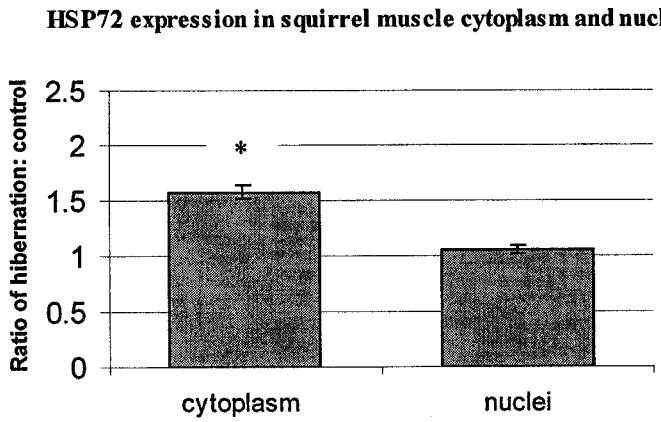


Fig. 4.7 Partial nucleotide sequences of (A) thirteen-lined ground squirrel (*Spermophilus tridecemlineatus*) and (B) little brown bat (*Myotis lucifugus*) hsp*73* and their deduced amino acid sequences. The partial cDNA sequences were amplified from squirrel kidney and bat brain by RT-PCR using degenerate primers. A single open reading frame was predicted from the 632bp of squirrel *hsp73* nucleotide sequence encoding a peptide of 210 amino acids and from the 634bp of bat *hsp73* nucleotide sequence encoding a peptide of 210 amino acids, respectively. "N" represents unknown nucleotide, and "X" represents unknown amino acid.

A)

```
1       GGAGACAAGTCTGAAAACGTTCAAGATTTGCTGCTGTTGGATGTCACTCCTCTTTCCCTT
1        G  D  K  S  E  N  V  Q  D  L  L  L  L  D  V  T  P  L  S  L

61      GGTATTGAAACTGCTGGTGGAGTCATGACTGTCCTCATCAAGCGCAATACCACCATTCCT
21       G  I  E  T  A  G  G  V  M  T  V  L  I  K  R  N  T  T  I  P

121     ACCAAGCAGACACAGACCTTCACAACTTACTCTGACAACCAGCCTGGTGTGCTCATTCAG
41       T  K  Q  T  Q  T  F  T  T  Y  S  D  N  Q  P  G  V  L  I  Q

181     GTATATGAAGGTGAGCGTGCCATGACCAAGGATAACAACCTACTTGGCAAGTTTGAACTC
61       V  Y  E  G  E  R  A  M  T  K  D  N  N  L  L  G  K  F  E  L

241     ACAGGCATACCCCCTGCACCCCGTGGTGTTCCTCAGATTGAAGTCACATTTGATATTGAT
81       T  G  I  P  P  A  P  R  G  V  P  Q  I  E  V  T  F  D  I  D

301     GCCAATGGCATCCTGAATGTTTCTGCTGTAGACAAGAGTACAGGAAAAGAAAACAAGATT
101      A  N  G  I  L  N  V  S  A  V  D  K  S  T  G  K  E  N  K  I

361     ACCATCACTAACGACAAAGGTCGTTTGAGCAAGGAAGACATTGAGCGCATGGTCCAGGAA
121      T  I  T  N  D  K  G  R  L  S  K  E  D  I  E  R  M  V  Q  E

421     GCTGAGAAGTACAAAGCTGAAGATGAAAAGCAGAGGGACAAGGTGTCATCCAAGAATTCG
141      A  E  K  Y  K  A  E  D  E  K  Q  R  D  K  V  S  S  K  N  S

481     CTCGAGTCCTATGCATTCAACATGAAAGCAACTGTTGAAGATGAGAAACTTCAAGGAAAG
161      L  E  S  Y  A  F  N  M  K  A  T  V  E  D  E  K  L  Q  G  K

541     ATAAATGATGAGGACAAACAGAAGATTCTTGACAAATGTAATGAAATCATCAACTGGCTG
181      I  N  D  E  D  K  Q  K  I  L  D  K  C  N  E  I  I  N  W  L

601     GATAAGAACCAGACTGCAGAGAAGGGGAAAAA
201      D  K  N  Q  T  A  E  K  G  K
```

B)

```
1       GGAGACAAATCTGAAAATGTCCAAGATCTGCTGCTGCTGGATGTCACACCTCTTTCCC
1        G  D  K  S  E  N  V  Q  D  L  L  L  L  D  V  T  P  L  S

59      TTGGCATTGAGACGGCTGGCGGCGTCATGACGGTGCTGATAAAGCGCAACACCACCATCC
20      L  G  I  E  T  A  G  G  V  M  T  V  L  I  K  R  N  T  T  I

119     CCACCAAGCAGACGCAGACCTTCACCACCTACTCCGACAACCAGCCAGGCGTGCTCATCC
40      P  T  K  Q  T  Q  T  F  T  T  Y  S  D  N  Q  P  G  V  L  I

179     AGGTTTATGAAGGTGAGCGTGCCATGACCAAGGATAACAACCTGCTTGGCAAGTTTGAAC
60      Q  V  Y  E  G  E  R  A  M  T  K  D  N  N  L  L  G  K  F  E

239     TCACAGGCATACCTCCTGCACCTCGTGGTGTTCCTCAGATTGAAGTCACTTTTGATATTG
80      L  T  G  I  P  P  A  P  R  G  V  P  Q  I  E  V  T  F  D  I

299     ATGCCAATGGCATCCTCAATGTCTCTGCTGTGGATAAGAGTACAGGAAAAGAGAACAAGA
100     D  A  N  G  I  L  N  V  S  A  V  D  K  S  T  G  K  E  N  K

359     TTACCATCACTAACGACAAGGGTCGTCTGAGCAAGGAAGACATTGAGCGCATGGTCCAAG
120     I  T  I  T  N  D  K  G  R  L  S  K  E  D  I  E  R  M  V  Q

419     AAGCTGAGAAGTACAAAGCTGAAGATGAGAAGCAGCGGGACAAGGTGTCTTCCAAGAATT
140     E  A  E  K  Y  K  A  E  D  E  K  Q  R  D  K  V  S  S  K  N

479     CACTTGAGTCCTATGCATTCAACATGAAAGCAACTGTTGAAGATGAGAAACTCCAAGGCA
160     S  L  E  S  Y  A  F  N  M  K  A  T  V  E  D  E  K  L  Q  G

539     AGATCACTGATGAGGACAAACAGAAGATTCTTGACAAGTGCAATGAAATCATCAACTGGC
180     K  I  T  D  E  D  K  Q  K  I  L  D  K  C  N  E  I  I  N  W

599     TTGATAAGAACCAGACTGCCGANAAGGGGAAAAA
200     L  D  K  N  Q  T  A  X  K  G  K
```

Fig. 4.8 Comparison of *hsp73* partial nucleotide sequences from ground squirrel (*Spermophilus tridecemlineatus*), bat (*Myotis lucifugus*), human and mouse sequences and homology tree showing the percent identity between the sequences. Genbank accession numbers are: human (*Homo sapiens*, AF352832) and mouse (*Mus musculus,* M19141). The nucleotide sequences were aligned using DNAMAN software (A) and the homology tree (B) generated by DNAMAN shows the percent identities between the four *hsp73* nucleotide sequences listed above. For the bat, human and mouse sequences dashes (-) and dots (.) replace those nucleotides that are identical with the squirrel sequence and that are lack in the nucleotide sequences, respectively.

A)

```
squirrel   GGAGACAAGTCTGAAAACGTTCAAGATTTGCTGCTGTTGGATGTCACTCCTCTTTCCCTT   60
bat        --------a--------t--c------c--------c----------a------------   60
human      --------------g--t-----------------c------------------------   60
mouse      --------------g--------g-----------c------------------------   60

squirrel   GGTATTGAAACTGCTGGTGGAGTCATGACTGTCCTCATCAAGCGCAATACCACCATTCCT   120
bat        --c------g--g-----c--c--------g--g--g--a--------c--------c--c   120
human      ------------------------------------------t---------------   120
mouse      ----------------c-------------------------------------c--c   120

squirrel   ACCAAGCAGACACAGACCTTCACAACTTACTCTGACAACCAGCCTGGTGTGCTCATTCAG   180
bat        ----------g----------c--c-----c----------a--c--------c---   180
human      ----------------------t--c--t----------------------t------   180
mouse      -----------------tc----c--c----------------------a---------   180

squirrel   GTATATGAAGGTGAGCGTGCCATGACCAAGGATAACAACCTACTTGGCAAGTTTGAACTC   240
bat        --t-------------------------------------g------------------   240
human      --t--------c--------------a--------------g------------------   240
mouse      --g----------aa-g--------------c--------g-----a-----c--g---   240

squirrel   ACAGGCATACCCCCTGCACCCCGTGGTGTTCCTCAGATTGAAGTCACATTTGATATTGAT   300
bat        ----------t--------t-------------------------t------------   300
human      ----------t----------a----------------------t-----c------   300
mouse      --------c--t--a----------g--------------g--t--t-----c--c---   300

squirrel   GCCAATGGCATCCTGAATGTTTCTGCTGTAGACAAGAGTACAGGAAAAGAAAACAAGATT   360
bat        --------------c-----c--------g--t----------------g---------   360
human      --------t--a--c-----c--------g----------g--------g---------   360
mouse      --------------c----------------t-----c--------g--g--------c   360

squirrel   ACCATCACTAACGACAAAGGTCGTTTGAGCAAGGAAGACATTGAGCGCATGGTCCAGGAA   420
bat        ----------------g------c-------------------------------a---   420
human      --t--------t-----g--c----------------------a--t------------   420
mouse      --------c--t-----g--c--c-----t--------t----------------a---   420

squirrel   GCTGAGAAGTACAAAGCTGAAGATGAAAAGCAGAGGGACAAGGTGTCATCCAAGAATTCG   480
bat        -------------------------g------c-------------t----------a   480
human      -------------------------g--------------------------------a   480
mouse      --------------g-----g-----g--------a--t-----t--c--------c--a   480

squirrel   CTCGAGTCCTATGCATTCAACATGAAAGCAACTGTTGAAGATGAGAAACTTCAAGGAAAG   540
bat        --t-------------------------------------------------c-----c---   540
human      --t----------c-------------------------------------------c---   540
mouse      --g----------c--------------------g--------------------c---   540

squirrel   ATAAATGATGAGGACAAACAGAAGATTCTTGACAAATGTAATGAAATCATCAACTGGCTG   600
bat        --c-c--------------------------------g--c--------------------t   600
human      --t--c----------------------g-----g----------t----------t   600
mouse      --c--------------------------------g--c-------------g-------   600

squirrel   GATAAGAACCAGACTGCAGAGAAGGGGAAAAA                              632
bat        ----------------c--n----------                               632
human      --------t--------t-------aag--tt                             632
mouse      -------------------------aag--tt                             632
```

B)

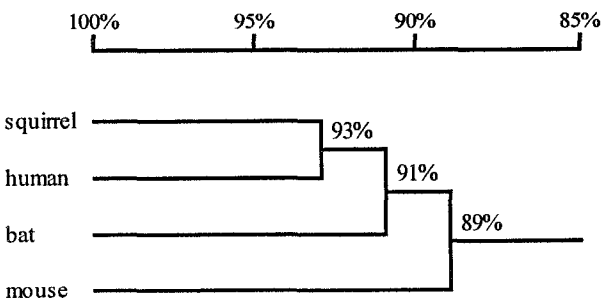


Fig. 4.9 Comparison of HSP73 partial amino acid sequences from ground squirrel (*Spermophilus tridecemlineatus*), bat (*Myotis lucifugus*), human and mouse sequences and homology tree showing the percent identity between the sequences. Genbank accession numbers are: human (*Homo sapiens*, AF352832) and mouse (*Mus musculus,* M19141). DNAMAN software was used to translate the nucleotide sequences, generate the alignment and compute the percent identities. Dashes (-) replace those amino acids that are identical as the squirrel. "X" represents the unknown amino acid.

```
squirrel   GDKSENVQDLLLLDVTPLSLGIETAGGVMTVLIKRNTTIPTKQTQTFTTYSDNQPGVLIQ     60
bat        ------------------------------------------------------------     60
human      ------------------------------------------------------------     60
mouse      ----------------------------------------------l-------------     60

squirrel   VYEGERAMTKDNNLLGKFELTGIPPAPRGVPQIEVTFDIDANGILNVSAVDKSTGKENKI    120
bat        ------------------------------------------------------------    120
human      ------------------------------------------------------------    120
mouse      ------------------------------------------------------------    120

squirrel   TITNDKGRLSKEDIERMVQEAEKYKAEDEKQRDKVSSKNSLESYAFNMKATVEDEKLQGK    180
bat        ------------------------------------------------------------    180
human      ------------------------------------------------------------    180
mouse      ------------------------------------------------------------    180

squirrel   INDEDKQKILDKCNEIINWLDKNQTAEKGK                                 210
bat        -t----------------------x---                                    210
human      ---------------------------ee                                   210
mouse      -----------------s----------ee                                  210
```

