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Molecular chaperones : protein folding, translocation, and assembly in the cell

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**MOLECULAR CHAPERONES: PROTEIN FOLDING,
TRANSLOCATION, AND ASSEMBLY IN THE CELL**

A THESIS

Presented to

The Faculty of the Department of Chemistry

San Jose State University

In Partial Fulfillment

of the Requirements for the Degree

Master of Arts

by

Bhavna McGowan

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ABSTRACT

MOLECULAR CHAPERONES: PROTEIN FOLDING, TRANSLOCATION, AND ASSEMBLY IN THE CELL

by Bhavna J. McGowan

Molecular chaperones is a term applied to a large group of unrelated proteins sharing a common function to guide and assist nascent polypeptides through the folding, assembly, and translocation pathway. These proteins include stress induced as well as constitutively expressed proteins. They help maintain the precursor protein in a "translocation-competent" conformation by binding to exposed hydrophobic and charged sites. In doing so, they prevent premature interactions with inter- or intra-polypeptide domains and with the environment from occurring and hence inhibit the pathway leading to aggregation. They do not convey any steric information and are released once the environment in the cell is conducive for the proper folding of the polypeptide into its native three dimensional functional conformation. Molecular chaperones play a significant role in the growth, survival, and regulation of a cell participating in cellular processes such as protein folding, translocation, import and export into various compartments, and immunological responses such as antigen presentation.

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GLOSSARY FOR ABBREVIATIONS

AZC	L-azetidine 2-carboxylic acids
cpn 60	chaperonin 60
Ct	Cytoplasmic
C-terminus	Carboxy-terminus
DHFR	Dihydrofolate reductase
DTT	Dithiothreitol
ER	Endoplasmic Reticulum
F1 β	Mitochondrial F1-ATPase β Subunit
Grps	Glucose regulated proteins
Hsp	Heat Shock proteins
MBP	maltose binding protein
MHC	Major histocompatibility complex
Mt	Mitochondrial
NEM	N-ethylmaleinimide
N-terminus	Amino-terminus
OmpA	Outer membrane protein A
OmpF	Outer membrane protein F
ppjF	Prepro-j factor
RBP	Ribose binding protein
Rubisco	Ribulose-1,5-bisphosphate carboxylase/oxygenase
smHsp	Small heat shock proteins
TCP1	Tailless complex polypeptide

I. INTRODUCTION

Proteins in the cell must be in their native, completely folded form in order to be functionally competent. The formation of the three dimensional structure requires the presence of a complete polypeptide sequence or a domain of approximately 70 amino acids. The polypeptide chains can begin folding as soon as they emerge from the ribosomes as nascent chains or from the lipid bilayer following membrane translocation.

As proteins emerge from the ribosomes as nascent polypeptide chains with their amino terminus end, they exist temporarily in an unfolded or partially folded state. The polypeptide chains that are destined for transport can only pass across membranes in an extended partially folded state in order to reach their proper compartment (Hartl et al., 1994). In such an extended conformation, potentially interactive surfaces such as hydrophobic pockets and charged sites which would otherwise be buried in the native state are exposed to solvent. This can lead to premature interactions with other inter- or intra-polypeptide domains and/or with the environment resulting in misfolding or aggregation (Ellis and van der Vies, 1991). The relatively high protein concentrations found in the cytosol increases the probability of incorrect folding reactions allowing the pathway to an aggregated state to kinetically outcompete that of the correct folding pathway (Landry and Gierasch, 1994). Although the primary sequence of a protein specifies the final three-dimensional structure, the cell needs additional information to help guide the polypeptide to its proper destination where it can fold properly and become functionally active (Hartl et al., 1994).