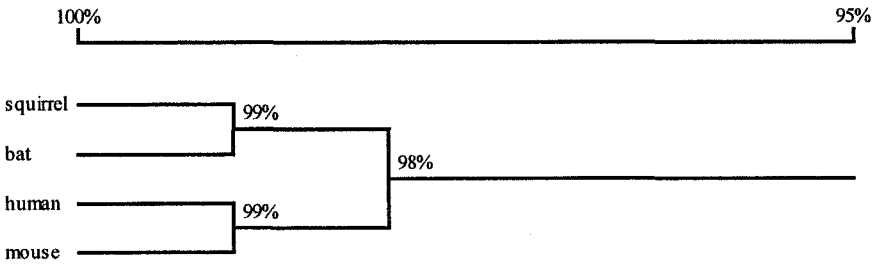


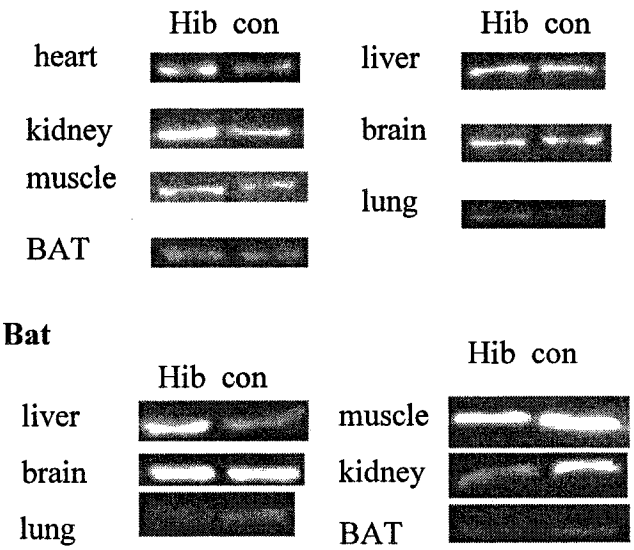
Fig. 4.10. The effects of hibernation on ground squirrel and bat HSP73 protein expression in different tissues.

A) Representative Western blots showing HSP73 protein levels in seven tissues of euthermic (con) and hibernating (hib) ground squirrels and bats.

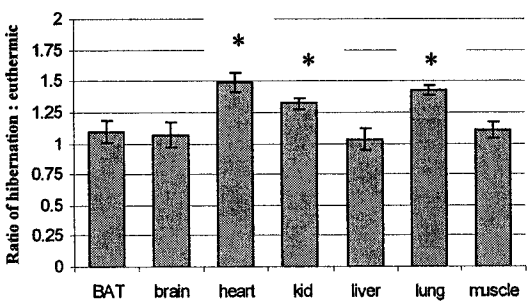
B) Histograms show the ratio of HSP73 protein levels in hibernating versus euthermic ground squirrels. Mean values (± SEM) were calculated from n = 3 trials. * - Mean value for hibernating sample is significantly different from the corresponding euthermic value, $P < 0.05$.

C) Histograms show the ratio of HSP73 protein levels in hibernating versus euthermic bats. Mean values (± SEM) were calculated from n = 3 trials. * - Mean value for hibernating sample is significantly different from the corresponding euthermic value, $P < 0.05$.

A)



B



C)

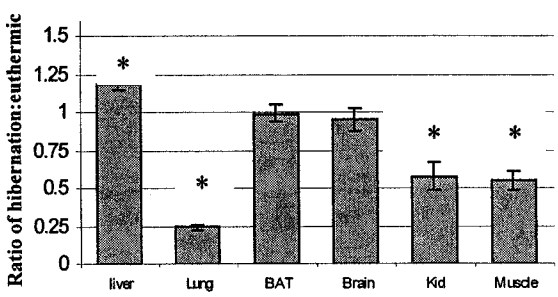
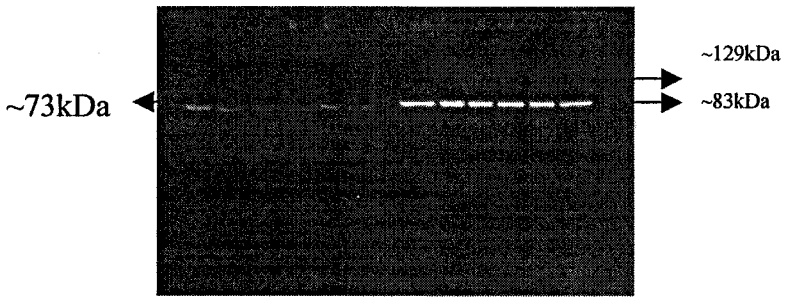
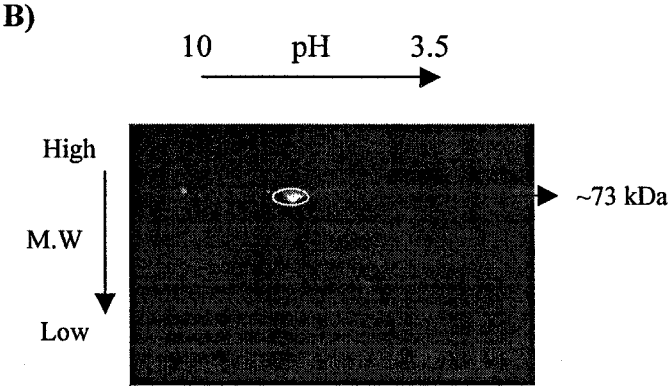


Fig. 4.11 Representative Western blots and 2D-PAGE Western blots showing HSP73 protein bands in ground squirrel BAT and muscle (A) and spots in liver (B) and bat liver (C), respectively.

A)



B)



C)

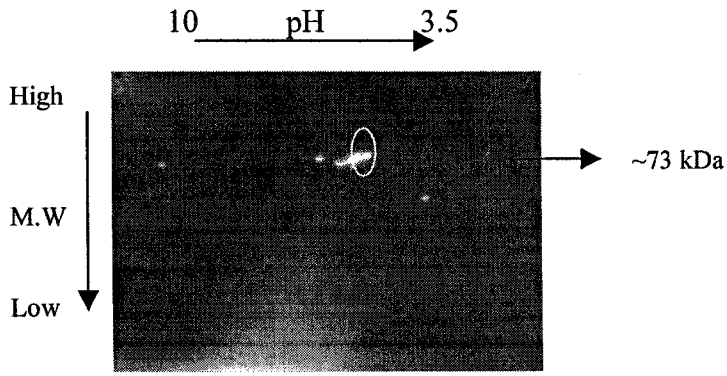
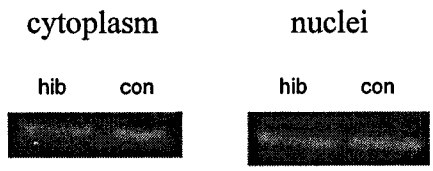


Fig.4.12 HSP73 protein distribution in nuclear versus cytoplasmic fractions as assessed after subcellular fractionation and Western blotting.

Representative Western blots show HSP73 levels in cytoplasmic and nuclear fractions of ground squirrel skeletal muscle from euthermic control (con) versus hibernating (hib) animals.

Histogram shows the relative expression levels of HSP73 (hibernating versus control) in cytoplasmic and nuclear fractions of squirrel muscle. Mean values (± SEM) were calculated from n = 3 independent trials. * - Mean value for hibernating sample was significantly different from the corresponding euthermic value, $P < 0.05$.

A)



B)

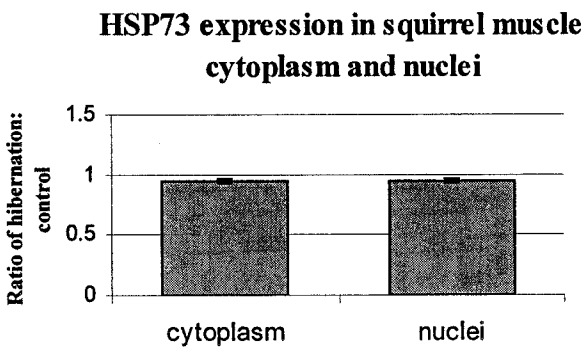


Fig. 4.13 Partial nucleotide sequences of (A) thirteen-lined ground squirrel (*Spermophilus tridecemlineatus*) and (B) little brown bat (*Myotis lucifugus*) *hsp40* and their deduced amino acid sequences. The partial cDNA sequences were amplified from squirrel heart and bat liver by RT-PCR using degenerate primers. A single open reading frame was predicted from the 452 bp of squirrel *hsp40* nucleotide sequence encoding a peptide of 150 amino acids and from the 649 bp of bat *hsp40* nucleotide sequence encoding a peptide of 216 amino acids, respectively. "N" represents unknown nucleotide, and "X" represents unknown amino acid.

A)

```
1          ACTTCCTTTGGAGATTTCCAGAGTGTATTTGATCAGCCTCAGGAATATATCATGGAGTTA
1           T  S  F  G  D  F  Q  S  V  F  D  Q  P  Q  E  Y  I  M  E  L

61         ACCTTCAATCAGGCTGCCAAGGGTGTCAACAAGGAGTTCACTGTGAACATCATGGACACC
21          T  F  N  Q  A  A  K  G  V  N  K  E  F  T  V  N  I  M  D  T

121        TGTGAGCGCTGCGACGGAAAAGGGAATGAGCCCGGAACCAAGGTGCAGCATTGTCACTAC
41          C  E  R  C  D  G  K  G  N  E  P  G  T  K  V  Q  H  C  H  Y

181        TGCGGGGGCTCTGGCATGGAAACCATCAATACAGGCCCTTTTGTGATGCGTTCCACCTGT
61          C  G  G  S  G  M  E  T  I  N  T  G  P  F  V  M  R  S  T  C

241        CGGAGATGCGGTGGCAGGGGCTCCATCATCACAACTCCCTGTGTGATCTGCAGAGGAGCA
81          R  R  C  G  G  R  G  S  I  I  T  T  P  C  V  I  C  R  G  A

301        GGACAAGCCAAGCAGAAAAAGCGAGTAGTGATCCCTGTGCCTGCAGGAGTCGAGGATGGC
101         G  Q  A  K  Q  K  K  R  V  V  I  P  V  P  A  G  V  E  D  G

361        CAAACTGTGAGGATGCCTGTGGGAAAACGAGAAATTTTTGTCACATTCAGGGTGCAGAAG
121         Q  T  V  R  M  P  V  G  K  R  E  I  F  V  T  F  R  V  Q  K

421        AGCCCTGTGTTCCGGAGGGACGGCGCAGACAT
141         S  P  V  F  R  R  D  G  A  D
```

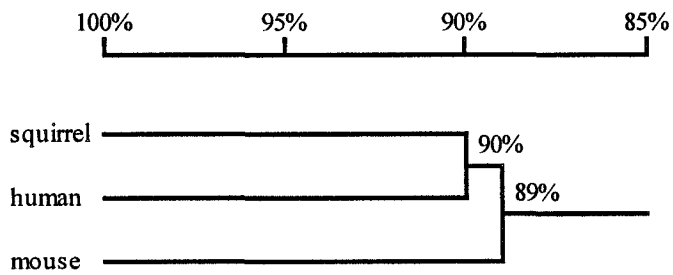
B)

```
1          GAGAGGAAGCAGTATGATGCCTACGGCTCCACTGGCTTTGATCCTGGGGCTGGTGGCTCT
1           E  R  K  Q  Y  D  A  Y  G  S  T  G  F  D  P  G  A  G  G  S

61         GGGCAGAGCTACTGGAAAGGAGGCCCCACCGTCGACCCAGAGGAGCTCTTCAGGAAGATC
21          G  Q  S  Y  W  K  G  G  P  T  V  D  P  E  E  L  F  R  K  I

121        TTTGGGGAATTCTCATCATCTTCCTTTGGAGATTTCCAGAGTGTATTCAGTCAGCCTCAG
41          F  G  E  F  S  S  S  S  F  G  D  F  Q  S  V  F  S  Q  P  Q

181        GAGTATATCATGGATTTGACATTCAATCAAGCTGCCAAGGGTGTCAACAAGGAGTTCACT
61          E  Y  I  M  D  L  T  F  N  Q  A  A  K  G  V  N  K  E  F  T

241        GTGAACATCACCGATACCTGTGAGCGGTGCAATGGCAAGGGGAATGAGCCTGGCACCAAG
81          V  N  I  T  D  T  C  E  R  C  N  G  K  G  N  E  P  G  T  K

301        GTGCAGCATTGCCACTACTGCGGTGGCTCCGGCATGGAAACCATAAATACGGGCCCTTTT
101         V  Q  H  C  H  Y  C  G  G  S  G  M  E  T  I  N  T  G  P  F

361        GTGATGCGCTCCACGTGTCGGAGATGTGGTGGCCGAGGCACCATCATCACAACTCCATGT
121         V  M  R  S  T  C  R  R  C  G  G  R  G  T  I  I  T  T  P  C

421        GTTATATGCAGAGGAACAGGAGAAGCCAAGCAGAAGAAGAAAGTGGTTATCCCTGTGCCT
141         V  I  C  R  G  T  G  E  A  K  Q  K  K  K  V  V  I  P  V  P

481        GCAGGAGTTGAGGATGGCCAGACTGTGAGGATGCCTGTAGGAAAAAGAGAAATTTTCATC
161         A  G  V  E  D  G  Q  T  V  R  M  P  V  G  K  R  E  I  F  I

541        ACGTTCAGGGTGGAGAAAAGCCCNGTGTTCCGAAGGGACNGCGCAAACATCCACTCTGAC
181         T  F  R  V  E  K  S  P  V  F  R  R  D  X  A  N  I  H  S  D

601        CTCTTTATTTTCCTAGCTCAGGCTCTTCTTGGGGGGACAGCCAAAACCC
201         L  F  I  F  L  A  Q  A  L  L  G  G  T  A  K  T
```

Fig. 4.14 Comparison of squirrel (*Spermophilus tridecemlineatus*) (A) and bat (*Myotis lucifugus*) (B) *hsp40* partial nucleotide sequences with human and mouse sequences, respectively, and homology tree showing the percent identity between the sequences. Genbank accession numbers are: human (*Homo sapiens*, NM_005147) and mouse (*Mus musculus,* NM_023646). The nucleotide sequences were aligned using DNAMAN software and the homology tree generated by DNAMAN shows the percent identities between the *hsp40* nucleotide sequences. Dashes (-) replace those nucleotides that are identical with the squirrel or bat sequences.

A)

```
squirrel   ACTTCCTTTGGAGATTTCCAGAGTGTATTTGATCAGCCTCAGGAATATATCATGGAGTTA    60
human      t----a----------------cc--g--------------------ct----------g    60
mouse      t--c-t-----t----------a---g--------------------c--------a--g    60

squirrel   ACCTTCAATCAGGCTGCCAAGGGTGTCAACAAGGAGTTCACTGTGAACATCATGGACACC   120
human      --a--------a-----a-----g-----------------c-----------------g   120
mouse      --a--------a---------------------a-----------------t-----t---   120

squirrel   TGTGAGCGCTGCGACGGAAAAGGGAATGAGCCCGGAACCAAGGTGCAGCATTGTCACTAC   180
human      ------------a----c--g-----c--------c-----------------c------   180
mouse      -----------t-----c--g-----c-----t--------a-----------c--t---   180

squirrel   TGCGGGGGCTCTGGCATGGAAACCATCAATACAGGCCCTTTTGTGATGCGTTCCACCTGT   240
human      --t--c-----c-----------------c-------------------------g---   240
mouse      --t--c-----g-----------t-----------g---------------------a---   240

squirrel   CGGAGATGCGGTGGCAGGGGCTCCATCATCACAACTCCCTGTGTGATCTGCAGAGGAGCA   300
human      a--------t------c-c-------------t-t-g---------g-------g------   300
mouse      --------t------c-----------------a----------g-------g------   300

squirrel   GGACAAGCCAAGCAGAAAAAGCGAGTAGTGATCCCTGTGCCTGCAGGAGTCGAGGATGGC   360
human      -------------------------ga-------------------------------   360
mouse      -----------------g-----c--gaca--t----------------t--a-----t   360

squirrel   CAAACTGTGAGGATGCCTGTGGGAAAACGAGAAATTTTTGTCACATTCAGGGTGCAGAAG   420
human      --g--c---------------------a-g--------ca-t--g--------------a   420
mouse      --g-----a--------a-----------------------------------------a   420

squirrel   AGCCCTGTGTTCCGGAGGGACGGCGCAGACAT                              452
human      --------------------------------                              452
mouse      ----------------------t--------                              452
```



B)

```
bat       GAGAGGAAGCAGTATGATGCCTACGGCTCCACTGGCTTTGATCCTGGGGCTGGTGGCTCT   60
human     a------------c--------------tg-a-----c----------ca-c-----c     60
mouse     a----------------c--t--------tg----------c-----ca-ca-ca-----   60

bat       GGGCAGAGCTACTGGAAAGGAGGCCCCACCGTCGACCCAGAGGAGCTCTTCAGGAAGATC  120
human     ca---t----------g----------t--g-----c--------g-----------     120
mouse     ------g---------g------t---t-t--t-----c------t-g-----------   120

bat       TTTGGGGAATTCTCATCATCTTCCTTTGGAGATTTCCAGAGTGTATTCAGTCAGCCTCAG  180
human     -----c--g--------c-----a---------------cc--g--tga----------   180
mouse     --------g--------t---c-t-----t----------a---g--tga----------   180

bat       GAGTATATCATGGATTTGACATTCAATCAAGCTGCCAAGGGTGTCAACAAGGAGTTCACT  240
human     --a--ct-------g--------------------a-----g----------------c   240
mouse     --a--c--------a-----------------------------------a---------   240

bat       GTGAACATCACCGATACCTGTGAGCGGTGCAATGGCAAGGGGAATGAGCCTGGCACCAAG  300
human     ----------tg--c--g--------c-----c----------c-----c---------   300
mouse     --------t-tg--------------c--tg-c----------c--------a-----a   300

bat       GTGCAGCATTGCCACTACTGCGGTGGCTCCGGCATGGAAACCATAAATACGGGCCCTTTT  360
human     --------------------t--c--------------------c--c--a---------   360
mouse     --------------t-----t--c-----g----------t--c-----a--g------   360

bat       GTGATGCGCTCCACGTGTCGGAGATGTGGTGGCCGAGGCACCATCATCACAACTCCATGT  420
human     --------t---------a----------------c---t---------t-t-g--c---   420
mouse     --------t-----a--------------------g---t----------a---c---   420

bat       GTTATATGCAGAGGAACAGGAGAAGCCAAGCAGAAGAAGAAAGTGGTTATCCCTGTGCCT  480
human     --gg-c-----g---g-----c-------------a---cg----a-g-----------   480
mouse     --gg-c-----g---g-----c-----------------cgc---aca--t---------   480

bat       GCAGGAGTTGAGGATGGCCAGACTGTGAGGATGCCTGTAGGAAAAAGAGAAATTTTCATC  540
human     --------c--------------c--------------g--------g----------t   540
mouse     ----------a-----t--------a---------a--g------c----------tg--  540

bat       ACGTTCAGGGTGGAGAAAAGCCCNGTGTTCCGAAGGGACNGCGCAAACATCCACTCTGAC  600
human     ------------c----------t--------g------g-----g----------c---   600
mouse     --a---------c----------t--------g------g-t---g----------g---   600

bat       CTCTTTATTTTCCTAGCTCAGGCTCTTCTTGGGGGGACAGCCAAAACCC             649
human     ----------cta----------------------a-------g-g---             649
mouse     ----------caa-------a---a----------c---------g---             649
```

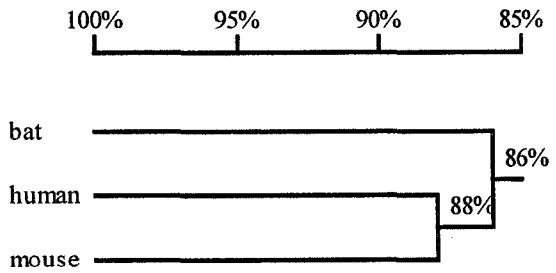
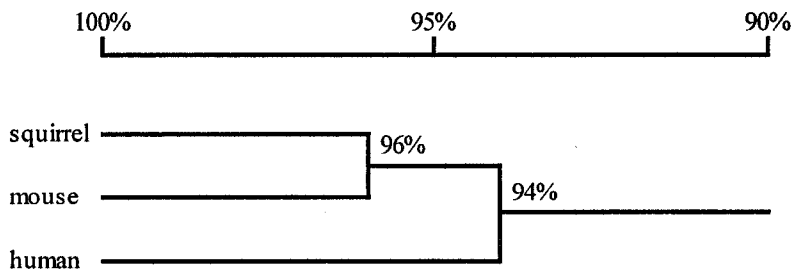


Fig. 4.15 Comparison of HSP40 partial amino acid sequences of ground squirrel (*Spermophilus tridecemlineatus*) (A) and bat (*Myotis lucifugus*) (B) with human and mouse sequences and homology tree showing the percent identity between the sequences. In (C) the alignment of all four amino acid sequences is shown for the 150 amino acids that they share in common. Genbank accession numbers are: human (*Homo sapiens*, NM_005147) and mouse (*Mus musculus,* NM_023646). DNAMAN software was used to translate the nucleotide sequences, generate the alignment and compute the percent identities. Dashes (-) replace those amino acids that are identical with the first sequence shown. "X" represents an unknown amino acid.

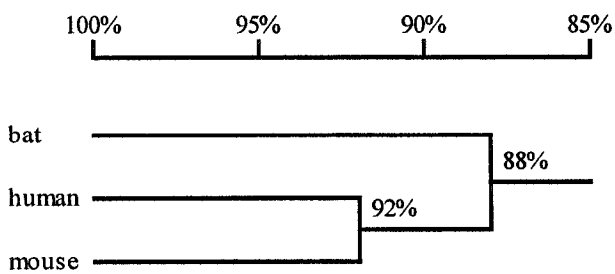
A)

```
squirrel   TSFGDFQSVFDQPQEYIMELTFNQAAKGVNKEFTVNIMDTCERCDGKGNEPGTKVQHCHY    60
human      s------t--------f-------------------------n---------------    60
mouse      sp-----n-------------------------------------------------    60

squirrel   CGGSGMETINTGPFVMRSTCRRCGGRGSIITTPCVICRGAGQAKQKKRVVIPVPAGVEDG   120
human      ------------------------------is---v------------m----------   120
mouse      --------------------------------n---v------------t----------   120

squirrel   QTVRMPVGKREIFVTFRVQKSPVFRRDGAD                               150
human      ------------i----------------                                150
mouse      ----------------------------                                 150
```



B)

```
bat        ERKQYDAYGSTGFDPGAGGSGQSYWKGGPTVDPEELFRKIFGEFSSSSFGDFQSVFSQPQ    60
human      k---------a------s--qh------------------------------t--d---    60
mouse      k---------a-----tss---g--r---s-----------------p-----n--d---    60

bat        EYIMDLTFNQAAKGVNKEFTVNITDTCERCNGKGNEPGTKVQHCHYCGGSGMETINTGPF   120
human      --f-e------------------m-------------------------------------   120
mouse      ----e------------------m------d-----------------------------   120

bat        VMRSTCRRCGGRGTIITTPCVICRGTGEAKQKKKVVIPVPAGVEDGQTVRMPVGKREIFI   180
human      -------------s--is---v---a-q-----r-m-----------------------   180
mouse      -------------s---n---v---a-q-----r-t----------------------v   180

bat        TFRVEKSPVFRRDXANIHSDLFIFLAQALLGGTAKT                         216
human      ----q--------g-d-------si---------ra                         216
mouse      ----q--------g-d-------si---i------a                         216
```



C)

```
bat        SSFGDFQSVFSQPQEYIMDLTFNQAAKGVNKEFTVNITDTCERCNGKGNEPGTKVQHCHY    60
squirrel   t---------d-------e------------------m------d---------------    60
human      -------t--d-----f-e------------------m----------------------    60
mouse      -p-----n--d-------e------------------m------d---------------    60

bat        CGGSGMETINTGPFVMRSTCRRCGGRGTIITTPCVICRGTGEAKQKKKVVIPVPAGVEDG   120
squirrel   ----------------------------s-----------a-q-----r------------   120
human      ----------------------------s--is---v---a-q-----r-m----------   120
mouse      ----------------------------s---n---v---a-q-----r-t----------   120

bat        QTVRMPVGKREIFITFRVEKSPVFRRDXAN                                 150
squirrel   -------------v----q--------g-d                                  150
human      ------------------q--------g-d                                  150
mouse      -------------v----q--------g-d                                  150
```