The cell must somehow create an environment where aggregation is prevented and proper folding of a polypeptide can occur in order for cellular processes to function normally. There are several mechanisms that a cell uses to accomplish this task. It can 1) block hydrophobic sites to avoid further interactions leading to aggregation, 2) create an environment in the cell to facilitate protein folding, 3) increase the kinetics of the reaction to the folded pathway to outcompete the reaction to the aggregated state, 4) prevent the chain from premature folding until the entire chain is synthesized, and 5) protect intermediates along a folding pathway from proteolysis and other interactions leading to misfolding and aggregation. These functions can be carried out by the cell with the aid of a group of structurally unrelated proteins called molecular chaperones (Agard, 1993; Landry and Gierasch, 1994).

Molecular chaperones are ubiquitous proteins consisting of several protein families. Their role lies in helping the cell combat the high risk of malfunction due to incorrect premature interactions that could occur while proteins are attempting to fold into their functional conformation (Hartl et al., 1994). They assist protein folding in the cell by maintaining newly synthesized proteins in a "translocation-competent" or "export-competent" state. The term "translocation-competent" or "export-competent" state refers to an intermediate in the folding pathway which is equivalent energetically to the unfolded state but is highly dynamic and compact. This is referred to as the "molten globule state." The molten globule state is similar to the native state in dimension and secondary structure but lacks stable tertiary structure. Hydrophobic surfaces that are normally buried within the interior of the native state are exposed in this state. This solvent exposure of hydrophobic surfaces renders these

intermediates prone to aggregation (Baldwin et al., 1989; Jaenicke et al., 1987).

The chaperones bind to and stabilize these hydrophobic patches and keep them from interacting with each other or the environment until the protein can assume its native structure. There is no threat of aggregation once a protein assumes its biologically competent three dimensional structure since all interactive surfaces are buried within the interior of the protein (Ellis and van der Vies, 1991).

The term "molecular chaperone" was originally coined by Laskey et al. (1978) to describe the function of nucleoplasmin found in *Xenopus oocytes*. Nucleoplasmin is a nuclear protein that is involved in nucleosome core assembly. It was found that interaction between excess nucleoplasmin and histones is needed prior to addition of DNA for proper formation of the nucleosome core. If DNA was mixed with the histones without nucleoplasmin, a precipitate resulted. It appeared that nucleoplasmin acted as a chaperone to guide assembly, but not as a component of the final structure. Nucleoplasmin can bind to the histones and reduce their positive charge and, in this way, prevent incorrect interactions with the DNA from occurring. It can then release the histones in a controlled fashion and transfer them to the assembled chromatin (Laskey et al., 1978).

The definition of molecular chaperones has been extended to describe any protein which can bind to and stabilize partially folded conformations of other proteins in a controlled fashion such that the proteins are released only when they are folded and not "prone" to aggregation. They do not convey any steric information and can only bind to specific structural features, such as hydrophobic sites, to prevent them from interacting prematurely. They are

specific to partially folded or unfolded proteins, yet can bind to any substrates with no preference for primary structures. They do not bind to native structures (Ellis and van der Vies, 1991).

Many cellular processes such as protein synthesis, protein transport, protein function, organelle biogenesis, and stress response require the aid of molecular chaperones to prevent misfolding and guide them to their proper destination and fate. This review will discuss the various molecular chaperone machines and the role they play in the many processes in the cell.

II. MOLECULAR CHAPERONE CLASSES

A. INTRODUCTION

Molecular chaperones are essential for cell life. Many different classes of chaperones have been identified in both prokaryotes and eukaryotes. They have been found in many cell compartments and function to perform tasks needed for in vivo folding such as guiding proteins through the folding pathway, regulating the rate of folding, and aiding oligomeric assembly. Many chaperones have also been shown to be induced in response to stress such as from heat and high temperature.

The various classes of molecular chaperones are presented in Table 1, defined on the basis of functionality. The members within each class are defined based on sequence similarity and the compartments with which they are associated. A brief description of the role they play within cells is also mentioned.