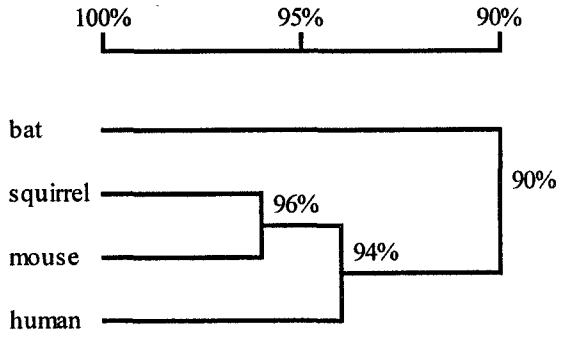


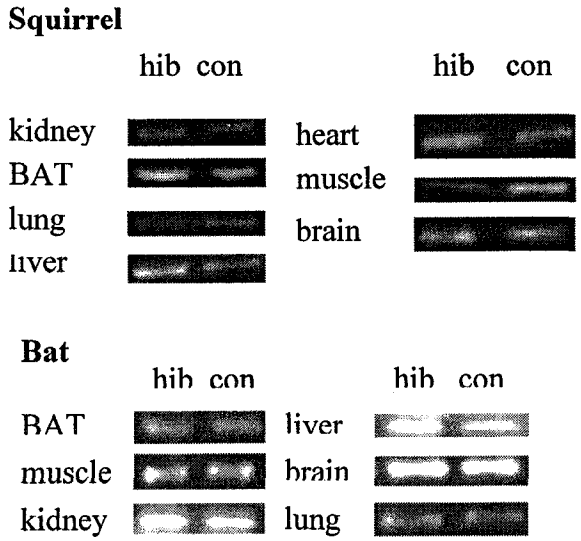
Fig. 4.16 The effects of hibernation on ground squirrel and bat HSP40 protein expression in different tissues.

A) Representative Western blots showing HSP40 protein levels in seven squirrel tissues and six bat tissues of euthermic (con) and hibernating (hib) animals.

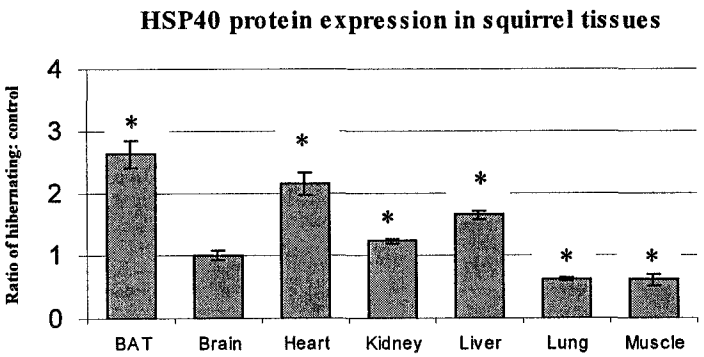
B) Histograms show the ratio of HSP40 protein levels in hibernating versus euthermic ground squirrels. Mean values (± SEM) were calculated from n = 3 trials. * - Mean value for hibernating sample is significantly different from the corresponding euthermic value, $P < 0.05$.

C) Histograms show the ratio of HSP40 protein levels in hibernating versus euthermic bats. Mean values (± SEM) were calculated from n = 3 trials. * - Mean value for hibernating sample is significantly different from the corresponding euthermic value, $P < 0.05$.

A



B)



C)

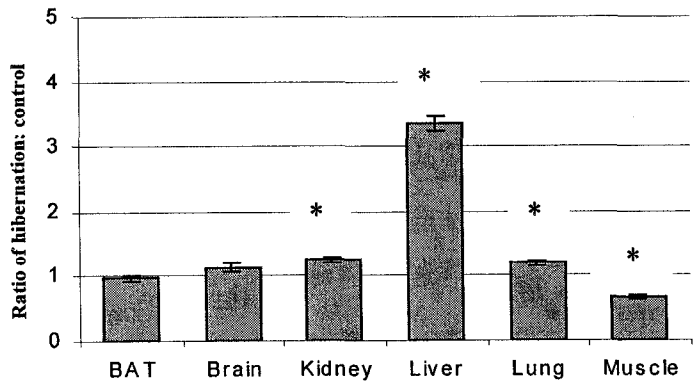


Fig. 4.17 Representative 2D-PAGE Western blot showing HSP40 protein spots in ground squirrel liver (A) and bat liver (B), respectively.

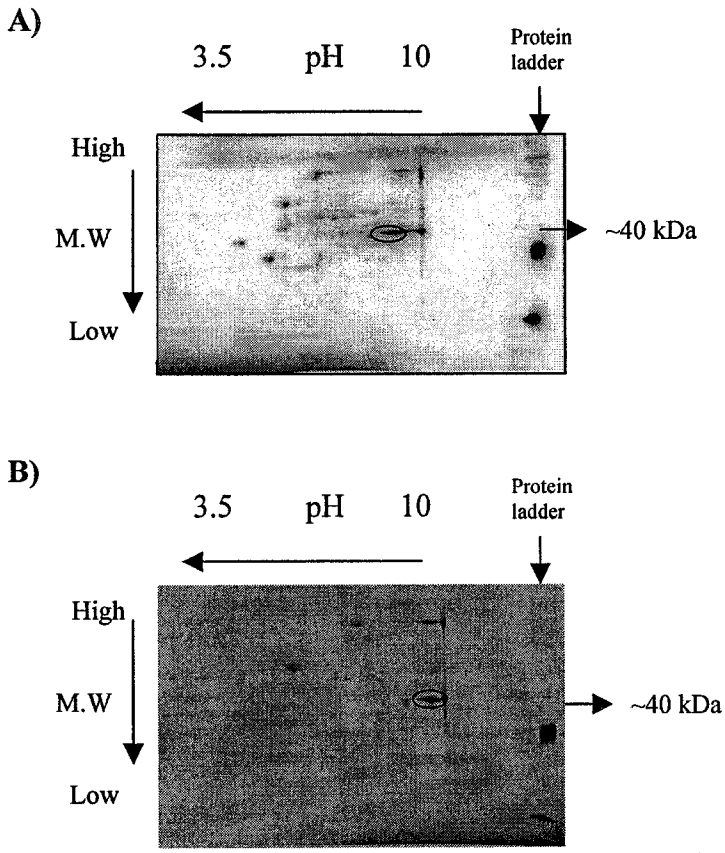
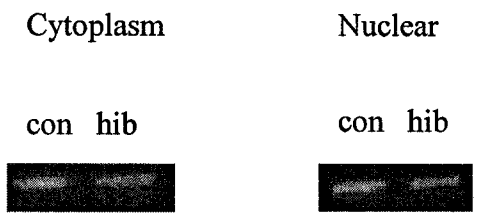


Fig.4.18 HSP40 protein distribution in nuclear versus cytoplasmic fractions as assessed after subcellular fractionation and Western blotting.

A) Representative Western blots show HSP40 levels in cytoplasmic and nuclear fractions of ground squirrel skeletal muscle from euthermic control (con) versus hibernating (hib) animals.

B) Histogram shows the relative expression levels of HSP40 (hibernating versus control) in cytoplasmic and nuclear fractions of squirrel muscle. Mean values (± SEM) were calculated from n = 3 independent trials. * - Mean value for hibernating sample was significantly different from the corresponding euthermic value, $P < 0.05$.

A)



B)

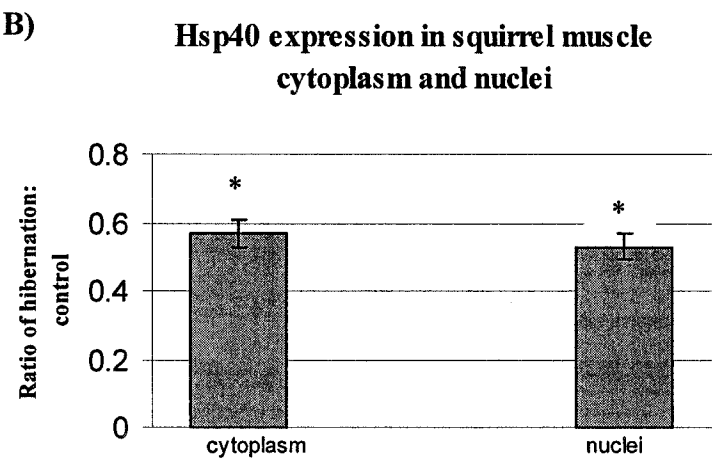


Fig. 4.19 Partial nucleotide sequences of thirteen-lined ground squirrel (*Spermophilus tridecemlineatus*) *hsp90α* (A) and *hsp90β* (B) and their deduced amino acid sequences. The partial cDNA sequences were amplified from squirrel kidney by RT-PCR using degenerate primers. A single open reading frame was predicted from the 620 bp of squirrel *hsp90α* nucleotide sequence encoding a peptide of 206 amino acids and from the 560 bp of squirrel *hsp90β* nucleotide sequence encoding a peptide of 186 amino acids, respectively. "N" represents unknown nucleotide, and "X" represents unknown amino acid.

A)

```
1        GGGAAGTGATCTACATGATTGAGCCCATCGATGAGTACTGTGTCCAACAGCTGAAGGAAT
1           E  V  I  Y  M  I  E  P  I  D  E  Y  C  V  Q  Q  L  K  E

61       TTGAGGGGAAGACTTTAGTGTCTGTCACCAAAGAGGGCTTGGAGCTTCCAGAAGATGAAG
20       F  E  G  K  T  L  V  S  V  T  K  E  G  L  E  L  P  E  D  E

121      AAGAGAAGAAGAAACAGGAAGAGAAAAAGACAAAGTTTGAAAACCTCTGTAAGATCATGA
40       E  E  K  K  K  Q  E  E  K  K  T  K  F  E  N  L  C  K  I  M

181      AGGACATCTTGGAGAAAAAGGTTGAAAAGGTGGTTGTGTCAAACCGATTGGTGACCTCTC
60       K  D  I  L  E  K  K  V  E  K  V  V  V  S  N  R  L  V  T  S

241      CATGCTGTATCGTCACAAGCACATACGGCTGGACAGCAAACATGGAAAGAATCATGAAAG
80       P  C  C  I  V  T  S  T  Y  G  W  T  A  N  M  E  R  I  M  K

301      CTCAAGCTCTCAGAGATAACTCTACAATGGGCTACATGGCAGCAAAGAAACACCTGGAGA
100      A  Q  A  L  R  D  N  S  T  M  G  Y  M  A  A  K  K  H  L  E

361      TAAATCCTGACCACTCCATTATTGAGACCTTGAGGCAAAAGGCAGAGGCTGACAAGAATG
120      I  N  P  D  H  S  I  I  E  T  L  R  Q  K  A  E  A  D  K  N

421      ATAAGTCTGTGAAAGATCTGGTCATCTTGCTGTACGAAACTGCTCTCCTGTCTTCTGGCT
140      D  K  S  V  K  D  L  V  I  L  L  Y  E  T  A  L  L  S  S  G

481      TCAGTTTGGAAGATCCCCAGACCCATGCTAACAGGATCTACAGGATGATCAAACTTGGTC
160      F  S  L  E  D  P  Q  T  H  A  N  R  I  Y  R  M  I  K  L  G

541      TAGGTATTGATGAGGATGATCCCACTGCTGACGACACCAGTGCTGCTGTAACAGAAGAGA
180      L  G  I  D  E  D  D  P  T  A  D  D  T  S  A  A  V  T  E  E

601      TGCCACCCCTGGAAGGGGAT
200      M  P  P  L  E  G  D
```