Nucleoplasmin was the first molecular chaperone identified. It was found to play a role in the assembly of the nucleosome core in eggs of the frog *Xenopus*. Heat shock proteins, proteins induced from environmental stresses such as an increase in temperature, were implicated next in their role as molecular chaperones. This class contains many families of proteins including Hsp 110, Hsp 90, Hsp 70, Hsp 60, TriC, and sHsps. Their roles include assisting protein folding, protein oligomerization, protein translocation and many other critical cellular processes. Two other molecular chaperones, Sec B and PapD, were identified in *E. coli*. Sec B plays a role in the *E. coli* secretion

machinery. PapD is a protein which is required for the assembly of pili, an adhesive structure necessary for bacterial infections. These various molecular chaperone machines participate in the folding, translocation, and assembly of functional proteins in the cell.

Table 1: Various Classes of Molecular Chaperones

CLASS	MEMBERS	FUNCTIONS
Nucleoplasmin and Related Proteins	Nucleoplasmin Protein XLNO-38 Nucleoplasmin S	Assembly of nucleosome and ribonucleoprotein particles in euk.
HSP 90 Proteins	HtpG Hsp 90 Hsp 83	Masking of steroid receptors; interacts with kinases; facil. protein folding
HSP 70 Proteins	DnaK Ssa1-4p Kar-2p BiP/Grp 78 Ssc1p ct-Hsp 70	Assembly of newly synthesized and transported proteins
HSP 60 Proteins	HSP 60 GroEL Rubisco binding prot.	Folding of nascent polypeptides and transported proteins
TriC	TCP1	Binding to unfolded protein; functions unknown
Small Hsps	Proteins with MW between 15 and 30 kDa	Present in normal and stressed cells; functions unknown
Sec B protein		Prot. transport in bacteria
PapD protein		Pilus assembly in bacteria

B. NUCLEOPLASMIN AND RELATED PROTEINS

Nucleoplasmin was the first molecular chaperone identified. Laskey et al. (1978) coined the term molecular chaperone to describe the function of nucleoplasmin in extracts of eggs of the toad *Xenopus*. Nucleoplasmin is found throughout the nucleoplasm and along the whole length of lampbrush chromosome loops and within the nucleoli. Laskey et al. (1978) found that the assembly of the nucleosome core could not occur without the presence of nucleoplasmin. They found that mixing separated DNA and histones (salt extracted) in the absence of nucleoplasmin at physiological ionic strength resulted in a precipitate. However, if nucleoplasmin was first mixed with the histones and then DNA was added, the formation of the nucleosome core was successful, as revealed by sedimentation, buoyant density, and beaded appearance in the electron microscope. Further study revealed that nucleoplasmin interacts only with the histones and it does not bind to DNA or to assembled nucleosome. They concluded that the function as a chaperone involves the shielding of positive charges of the histone monomers so that incorrect interactions with DNA are minimized .

Many other nuclear proteins have also been identified that have chaperoning functions. The protein XLNO-38 was found in the granular component of the nucleolus (Schmidt-Zachmann et al., 1987). Their studies comparing the first 124 amino acids of XLNO-8 with the amino terminal region of nucleoplasmin showed 61% sequence homology. They found this protein to be associated with both large and small preribosomal subunit particles implying the possible role in assembly of basic proteins on ribosomal RNA precursors.

Nucleoplasmin S has been purified from *Xenopus* kidney cell line. Cotten and Chalkey (1987) showed that the protein crossreacts with monoclonal antibodies targetted against protein XLNO-38. The relationship of nucleoplasmin S to nucleoplasmin and protein XLNO-38 is not yet known.

C. HEAT SHOCK PROTEINS

One of the largest families of molecular chaperones is the heat shock proteins (Hsps) and consists of many classes of proteins that differ in their mode of induction and apparent molecular mass on SDS gels. These proteins are both induced as a response to stress such as abnormal temperature as well as expressed constitutively. They were first discovered through studies on *Drosophila melanogaster*. It was found that they were expressed in response to exposure of fly larvae to high temperatures; they obtained their name due to the nature of their induction (Tissieres et al., 1974). It was later found that other stimulus such as exposure to uncouplers of oxidative phosphorylation, inhibitors of electron transport, certain heavy metal ions, ethanol, and cell anoxia all resulted in an increase in the expression of these proteins (Welch et al., 1986).

Heat shock proteins are ubiquitous and represent many structurally unrelated classes of proteins. They are present in all organisms and in many cell compartments. The naming of Hsps is based on their apparent molecular mass on SDS gels and the organisms in which they are found. The classes include Hsp 110, 90, 70, and 60 referring to heat inducible protein of 110, 90, 70, and 60 kDa, respectively. Hsps from different organisms are named differently within each class. In higher eukaryotic cells, the heat shock proteins are referred to as glucose regulated proteins (grps) since they were expressed as a result of glucose starvation. They are also defined by their apparent masses on SDS gels. These include grp 94 of the Hsp 90 class and grp 78 (also referred to as BiP) of the Hsp 70 class. In bacteria, the heat shock proteins

are named according to the bacterial host functions essential for bacteriophage growth. For example, Dna K, a Hsp 70 class protein found in *E.coli*, is named because it is required for bacteriophage DNA replication whereas GroEL/GroES, Hsp 60 class members found in *E.coli*, is needed for assembly of bacteriophage head and tail components. In yeast cells, the Hsps are referred to by a genetic based nomenclature. These include Ssa1-4p, of the Hsp 70 class (Georgopoulos and Welch, 1993).

In all organisms, heat shock proteins are induced rapidly in response to an environmental insult. Environment insults include stresses such as heat, various inhibitors of glycosylation, agents that perturb calcium homeostasis (calcium ionophores), different inducing agents (DTT), as well as diseased states (viral infections, fever, inflammation, cardiac hypertrophy, tissue trauma, metabolic disease and cancer). Different organisms induce these proteins at different temperatures. For example, *Drosophila melanogaster*, thermophilic bacteria, arctic fishes, mammals, and soybeans induce different classes of Hsps at 33-37°C, 60°C, 5-10°C, fever temperatures, and 100°F, respectively (Lindquist and Craig, 1988).

Many studies reveal the protective and defensive roles that Hsps play in cell survival. Bacteria and yeast cells cannot survive at high temperatures when the genes encoding the major stress proteins are deleted (Tikawa and Ryu, 1979). Mammalian cells cannot survive heat shock once treated with antibodies specific to stress proteins (Welch, 1992). It was also found that cells are killed rapidly if immediately subjected to very high temperatures, whereas if the cells are first pretreated to a mild temperature and then subjected to higher temperatures, the cells die more slowly (Gerner and Scheider, 1975; Henle and

Leeper, 1976). This suggests that heat shock proteins are induced by moderate stress and serve to protect the organisms from continued or a more severe stress. Heat shock proteins have also been found to be immunodominant antigens in many infections. In many organisms such as *Mycobacterium leprae* (agent in leprosy), *Coxiella burnetti* (Q fever), *Plasmodium falciparum* (malaria), *Schistosoma mansoni* (schistosomiasis), circulating antibodies and activated T cells are specific to major heat shock proteins (Lindquist and Craig, 1988). This suggests that at higher temperatures, these proteins must be present in abundance and thus are processed by macrophages as major foreign antigens for presentation to lymphocytes.

Another role implicated for Hsps is that of a chaperone which functions to help the cell combat the problem of aggregated proteins and abnormally folded proteins. There are many studies that lead to this hypothesis. Ananthan et al. (1986) found that induction of denatured proteins into living cells resulted in an increased expression of Hsps. Finley et al. (1984) also observed an increase in the expression of Hsps in *S. cerevisiae* with mutations in components involved with proteolytic pathways. *Drosophila* cells expressing high levels of mutant actin exhibited high constitutive Hsps as revealed by Karlik et al. (1984) and Hiromi et al. (1986). Edington et al. (1989) found that cells first incubated with agents known to stabilize protein conformation (glycerol or deuterium oxide) and then subjected to heat shock expressed much lower levels of Hsps as compared to cells without these agents. The role of the various classes of Hsps as molecular chaperones is discussed below.