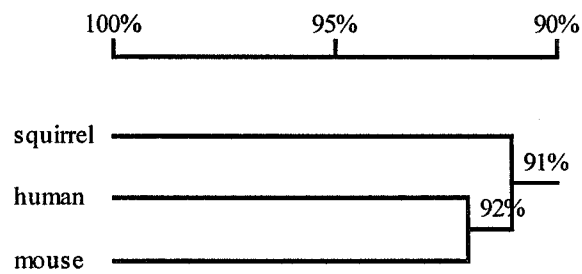
B)

```
  1      GGTGGGATTTATTCTGCTTATCTAGTGGCAGAGAAAGTGGTTGTGATCACAAAGCATAAT
  1       G  G  I  Y  S  A  Y  L  V  A  E  K  V  V  V  I  T  K  H  N

 61      GATGATGAACAGTATGCTTGGGAGTCTTCTGCTGGGGGATCTTTCACTGTGCGGGCTGAT
 21       D  D  E  Q  Y  A  W  E  S  S  A  G  G  S  F  T  V  R  A  D

121      CATGGTGAGCCCATGGCCGGGGTACTAAAGTCATTCTCCACCTCAAAGAAGACCAGACA
 41       H  G  E  P  I  G  R  G  T  K  V  I  L  H  L  K  E  D  Q  T

181      GAGTACTTGGAGGAGAGGCGTGTCAAAGAAGTGGTGAAGAAGCACTCACAGTTCATAGGC
 61       E  Y  L  E  E  R  R  V  K  E  V  V  K  K  H  S  Q  F  I  G

241      TATCCAATCACCCTTTATTTGGAGAAGGAACGAGAGAAGGAAATCAGTGATGATGAGGCA
 81       Y  P  I  T  L  Y  L  E  K  E  R  E  K  E  I  S  D  D  E  A

301      GAGGAAGAGAAAGGTGAGAAAGAAGAGGAAGATAAAGATGATGAGGAGAAACCCAAGATT
101       E  E  E  K  G  E  K  E  E  E  D  K  D  D  E  E  K  P  K  I

361      GAAGATGTGGGCTCAGATGAGGAGGATGACACTAGTAAGGATAAGAAGAAGAAAACAAAG
121       E  D  V  G  S  D  E  E  D  D  T  S  K  D  K  K  K  K  T  K

421      AAGATTAAGGAGAAATATATTGATCAAGAAGAACTGAACAAGACCAAGCCCATTTGGACC
141       K  I  K  E  K  Y  I  D  Q  E  E  L  N  K  T  K  P  I  W  T

481      AGAAACCCTGATGACATCACTCAGGAAGAATATGGAGAATTCTACAAGAGCCTAACCAAT
161       R  N  P  D  D  I  T  Q  E  E  Y  G  E  F  Y  K  S  L  T  N

541      GATTGGGAAGANCACTTGGC
181       D  W  E  X  H  L
```

Fig. 4.20 Comparison of squirrel *hsp90α* (A) and *hsp90β* (B) partial nucleotide sequences with human and mouse *hsp90α* and *hsp90β* nucleotide sequences, respectively. Genbank accession numbers for *hsp90α* are human (*Homo sapiens*, NM_005348) and mouse (*Mus musculus*, NM_010480) and for *hsp90β* are human (*Homo sapiens*, NM_007355) and mouse (*Mus nusclulus*, NM_008302), respectively. The nucleotide sequences were aligned using DNAMAN software and the homology tree generated by DNAMAN shows the percent identities between different nucleotide sequences. Dashes (-) replace those nucleotides that are identical with the squirrel sequence.

A)

```
squirrel   GGGAAGTGATCTACATGATTGAGCCCATCGATGAGTACTGTGTCCAACAGCTGAAGGAAT   60
human      ta----------t-------------t-----------------------------      60
mouse      ta-----a--t--t-------------t--------t-----g----------------      60

squirrel   TTGAGGGGAAGACTTTAGTGTCTGTCACCAAAGAGGGCTTGGAGCTTCCAGAAGATGAAG   120
human      ----------------------a----------a---c----a--------g-------   120
mouse      -------c-----c--g--------t--------a--ac----a----------------   120

squirrel   AAGAGAAGAAGAAACAGGAAGAGAAAAAGACAAAGTTTGAAAACCTCTGTAAGATCATGA   180
human      -------a-----g-------------a----------g--------c--a-------   180
mouse      -g--a-----------------------------a-----g--------c--a--t----   180

squirrel   AGGACATCTTGGAGAAAAAGGTTGAAAAGGTGGTTGTGTCAAACCGATTGGTGACCTCTC   240
human      -a-----a----------a-----------------------------------a----   240
mouse      -a--t--t--------g-----------------------------c-------a--c-   240

squirrel   CATGCTGTATCGTCACAAGCACATACGGCTGGACAGCAAACATGGAAAGAATCATGAAAG   300
human      ----------t-------------t--------------------g-------------   300
mouse      -g--------t-------------t--g-----------------g-------------   300

squirrel   CTCAAGCTCTCAGAGATAACTCTACAATGGGCTACATGGCAGCAAAGAAACACCTGGAGA   360
human      -------c--a-----c-----a--------t---------------------------   360
mouse      -------c--------c-----a--------t---------------------------   360

squirrel   TAAATCCTGACCACTCCATTATTGAGACCTTGAGGCAAAAGGCAGAGGCTGACAAGAATG   420
human      ----c--------t-----------------a--------------------t-----c-   420
mouse      ----------t-------------a-----a---------------------------   420

squirrel   ATAAGTCTGTGAAAGATCTGGTCATCTTGCTGTACGAAACTGCTCTCCTGTCTTCTGGCT   480
human      -c----------g-----------------t--t--------g----------------   480
mouse      -c--a--------g--------------------t--------a-----a----------   480

squirrel   TCAGTTTGGAAGATCCCCAGACCCATGCTAACAGGATCTACAGGATGATCAAACTTGGTC   540
human      -----c----------------a-------------------------------------   540
mouse      -----c-----------------------------------------------g-------   540

squirrel   TAGGTATTGATGAGGATGATCCCACTGCTGACGACACCAGTGCTGCTGTAACAGAAGAGA   600
human      -g----------a-----c--t--------t--t-----------------t-----a-   600
mouse      ----------------------t----tg--t--------------------t-----a-   600

squirrel   TGCCACCCCTGGAAGGGGAT                                          620
human      ----------t-----a---                                          620
mouse      ----t-----------a---                                          620
```



B)

```
squirrel   GGTGGGATTTATTCTGCTTATCTAGTGGCAGAGAAAGTGGTTGTGATCACAAAGCATAAT    60
human      -t---ct----------c--ct-g-------------------------------c--c    60
mouse      -tc--at-c--c--g--c--------t----------------------g-----c---    60

squirrel   GATGATGAACAGTATGCTTGGGAGTCTTCTGCTGGGGGATCTTTCACTGTGCGGGCTGAT   120
human      ----------------------------------a--t--c----------t-----c   120
mouse      --------g--------c--------g-----g--t--c--c-----c--c-----a--c   120

squirrel   CATGGTGAGCCCATTGGCCGGGGTACTAAAGTCATTCTCCACCTCAAAGAAGACCAGACA   180
human      ------------------a-------c-----g--c-----t--t--------t------   180
mouse      --------------------------c-----g--c--t---------------------   180

squirrel   GAGTACTTGGAGGAGAGGCGTGTCAAAGAAGTGGTGAAGAAGCACTCACAGTTCATAGGC   240
human      ------c-a--a--------g----------a----------t--t-------------   240
mouse      ------------------a-g-----g--------------a--t--g-------------   240

squirrel   TATCCAATCACCCTTTATTTGGAGAAGGAACGAGAGAAGGAAATCAGTGATGATGAGGCA   300
human      -----c----------------------------------------t--------------   300
mouse      -----c--------c-----------------g--------g------------------   300

squirrel   GAGGAAGAGAAAGGTGAGAAAGAAGAGGAAGATAAAGATGATGAGGAGAAACCCAAGATT   360
human      ----------------------------------------a--a------------c   360
mouse      ----------------------g----------g--g----------g--t-------   360

squirrel   GAAGATGTGGGCTCAGATGAGGAGGATGACACTAGTAAGGATAAGAAGAAGAAAACAAAG   420
human      ----------t------------------gcg----------------------t---   420
mouse      ----------a--c--------a-------gcg-c--a--c-----a------------   420

squirrel   AAGATTAAGGAGAAATATATTGATCAAGAAGAACTGAACAAGACCAAGCCCATTTGGACC   480
human      -----c--a--------c--------g--------a--------------t---------   480
mouse      -----c--a-----g--c-----c--g--g--g----------a-----t--c-------   480

squirrel   AGAAACCCTGATGACATCACTCAGGAAGAATATGGAGAATTCTACAAGAGCCTAACCAAT   540
human      --------------------c--a--g--g----------------------c--t---   540
mouse      --------g----------g-----g--g-----c--------t--------c------   540

squirrel   GATTGGGAAGANCACTTGGC                                         560
human      --c--------c--------                                         560
mouse      --c-----g--c--------                                         560
```

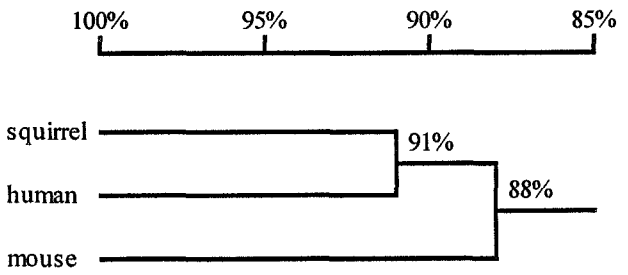
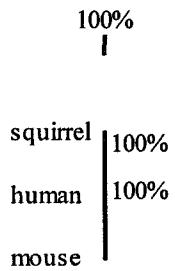


Fig. 4.21. Comparison of ground squirrel (*Spermophilus tridecemlineatus*) HSP90α (A) and HSP90β (B) partial amino acid sequences with human and mouse HSP90α and HSP90β sequences, respectively. Genbank accession numbers for HSP90α are: human (*Homo sapiens*, NM_005348) and mouse (*Mus musculus*, NM_010480) and for *hsp90β* are human (*Homo sapiens*, NM_007355) and mouse (*Mus nusclulus*, NM_008302), respectively. DNAMAN software was used to translate the nucleotide sequences, generate the alignment and compute the percent identities. Dashes (-) replace those amino acids that are identical to those in the squirrel sequence.

A)

```
squirrel   EVIYMIEPIDEYCVQQLKEFEGKTLVSVTKEGLELPEDEEEKKKQEEKKTKFENLCKIMK   60
human      ------------------------------------------------------------   60
mouse      ------------------------------------------------------------   60

squirrel   DILEKKVEKVVVSNRLVTSPCCIVTSTYGWTANMERIMKAQALRDNSTMGYMAAKKHLEI  120
human      ------------------------------------------------------------  120
mouse      ------------------------------------------------------------  120

squirrel   NPDHSIIETLRQKAEADKNDKSVKDLVILLYETALLSSGFSLEDPQTHANRIYRMIKLGL  180
human      ------------------------------------------------------------  180
mouse      ------------------------------------------------------------  180

squirrel   GIDEDDPTADDTSAAVTEEMPPLEGD                                     206
human      -------------------------                                     206
mouse      --------v----------------                                     206
```



B)

```
squirrel   GGIYSAYLVAEKVVVITKHNDDEQYAWESSAGGSFTVRADHGEPIGRGTKVILHLKEDQT   60
human      v-f---------------------------------------------------------   60
mouse      v-f---------------------------------------------------------   60

squirrel   EYLEERRVKEVVKKHSQFIGYPITLYLEKEREKEISDDEAEEEKGEKEEEDKDDEEKPKI  120
human      ------------------------------------------------------------  120
mouse      ------------------------------------------------e-----------  120

squirrel   EDVGSDEEDDTSKDKKKKTKKIKEKYIDQEELNKTKPIWTRNPDDITQEEY           171
human      ----------sg--------------------------------------           171
mouse      ----------sg--------------------------------------           171
```

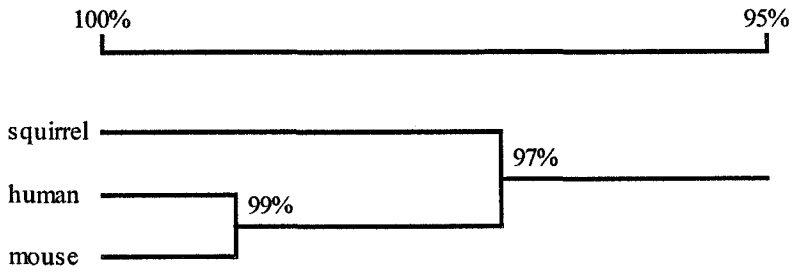
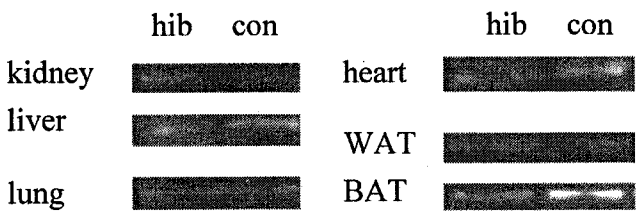


Fig. 4.22. The effects of hibernation on ground squirrel HSP90α/β protein expression in different tissues.

A) Representative Western blots showing HSP90α/β protein levels in six tissues of euthermic (con) and hibernating (hib) ground squirrels.

B) Histograms show the ratio of HSP90α/β protein levels in hibernating versus euthermic ground squirrels. Mean values (± SEM) were calculated from n = 3 trials. * - Mean value for hibernating sample is significantly different from the corresponding euthermic value, $P < 0.05$.

A)



B)

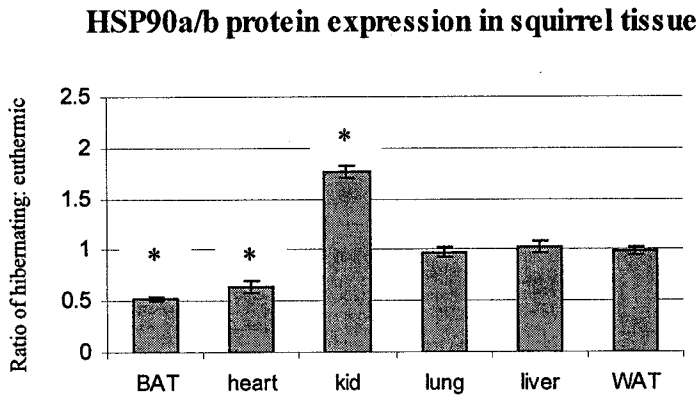


Fig. 4.23 Representative 2D-PAGE Western blots showing HSP90α/β protein spot in ground squirrel liver.

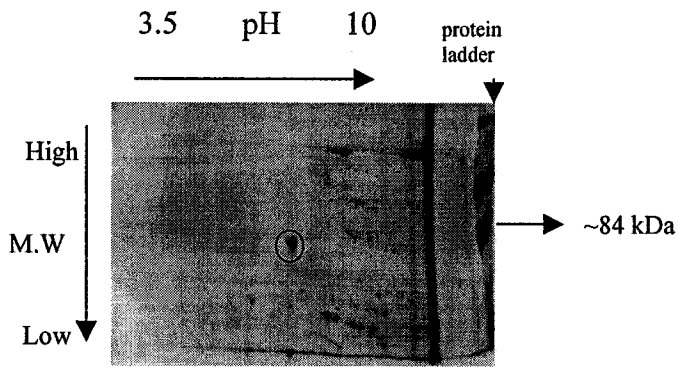


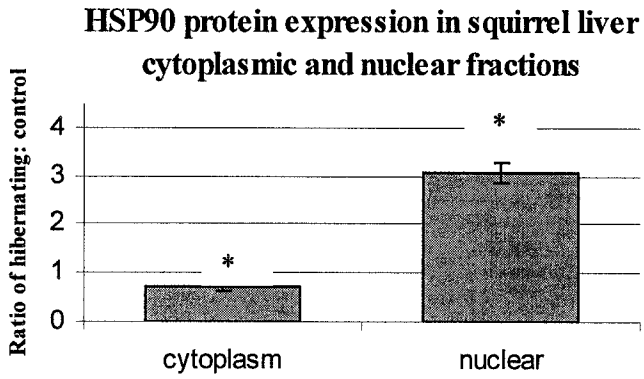
Fig.4.24 HSP90α/β protein distribution in nuclear versus cytoplasmic fractions of ground squirrel liver as assessed after subcellular fractionation and Western blotting.

A) Representative Western blots show HSP90α/β levels in cytoplasmic and nuclear fractions from euthermic control (con) versus hibernating (hib) animals.

B) Histogram shows the relative expression levels of HSP90α/β (hibernating versus control) in cytoplasmic and nuclear fractions of squirrel liver. Mean values (± SEM) were calculated from n = 3 independent trials. * - Mean value for hibernating sample was significantly different from the corresponding euthermic value, $P < 0.05$.

A)

B)



CHAPTER 5
DISCUSSION

Hibernators can maintain their body temperatures near 0°C for several weeks without injury whereas nonhibernating mammals (such as man) typically die of hypothermia when core body temperature falls below about 20-25°C (Breukelen and Martin, 2002; Hirvonen, 1979). With profoundly reduced metabolism, reduced heart rate and extremely low body temperature, hibernators can save up to 90% of the energy that would otherwise be needed to remain euthermic over the winter months. The low body temperatures of the hibernating state could disturb normal cellular processes and one of the consequences could be an increase in misfolded and unfolded proteins. Stress conditions that are damaging to cellular proteins can trigger the increased expression of several highly conserved proteins such as GRPs and HSPs.

During hibernation, overall metabolic rate is strongly suppressed as a result of the strong inhibition of many metabolic processes; one of the prominent functions that is strongly decreased is protein biosynthesis. At least two factors contribute to the general inhibition of protein synthesis in hibernation: (a) phosphorylation-mediated inhibition of selected ribosomal initiation and elongation factors including eukaryotic initiation factor 2 (eIF2) and eukaryotic elongation factor 2 (eEF2) (DeGracia *et al.*, 2002), and (b) disaggregation of polysomes (Storey and Storey, 2004). Although the mRNA transcript levels for most genes are not affected in squirrel and bat tissues during hibernation (Eddy and Storey, 2002; Hittel and Storey, 2002) and protein translation slows dramatically during torpor (Breukelen and Martin, 2002), the expression of selected genes is up-regulated as evidenced by elevated mRNA and/or protein levels (Andrews *et al.*, 1998; Boyer *et al.*, 1988; Fahlman *et al.*, 2000; Gorham *et al.*, 1998; Hittel and Storey, 2001). The present study showing that Grps and Hsps increase in tissue-specific patterns during

hibernation indicates that these proteins are part of a select group of proteins that are up-regulated during hibernation. Enhanced levels of these chaperones would potentially help to stabilize other cellular proteins during hibernation.

To explore the molecular basis of mammalian hibernation, RT-PCR and Western blot techniques were employed to analyze the expression of some well-known shock proteins in order to determine whether the altered expression of HSPs and GRPs could contribute to the biochemical phenotype of hibernation. The results showed enhanced expression of proteins in both of these families during hibernation as evidenced by changes in mRNA (Grps) and/or protein (Grps, Hsps) levels.

5.1. Glucose regulated proteins

5.1.1. Analysis of the structures and similarity of GRP75, GRP94 and GRP170

Grps are mainly located in ER and/or mitochondria where they function as chaperones to bind to newly synthesized, unfolded, and /or incompletely glycosylated proteins in the lumen of the ER and mitochondria and are also involved in peptide import (Easton *et al.*, 2000; Ran *et al.*, 2000). Studies to date indicate that GRP families are evolutionarily conserved and present results agree with this. In comparison with other nonhibernator mammals, the partial amino acid sequences of squirrel and bat GRP75 had very high similarities up to 99% and 97%, respectively (Fig. 3.3 B). The squirrel GRP94 partial amino acid sequence was also highly conserved with 98% identity with the human and mouse sequences (Fig. 3.11). Ground squirrel and bat partial GRP170 sequences also showed 96% and 94% identities, respectively, with mouse and human sequences (Fig.3.18 A and B). Hence, all three GRPs in squirrels and/or bats show high

conservation of sequence as compared with other mammals. Furthermore, there were only a few unique amino acid substitutions that were specific to the proteins from hibernators. No unique amino acid substitutions were seen in the GRP75 sequences of either ground squirrels or bats as compared with the other mammals. Ground squirrel GRP94 showed two unique changes as compared with the sequence of human, mouse and cow GRP94: a lysine at residue 160 replacing asparagine and a serine at residue 169 replacing glycine in the other sequences. Compared with human, mouse and rat sequences, ground squirrel GRP170 showed a substitution of a threonine replacing serine at residue 28 whereas bat GRP170 showed two differences, a glutamine replacing histidine at residue 131 and a glutamate replacing alanine at residue 137. However, the two hibernator species clearly do not have the same substitutions and, therefore, it may be difficult to characterize these substitutions as being important for low temperature function of the protein. Future studies would have to purify GRP 94 and GRP170 from hibernators and analyze physical and functional properties of the proteins at high and low temperatures in comparison with the homologous proteins from nonhibernating mammals.

5.1.2. Hibernation responsive transcription and/or translation Grp75

GRP75 is a well-studied chaperone protein that participates in protein folding in mitochondria and the endoplasmic reticulum. The data for squirrel tissues show that GRP75 protein was elevated in brain and liver of hibernating animals although *grp75* message was stable during hibernation. Hence, the expression patterns at transcription and translation levels do not match. The explanation for this may be the differential

distribution of individual mRNA species between polysome and monosome fractions. During hibernation, an overall disaggregation of polysomes occurs, which is one factor in the inhibition of protein synthesis. However, not all polysomes disappear during hibernation, and some selected mRNA types remain with the polysome fraction and are preferentially translated during hibernation (Hittel and Storey, 2002). This may account for the enhanced levels of GRP75 protein in brain and liver despite a lack of change in *grp75* message. Alternatively, since protein degradation is also suppressed during hibernation (Storey and Storey, 2004), a reduced rate of GRP75 degradation would also lead to a net elevation of the protein during hibernation. In squirrel muscle and heart, the opposite expression pattern was seen – although mRNA expression was stable, protein levels decreased. This indicates a reduced need for this protein in muscles during hibernation and may result from differential rates of the synthesis versus degradation of this protein in muscles during hibernation. Transcript levels of *grp75* were strongly elevated in squirrel BAT during hibernation (by 2.8-fold) and also increased slightly in kidney, but GRP75 protein content stayed constant in these two tissues during hibernation. Stimulation of *grp75* in BAT during hibernation could occur as a result of an accumulation of misfolded proteins in cells at low temperature (Lee, 2001). Up-regulation of GRP75 may also help to minimize oxidative damage during torpor (Carey *et al.*, 2000). The unfolded protein response is known to include induction of some molecular chaperone genes such as *grp75*. The signals and transcription factor involved in this induction of *grp75* in BAT remain to be determined. Once synthesized, *grp75* gene transcripts are likely sequestered into the monosome fraction which is translationally silent so that the protein is not synthesized during torpor. However, such a

pattern would allow rapid rates of synthesis of GRP75 protein when the ground squirrels arouse from torpor. BAT is the key organ providing thermogenesis to rewarm the hibernator during arousal from torpor and enhanced rates of protein synthesis during the arousal process may be necessary to support this function. Early synthesis of GRP75 during arousal, from pre-existing stored transcripts may be important for rapidly elevating the levels of this chaperone which could then aid the folding of other newly synthesized proteins, particularly in the mitochondria where thermogenesis is centered.

In bats, an organ-specific pattern of response by *grp75* transcripts and GRP75 protein was again seen but differed substantially from the ground squirrels. Both mRNA and protein were slightly increased in muscle and brain of hibernating bats, compared with euthermic animals. Hence, gene and protein expression patterns correlated well in these two organs and this argues for a necessary function for elevated GRP75 in these organs of torpid animals. In bat liver and lung, *grp75* was relatively stable but protein fell somewhat, suggesting a reduced need for GRP75 chaperone action in these organs during hibernation.

GRP75 can bind other stress proteins like GRP94 (Takano *et al.*, 2001) and therefore it might be expected that the two proteins would be up-regulated together in situations where they were needed. Indeed, there is evidence of this from the present results. In ground squirrels, GRP75 protein was significantly elevated in brain and liver during hibernation (Figure 3.6). GRP94 protein was also elevated in these organs (as well as in lung and muscle) (Figure 3.13). Furthermore, *grp75* and *grp94* mRNA levels were both elevated in ground squirrel BAT and, although protein was not increased in BAT, the increased message may support enhanced protein synthesis during arousal and

interbout periods. All of these results suggest coordinate regulation of GRP75 and GRP94.