1. Heat Shock Protein 110

Not much is known about the Hsp 110 family of heat shock proteins. It has only been characterized in mammalian cells. Hsp 110 of murine cells is a constitutively expressed protein found within the fibrillar region of the nucleolus. This region is well known as the site of ribosomal RNA transcription and processing. The synthesis of Hsp 110 increases fivefold after stress induction. After heat shock, it forms a ring-like structure at the nucleolar periphery. When the cells are treated with RNase¹, it is released from the nucleus. This suggests the possible interaction of the protein with RNA or complex of proteins that bind RNA (Subject et al., 1983).

Another protein, with molecular weight of 110 kDa was also discovered in the fibrillar component of segregated nucleoli and called C23 (Shyy et al., 1986). It contains several phosphorylated and highly acidic regions. Comparison of C23 with Hsp 110 shows that they share a slight degree of homology but are two distinct proteins. C23 may possibly represent a second member of the Hsp 110 family.

The implication of possible associations of Hsp 110 protein with the nucleolar region suggests that its role is in protecting the ribosome and aiding in the recovery of normal nucleolar transcription events after heat shock. More research is needed to characterize this family of proteins.

¹ RNase-enzyme which cleaves the ester bond between the phosphate and the 5' carbon of the ribose of a pyrimidine nucleotide.

2. Heat Shock Protein 90

The heat shock 90 family of proteins is the most abundant and highly conserved of the Hsps. They have been found in many organisms including bacteria, yeast, and mammals and have molecular weights ranging from 83-92 kDa (based on SDS gels). They represent 1-2% of total soluble protein in cells under nonstress conditions and their concentrations increase under stress conditions. They are predominantly located in the cytosol; however, a homologue has been identified in the endoplasmic reticulum of mammals (Perdew and Whitelaw, 1991).

Two Hsp 90 genes have been isolated, cloned, and sequenced in yeast cells, *S. cerevisiae*. One is constitutively expressed (Hsc 83) and the other is induced 10-15 fold by heat (Hsp 83). Only one Hsp 90 homologue has been found in *E. coli* (HtpG). The *E. coli* protein is 40% homologous to the eukaryotic proteins. In mammals, two Hsp 90 proteins have been cloned. One is found in the cytosol (Hsp 83) and the other is located in the endoplasmic reticulum (Grp 84) (Lindquist and Craig, 1988).

Since the amino acid sequence has been highly conserved through evolution, Hsp 90 must have some critical cell function in both normal and stressed cells. Biochemical analysis revealed that it has no enzymatic activity, but it does have an ATPase activity ranging from 0.3min⁻¹ to 140min⁻¹ (Becker and Craig, 1994; Jakob and Buchner, 1994). Hsp 90 has been found associated with many different proteins including progesterone and glucocorticoid receptors, Ah receptor, actin, tubulin, oncogenic tyrosine kinases, and initiation factor E1F-2 α kinase. It apparently acts as a chaperone that

guides the folding pathway of proteins and possibly aids in the transport of some proteins such as steroid receptors (Perdew and Whitelaw, 1991).

Cytosolic Hsp 90

The cytosolic Hsp 90 protein, also referred to as Hsp 83, has a molecular weight ranging from 87 to 92 kDa (based on SDS gels). It exists as a dimer with an extended rodlike structure in purified form as indicated by its large Stoke's radius. However, through biochemical analysis of whole cell lysates by gel filtration, Welch et al. (1991) revealed that the protein exists as a heterogeneous structure containing multiple isoforms, some of which are phosphorylated. In higher eukaryotes, it exists as two homologous isoforms. These isoforms are referred to as alpha and beta and are produced in equal amounts.

Some differences exist between the Hsp 90 protein in *E. coli*, yeast, and mammals. Sequence comparison of different members of the Hsp 90 family are described in Figure 1 (Jakob and Buchner, 1994). In eukaryotic cells, Hsp 90 proteins contain a large number of acidic residues which results in regions of extreme high negative-charge density as well as a smaller region of high negative-charge density toward the carboxy terminus. The *E. coli* homologue HtpG only contains a smaller region of high negative-charge density toward the carboxy terminus. These terminal regions are usually different in all eukaryotic cells except for the four most terminal amino acids, Glu-Glu-Val-Asp. These are identical in all eukaryotic Hsp 90s. In trypanosomes, Gln replaces the second Glu. Interestingly, this sequence is also found in the carboxy terminus in the Hsp 70 family of heat shock proteins.

Figure 1: Sequence comparison of different members of the Hsp 90 family. The sequences were aligned using the program PileUp from the GCG package. Sequences were obtained from the Swissprot database. Indicated regions at the amino and carboxyl terminus of Grp 94 represent the signal sequence and the ER-retention motif KDE_L, respectively. The amino acid sequences of highly charged regions (indicated by shaded boxes) are given for comparison. Charged residues are underlined. This figure was copied with permission (Jakob and Buchner, 1994).

Another difference between yeast cells and *E. coli* cells is that the genes for both Hsp 83 and Hsc 83 are needed for cell viability in yeast cells whereas the HtpG gene is not needed for cell viability in *E. coli*. Borkovich et al. (1989) found that if individual genes of yeast cells were mutated, the cell survived whereas the double mutation was lethal. The single mutations in yeast cells show some interesting properties. They grew well at 25 °C but not at above 37 °C. The single mutant also cannot withstand exposure to extreme temperature. During exposure to 50 °C, these cells died faster than the wild type. However, this is true only with cells grown on acetate and not for those grown on glucose medium. Reasons for these results are unclear, but may indicate that Hsp 90 plays a role in thermotolerance only in respiring cells and may have another role in fermenting cells. Bardwell and Craig (1987) studied the deletion of the HtpG gene in *E. coli* and found that it does not kill the cells, as it did with yeast cells. However, the mutants do not grow as well at high temperature. The yeast Hsp 90 is an essential protein at all temperatures whereas HtpG is not.

Three major functions have been identified for Hsp 90: 1) interaction with the folding pathway of other proteins, 2) interaction with kinases, and 3) interaction with receptors. Figure 2 shows the current model for Hsp 90 function (Jakob and Buchner, 1994).

Hsp 90 proteins have been found to guide the *in vitro* folding of citrate synthase, MyoD, and casein kinase II. Through *in vitro* refolding experiments with citrate synthase, Wiech et al. (1992) revealed that association of Hsp 90 with the protein prevented its aggregation during the refolding. In order to study the specificity of the reaction, other proteins such as bovine serum albumin,

Figure 2: Hypothetical model for the function of Hsp 90. (a) Interaction with receptors. (b) Interaction with v-Src. (c) Interaction of isolated Hsp 90 with folding protein *in vitro*. The general scheme is that Hsp 90 [complexed with other proteins in (a) and (b)] recognizes and binds immature, ligand-free or non-native form of a protein and chaperones it to its native/functional form. Which conformational state of the substrate is recognized is not clear at present. The model does not aim to represent the precise composition of the Hsp 90 complexes. In the case of receptors, Hsp 70 might not be present in stoichiometric amounts, as indicated by its dotted outline. However, it should be noted that the composition of the complexes seems to be different in the case of receptors and kinases and also before and after association with the substrate protein. The effect of the Hsp 90 complex on protein folding *in vitro* has not been addressed yet. CS, citrate synthase. This has been copied with permission (Jakob and Buchner, 1994).