5.1.3. Hibernation responsive transcription and/or translation Grp94

GRP94 is the most abundant protein of the ER lumen (Csermely *et al.*, 1998) and possibly has a very similar structural and functional organization of its domains to that of HSP90. Therefore, it may have a N-terminal domain that can bind ATP/ADP and target protein, C-terminal domain that can bind target protein and peptide, and highly charged hinge region that contains nuclear localization signal and can bind target protein (Csermely *et al.*, 1998). GRP94 is a hydrophobic protein and forms dimers (Nomoto *et al.*, 1996) which show a tail-to-tail organization of two GRP94 molecules because of the hydrophobic interactions (Wearsch and Nicchitta, 1996). GRP94 is phosphorylated by many protein kinases (Cala and Jones, 1994; Wearsch and Nicchitta, 1997) and is also a calcium-binding protein with 4 high-affinity and about 10 low-affinity calcium-binding sites (Van *et al.*, 1989). Csermely *et al.* (1998) think that calcium may have an important role in the regulation of GRP94 functions because of the high calcium concentration of the ER. GRP94 is also a glycoprotein and its glycosylation pattern tends to change under stress, characterized by the increased resistance to endoglycosidase H digestion (Booth and Koch, 1989). Csermely *et al.* (1998) suggest that the status of GRP94 glycosylation may play an important role in the regulation of ER chaperone activity after stress. Additionally, GRP94 can specifically bind many kinds of peptides (Wearsch *et al.*, 1998). The GRP94-peptide binding can be activated by stress and coincident with a stable,

tertiary conformational change (Wearsch *et al.*, 1998). GRP94 is an ATP binding protein as well (Clairmont *et al.*, 1992).

The transcription and translation of GRP94 was examined in the organs of euthermic versus hibernating squirrels. Overall, the data show that neither mRNA nor protein levels were greatly affected during hibernation. Squirrel brain showed a consistent small increase (about 1.2-fold) in both mRNA and protein, which suggests a need for the protective action of GRP94 in brain during hibernation. GRP94 protein was also slightly elevated (1.2-1.3 fold) in liver, lung and muscle during hibernation although mRNA levels remained the same or lower. This could suggest a difference in the rates of GRP94 protein synthesis versus degradation during hibernation so that a net small accumulation of the protein occurs. The expression pattern in BAT was comparable to the response seen for GRP75; that is, *grp94* mRNA increased during hibernation but GRP94 protein was unaffected. Again, this might result from sequestration of *grp94* mRNA into the monosome fraction during torpor so that rapid protein translation can occur during arousal.

5.1.4. Hibernation responsive transcription and/or translation Grp170

GRP170 is localized in the ER and is induced under ischemia/hypoxia which Pahl (1999) thinks may trigger an ER stress response. GRP170 was originally purified and cloned from cultured rat astrocytes exposed to hypoxia and has protective effects in neurons (Tamatani *et al.*, 2001). Secondary structure predictions of GRP170 suggest that it contains an apparent ATPase domain, β-stand domain (peptide binding), and long loop followed by a helical domain (Easton *et al.*, 2000). The reported functions of GRP170 are

mainly in the protection of ER under anoxic and ischemic stress (Tsukamoto *et al.*; Tsukamoto *et al.*, 1998; Tamatani *et al.*, 2001; Miyazaki *et al.*, 2002), low pH, and glucose starvation (Kobayashi and Ohta, 2005). The intracellular functions may be in protein folding and /or assembly. GRP170 has been described as an ER resident glycoprotein and may play a role in immunoglobulin folding and assembly in conjunction with GRP78 and GRP94 (Lin *et al.*, 1993). GRP170 is also an efficient ATP-binding protein (Dierks *et al.*, 1996).

Grp170 was examined in both squirrel and bat tissues. Compared with the other two GRPs studied, the mRNA expression level of *grp170* was very low in both euthermic and hibernating tissues; indeed, in some tissues it was not detected due to the low level expression. Significant decreases occurred in both squirrel and bat lung and kidney *grp170* transcripts during hibernation, which suggests a reduced requirement for GRP170 in these organs during torpor. In squirrel heart, however, *grp170* transcripts were significantly increased during hibernation. Grp170 is also called oxygen regulated protein 150 (ORP150) and is known to be responsive to the hypoxia-inducible transcription factor (HIF-1). When DNA array screening was used to search for hibernation-responsive genes in ground squirrel heart, both the alpha and beta subunits of HIF-1 as well as ORP150 and prolyl hydroxylase were identified as putatively up-regulated in hibernation (Storey, 2003). Recent studies have shown that HIF-1α is up-regulated in selected organs of ground squirrels during hibernation (Morin and Storey, 2005). Hypoxia leads to a drop in Tb in many mammalian species. Hibernating species show an even more pronounced drop in Tb in response to hypoxia and a hypoxia-hypothermia connection has been proposed as a part of the mechanism of regulating the drop in Tb during hibernation. The

upregulation of *grp170* in heart play a part in this hypoxia-hypothermia connection (Renata *et al.*, 2001).

The subcellular distribution of Grp75 and Grp94 in cytoplasmic and nuclei was also analyzed in squirrel skeletal muscle. Results show that both proteins are located in both cytoplasmic and nuclear fractions, which suggests that both proteins also have roles to play in nuclei.

5. 2. Heat shock proteins

5.2.1. Analysis of the structure and similarity of HSP40, HSP72, HSP73 and HSP90

Proteins in the Hsp40 chaperone family play an important role as molecular chaperones in assisting protein folding under both normal and stressed conditions (Kelley, 1998; Hennessy *et al.*, 2005). Hsp40 proteins contain a J domain which features a ~70-amino-acid-residue signature and is comprised of four alpha-helices and a loop region with a fixed tripeptide of histidine, proline and aspartic acid (HPD motif). The Helix II and the HPD motif have very important roles in recruiting co-chaperone (Hsp70) partners and accelerating the ATP-hydrolysis step of the chaperone cycle (Hennessy *et al.*, 2005). Hsp40 functions as a molecular chaperone by forming dimers and the C-terminal fragment of Hsp40 is responsible for dimerization (Wu *et al.*, 2005; Borges *et al.*, 2005). The sequence of Hsp40 is typically highly conserved and my study shows that this is also true of hibernating mammals in comparison with nonhibernators. Thus, the squirrel partial Hsp40 amino acid sequence showed 96% and 94% identity, respectively, with that of the mouse and human sequences, while the bat partial Hsp40 had 88% identity with human and mouse (Fig. 4.15 A and B). When the sequence segments that

were shared in common between bat, squirrel, human and mouse were compared (Fig. 4.15C), it can be seen that bat Hsp40 showed 10 substitutions that were not seen in the squirrel, mouse or human sequences. However, only 1 substitution was shared in common by the two hibernators – isoleucine at position 96 replacing valine in the nonhibernators. Hence, there is not good evidence in the case of Hsp40 for sequence changes that might support low temperature function of the hibernator protein.

Hsp72 and Hsp73 are members of HSP70 family. Hsp72 is a major inducible member of the heat shock protein family and can protect cells against many cellular stresses including heat shock. Hsp73 is the constitutively synthesized protein. Both Hsps have several functional domains. The peptide-binding domain is located in the carboxyl portion which plays an important role in protecting cells from thermal stress by binding to unfolded or partially folded polypeptides (Li *et al.*, 1995). All HSP70 family members bind ATP (Snoeckx *et al.*, 2001). The amino-terminal domain contains ATPase activity. Lewis and Pelham (1985) showed evidence that Hsp72 bound tightly first to some nuclear component(s) and then to nucleoli after heat shock but were released rapidly from these binding sites when ATP was present even at very low levels. They suggest that ATP-driven cycles of binding and release of hsp70 proteins help to solubilize aggregates of proteins or RNPs that form after heat shock. In addition to the binding sites, both proteins carry nuclear localization signals (NLS) (Snoeckx *et al.*, 2001; Lamian *et al.*, 1996). Most Hsp70 family members are well conserved across phylogeny. In this study, the results show that squirrel and bat Hsp72 amino acid partial sequences shared 99% and 96% identities, respectively, with human, dog and cow sequences whereas Hsp73 shared 98% identities with human and mouse, respectively. This suggests that hibernator Hsp72

and Hsp73 share all the common functional domains of the proteins in nonhibernating mammals. Furthermore, there were no cases where the Hsp72 sequences from the two hibernators showed a common substitution that was different from the sequences of nonhibernators. However, Hsp73 showed two substitutions that were shared by ground squirrels and bats (glycine 209, lysine 210) replacing two glutamate residues in the nonhibernating mammals (Fig. 4.9).

Hsp90, one of the most abundant cytosolic Hsps, is a highly conserved and essential stress protein that is expressed in all eukaryotic cells. Three important functions of Hsp90 are related with its conformational transitions. The most conserved domain of Hsp90 is the nucleotide-binding pocket near the N-terminus which binds to ATP and ADP (Grenert *et al.*, 1997). The binding of ATP induces dimer interaction near the N-terminal domains of the Hsp90 homodimer (Prodromou *et al.*, 2000). In addition to the N-terminal, the C-terminal site also has a nucleotide-binding site (Marcu *et al.*, 2000) which interacts with that in the N-terminal site in an coordinated fashion (Pratt and Toft, 2003) for regulating the conformational state of Hsp90. Hsp90 has been found to interact with co-chaperones including the proteins that contain TPR (tetratricopeptide repeat) domains like Hop; the interaction occurs near the C-terminus of Hsp90 and forms a complex with the co-chaperone (Pratt and Toft, 2003). The mechanism regarding how Hsp90 interacts with its substrate remains unclear. One possibility is that multiple sites for substrate-binding are located in Hsp90. At least two regions of Hsp90, one near the N-terminus containing a peptide binding site that seems to preferentially bind peptides longer than 10 amino acids and one near the C-terminus binding to partially folded proteins in an ATP-independent way potentially regulated by co-chaperones, have been

shown to prevent the aggregation of denatured polypeptides (Young *et al.*, 1997; Scheibel *et al.*, 1998). Hsp90 also plays an important role in folding various nuclear hormone receptors and a number of protein kinases, all of which are involved in signaling. The chaperone complex of Hsp90·Hop·Hsc70·p23 folds steroid receptors and aids the maturation of the receptors in which Hsp90 plays a crucial role as a central organizer of the "early" (Hsc70- and Hop-containing) and "late" (p23-containing) complexes (Csermely *et al.*, 1998). Like other chaperones, Hsp90 protein is highly conserved. Our study shows that one of the isoforms, Hsp90α, had 100% identity among squirrel, human and mouse partial amino acid sequences whereas Hsp90β had 97% identity between squirrel and human/mouse partial amino acid sequences. This indicates that squirrel Hsp90 has the same domains and functional groups as does the protein in other organisms. Hsp90β showed 4 unique substitutions as compared with the human or mouse sequences: these were glycine 1, isoleucine 3, threonine 131 and serine 132 replacing valine, phenylalanine, serine and glycine, respectively.

5.2.2. Hsp40, Hsp72, Hsp73 and Hsp90 in squirrel hibernation

The main function of Hsp chaperones is to aid unfolded and misfolded proteins to enter or resume their normal conformation, to prevent the aggregation of "sticky" protein-folding intermediates to refold from folding traps by controlled binding and release, and transport the proteins to their destination (Snoeckx *et al.*, 2001). During hibernation, small mammals experience low body temperatures which would induce conformational changes in various proteins, sometimes with damaging consequences for protein function. Increased numbers of functionally compromised proteins could cause

the induction of molecular chaperones like heat shock proteins to either retain or restore the functional conformations of proteins. In our present study, the four Hsps studied were all elevated in some squirrel and/or bat tissues during hibernation.

Ydj1 is the major type I Hsp40 (heat-shock protein 40) family member in yeast. Ydj1 itself can function as a molecular chaperone to bind non-native polypeptides and suppress protein aggregations in vitro (Li and Sha, 2005) and the over-expression of another member of Hsp40, HDJ2, alone also significantly reduced astrocyte injury after both GD (glucose deprivation) and OGD (oxygen-glucose deprivation) (Qiao, 2003). Although the mechanisms by which Hsp40 functions as a molecular chaperone to recognize and bind non-native polypeptides is not understood, Li *et al.* (2003) found a crystal structure of the yeast Hsp40 Ydj1 complexed with its peptide substrate. In the complex, the Ydj1 peptide binding fragment contains three domains and the peptide substrate binds Ydj1 by forming an extra beta strand with domain I of Ydj1, while the leucine residue in the middle of the peptide substrate GWLYEIS inserts its side chain into a hydrophobic pocket formed on the molecular surface of Ydj1 domain I. Structure-based mutagenesis studies show that the hydrophobic pocket located on Ydj1 domain I may play a major role in mediating the interactions between Ydj1 and the peptide substrate (Li and Sha, 2005). Since the Hsp40 protein sequence is well conserved, the significantly up-regulated Hsp40 protein in squirrel BAT, liver, kidney and heart may have the same function as Ydj1 in yeast and HDJ2 in mouse to bind non-native polypeptides and suppress protein aggregations caused by low temperature during hibernation.

In addition to binding non-native polypeptides and suppressing protein aggregations by Hsp40 itself, another molecular chaperone activity for Hsp40 is to

facilitate Hsp70 to refold non-native polypeptides. The molecular chaperones, Hsp72 and Hsp73, are ubiquitous molecular chaperones that possess weak ATPase activity which can be substantially stimulated by peptide binding (Flynn *et al.*, 1989; Flaherty *et al.*, 1990). These chaperones contain three interdependent domains, which are a highly conserved 44-kDa N-terminal ATPase domain, an 18-kDa peptide binding domain, and a 10-kDa C-terminal helical lid domain. Lee e*t al.* (2004) found that cytosolic chaperone pairs of the Hsp70 family and their DnaJ homolog co-chaperones prevent nitric oxide-mediated apoptosis and heat-induced cell death, and they also found that the damage to rat liver by tetrachloride significantly induced both mRNA and protein expression of the cytosolic chaperones, Hsp40 and Hsp72. Ydj1 can pair with yeast Hsp70 Ssa1 to facilitate protein translocation and protein folding (Li and Sha, 2005). In mammals, DjA4 (another type I of DnaJ/Hsp40 homolog) is highly expressed in heart and testis, and the coexpression of Hsp70 and DjA4 protects against heat stress-induced cell death (Hafizur *et al.*, 2004); DjA4 binds Hsp70 and promotes its ATPase activity, and the energy from the hydrolysis of ATP is used to fold the dysfunctional proteins generated under heat stress. In addition to the functions mentioned above, Hsp72 and Hsp73 both play a role in protein degradation. Previously studies show that Hsp70 guides misfolded proteins to lysosomes (Agarraberes *et al.*, 1997; Chiang *et al.*, 1989) and in lysosomes, Hsp73 assists in protein degradation by transferring them into this organelle (Agarraberes *et al.*, 1997; Terlecky *et al.*, 1992). The elevated Hsp40 and Hsp73 protein expression in squirrel heart and kidney during hibernation may behave in this same way to help protect these organs from low temperature induced damage to proteins during hibernation and/or to transport misfolded proteins to lysosomes for degradation.

Hsp90 is another cytosolic stress protein. It contains ATP and ADP binding pockets near the N-terminus which act as a "molecular clamp" whose opening and closing by transient N-terminal dimerization are directly coupled to the ATPase cycle (Grenert *et al.*, 1997; Prodromou *et al.*, 2000). Recent studies indicate the presence of yet another nucleotide-binding site near the C-terminus of hsp90 (Garnier *et al.*, 2002; Marcu *et al.*, 2000; Soti *et al.*, 2002). ATP binding and hydrolysis is used to regulate the conformational states of the Hsp90 during its activities, and ATP is essential for the formation of an hsp90 state that is able to bind its co-chaperones like p23 (Grenert *et al.*, 1997). Although Hsp90 is an essential molecular chaperone that is critical for the activity of diverse cellular proteins, it is unable to bind biologic substrates such as steroid receptors on its own and requires the assistance of several other proteins (Pratt and Toft, 2003); therefore, the multiple sites for substrate binding to Hsp90 are needed. So far, three regions of hsp90, one near the N-terminus, one near the C-terminus, and a middle region, have been shown to prevent the aggregation of denatured proteins (Young *et al.*, 1997; Scheibel *et al.*, 1998; Johnson *et al.*, 2000). The Hsp90 complexes related to steroid receptor function and trafficking includes Hsp90 itself, Hsp70, Hsp40, Hop and p23. In these complexes, Hsp70 is required for the assembly of signalling protein·Hsp90 heterocomplexes, and these two chaperones likely interact directly with each other during opening of the steroid-binding cleft in the GR (Morishima *et al.*, 2001). Hop (Hsp-organizing protein), a 60-kDa protein, directly contacts Hsp90 and Hsp70 (Chen *et al.*, 1996). p23 is present to bind to the ATP-dependent conformation of hsp90 and stabilize its association with the receptor (Dittmar *et al.*, 1997). Hsp40 is a component of the multiprotein hsp90-based chaperone system where it potentiates GR.hsp90

heterocomplex assembly (Dittmar *et al.*, 1998). The chaperone activities of Hsp90 reported are almost exclusively related to the folding of various nuclear hormone receptors and a number of protein kinases, all of which are involved in signalling (Csermely *et al.*, 1998). The increases in Hsp40, Hsp73 and Hsp90 seen in squirrel kidney may be related to the coordinated formation of Hsp90 complexes and the folding of various nuclear hormone receptors and/or protein kinases during hibernation. Another novel role for Hsp90 was recently found; Hsp90 plays a role in the essential cellular functions of transcription and DNA repair by the interaction of an ATP-dependent DNA helicase (Flom *et al.*, 2005). The up-regulation of Hsp90 in squirrel kidney may also have the same function in folding the denatured enzymes during hibernation.

Compared with euthermic controls, Hsp90 was significantly elevated in the squirrel liver nuclear fraction and reduced in the cytoplasmic fraction, which indicates that during squirrel hibernation, a large portion of Hsp90 was transported to the nucleus from the cytoplasm to function as a nuclear chaperone during hibernation to bind denatured proteins and/or new synthesized proteins.

5.2.3. Hsp40, Hsp72, Hsp73 and Hsp90 in bat hibernation

In bat tissues, Hsp40 protein levels were strongly enhanced in liver during hibernation, rose slightly in kidney and lung and decreased in muscle. Hsp72 was slightly up-regulated in brain and liver but down-regulated or stable in other tissues during hibernation. Hsp73 was slightly enhanced in liver, but strongly reduced in lung. The notable increases in all three Hsps bat liver could perhaps suggest the importance of

chaperones both for stabilizing proteins within this central biosynthetic organ and for the correct folding of the many of the proteins that liver produces for export.

The cytosolic yeast Hsp40 Ydj1 contains a conserved zinc finger-like region (ZFLR), which has two zinc-binding domains (ZBD) that help regulate and specify Hsp70 function (Fan *et al.*, 2005). ZBDII is essential for Ydj1 to cooperate with Hsp70 to suppress protein aggregation and, therefore, it is required for yeast to survive heat stress. But for protein folding, both ZBDI and ZBDII are required for Hsp70 to capture non-native polypeptides from Ydj1. In this study, Hsp40, Hsp72, and Hsp73 were all up-regulated in bat liver. The up-regulation of Hsp40 may bind non-native polypeptides and then cooperate with either Hsp72 or Hsp73 to fold the polypeptides by stimulating ATP hydrolysis in the ATPase domain and capturing the client protein in the peptide-binding domain of Hsp70/DnaK. The J-domain of DnaJ simultaneously stimulates ATP hydrolysis in the ATPase domain and capture of the client protein in the peptide-binding domain of DnaK (Landry, 2003). During hibernation, the fine structure of the kidney cortex is well preserved, whereas most apparent ultrastructural changes take place in glomerular endothelial cells and podocytes (Zancanaro *et al.*, 1999). The finding of only minor ultrastructural changes in the kidney of arousing dormice undergoing 'reperfusion' further suggests that the whole process is finely regulated to prevent lesion. The up-regulation of Hsp40 in bat kidney may contribute to the maintenance and preservation of cell and organ structure in hibernation. Lung experiences long periods of ischemia and hypoxia in torpor (Mellen *et al.*, 2002), and so does brain, which may induce the molecular chaperone Hsp40 protein in bat lung and Hsp70 in bat brain, respectively, to fold the misfolded proteins resulting from stress.

5.3. A comparison of Grp and Hsp responses to hibernation in ground squirrels and bats

Overall, for most Grps studied, the changes in gene expression were more evident in ground squirrel tissues than in bat tissues during hibernation. For example, at the mRNA level, *Grp75* was strongly up-regulated in ground squirrel BAT whereas it was only slightly up-regulated in some bat tissues. *Grp170* transcripts were also strongly up-regulated in ground squirrel heart during torpor but transcripts were not elevated in any bat tissue examined. At the protein level, Grp75 increased strongly in ground squirrel brain but was only slightly elevated in bat brain and muscle.

Similar findings were noted for Hsps. Hsp40 protein was highly expressed in ground squirrel BAT, heart and liver during hibernation whereas levels increased strongly only in liver of bats during hibernation. Hsp72 increased substantially in ground squirrel muscle but rose only slightly in bat brain and liver during hibernation. Hsp73 was moderately expressed in ground squirrel heart, kidney and lung but only slightly increased in bat liver during hibernation. These results may indicate that bat organs experience less stress than do ground squirrel organs during hibernation. However, it may also be that constitutive levels of shock proteins (and shock protein mRNA) in bat tissues are high enough that there is not a need for up-regulation of the genes/proteins during each hibernation bout.

The patterns of expression of some of the shock proteins were similar in ground squirrel and bat tissues. For instance, Hsp40 was stable in brain, up-regulated in kidney and liver, and down-regulated in muscle of both ground squirrels and bats. This suggests that Hsp40 expression may be independent of species but respond to the common needs

of different organ types during hibernation. Hsp40 can function as a molecular chaperone alone and can also facilitate the binding of Hsp70s to other proteins.

Hsp72 is thought to be highly inducible whereas Hsp73 is constitutively expressed. The present analysis shows that Hsp72 is highly expressed in tissues of both control and hibernating animals and levels were elevated only in muscle of hibernating ground squirrels and in brain and liver of hibernating bats. This indicates that Hsp72 is sufficiently expressed in both stress and non-stress cells of hibernators. Hsp73 was upregulated in ground squirrel heart and lung but strongly suppressed in bat lung, kidney and muscle. This demonstrates that Hsp73 is actually inducible and its chaperone function is species specific.

In the present study, all the proteins except Grp170 were found to reside in both the cytoplasm and the nuclear fraction in ground squirrel muscle or liver. Both Hsp72 and Hsp73 carry nuclear localization signals (NLS) (Snoeckx *et al.*, 2001). Tuijl *et al.* (1991) and Welch *et al.* (1984) reported stress-mediated translocation of both Hsc70 and Hsp72 into the cellular nucleus, in particular to the nucleolus, which suggests a specific and unique role in the repair and protection of these cellular structures (Collier *et al.*, 1986).

5.4. Conclusions

Based on the above results, I conclude that Grps and Hsps indeed play a role in mammalian hibernation, supporting organ specific needs during torpor-arousal cycles by assisting in protein folding/refolding, degradation, and inhibition of irreversible aggregation of denatured proteins. However, although the elevated protein levels imply a

functional need for these proteins in hibernation, full proof that Hsps have protective roles in hibernation remains to be determined.

5.4. Perspectives

The data in the present thesis has documented a role for shock proteins in mammalian hibernation but considerable work remains to be done to fully understand what that role is as some actions and/or targets of Hsps and Grps might be quite different from their known functions under other stress conditions (e.g. heat) in nonhibernating species. Further studies could address the following topics.

1. To further characterize the role and control of HSps and Grps in hibernation more work could be done on the transcriptional regulation of the genes. For example, a detailed time course of the response/levels of the HSF transcription factor that control Hsp gene transcription should be done along with quantitative analysis of Hsp mRNA levels in response to hibernation. This would (presumably) confirm that HSF is the regulatory factor involved in hibernation-responsive expression of Hsps and then further experiments could be designed to examine the upstream second messengers and protein kinases that control HSF. Knowledge of the signaling pathway that is regulating HSF in hibernation would provide ideas about of how and why Hsps are responding to hibernation.

2. Mammalian heat shock proteins are usually induced at high body temperatures (39°C to 42°C) and function at euthermic (or higher) body temperatures. Protein structure and function is affected by temperature change because the hydrophobic and hydrophilic weak bonds that determine protein conformation are

differentially affected by temperature change. Hence, it is perhaps possible that the chaperone effects that Hsps or Grps have at euthermic body temperatures could be quite different to their actions/effects at cold body temperatures during hibernation. Hsps could be isolated from the tissues of hibernators (or produced by overexpression of complete Hsp genes generated from cDNA library screening). Their function at 5°C versus 37°C should be analyzed by examining and comparing their ability to re-fold denatured enzymes or other proteins *in vitro* at the two temperatures. Similarly, high versus low temperature functions of Grps could be analyzed with similar procedures.

3. The present study shows that HSPs and GRPs are elevated in selected tissues of hibernators but each tissue is composed of different cell types. Studies could be done to determine whether shock protein responses occur throughout an organ or occur only in a specific cell type within the tissue.

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