lysozyme, and antibodies were added to the reaction. The additional proteins imparted no significant effect indicating that the association of Hsp 90 with citrate synthase is a specific reaction. Addition of ATP also had no effect on the reaction. By suppressing aggregation, Hsp 90 allows the protein to proceed through the folding pathway and, by doing so, increases the probability that the protein molecules reach their native, functional state. The yield of folded protein was found to be proportional to the concentration of Hsp 90 as long as citrate synthase is in excess. If Hsp 90 is in excess, the folding kinetics are slower. Similar guidance of *in vitro* refolding by Hsp 90 was also seen with the muscle specific protein MyoD and casein kinase II. Shaknovich et al. (1992) observed an increase in the DNA binding activity of native MyoD in the presence of Hsp 90. Cavarelli et al. (1993) examined Hsp 90 in low salt buffers and found it to prevent the aggregation reaction of casein kinase II. BSA and ATP again had no effect on the refolding of the proteins mediated by Hsp 90 (Hendrick et al., 1993; Wiech et al., 1992).

Hsp 90 has also been found to be associated with protein kinases. This includes tyrosine kinase of various transforming proteins and E1F2- α kinase, a heme regulated eukaryotic initiation factor. Welch (1992) studied tyrosine kinase of the transforming protein of Rous Sarcoma virus pp60v-SRC and found it to spontaneously associate with Hsp 90 and a 50 kDa phosphoprotein upon synthesis. Hsp 90 remains bound to the protein until it becomes attached to the plasma membrane. Once the Hsp 90 is released, the enzyme is phosphorylated (on tyrosine) and then transported across the membrane where it functions as a kinase. For these reasons, Hsp 90 is thought to act as a chaperone in maintaining the structural form of the kinase to allow

transport across membrane. It also plays a role in the maturation of v-SRC.

Studies by Jakob and Buchner (1994) revealed that cells with low levels of Hsp 90 contain a lower amount of active, membrane-associated v-SRC molecules.

Further studies have shown association of Hsp 90 with other transforming proteins with kinase activity. These are the *fps* gene product from Fuginami and PRCC II sarcoma viruses, the *yes* gene product from Yamuguchi 73 sarcoma virus, the *ros* gene product from UR-2 sarcoma virus, and the *fes* gene product from Synder-Theilen feline sarcoma virus (Welch, 1992).

Hsp 90 is also found associated with other cellular kinases such as E1F2-alpha kinase, a heme-regulated eukaryotic initiation factor. In this association, the Hsp 90 protein stimulates the kinase by increasing phosphorylation of E1F2-alpha and inhibiting protein synthesis in reticulocyte lysates. Association with Hsp 90 increases the kinase activity when the cells are subjected to a heat shock treatment (Welch, 1992). The exact role of Hsp 90 in regulating changes in protein synthesis that occur in a cell after stress are unknown.

Hsp 90 has also been found associated with actin and tubulin. Its association with actin is dependent on calcium and is regulated by calmodulin. It is thought that possibly this may be how Hsp 90 is transported to the nucleus. The transport of Hsp 90 to nucleus during heat shock correlates with the rearrangement of actin filaments found in larger quantities in nuclei of heat-shocked cells.

Hsp 90 plays a role in regulating receptors. Steroid receptors are soluble proteins that can shuttle between the cytosol and nucleus. The Hsp 90 binds to newly synthesized receptors immediately after completion of

translation. Upon binding to Hsp 90, the receptors can bind to particular hormones (glucocorticoid, progesterone, estrogen, testosterone) and activate transcription from specific DNA sequences. It is generally thought that Hsp 90 in the absence of steroid hormone maintains the steroid receptor in an inactive or non-DNA-binding form. It may do so by interacting directly with the DNA binding domain or with a region within the receptor necessary for receptor dimerization. Becker and Craig (1994) determined that the Hsp 90-receptor complex is dissociated by hormone and high salt ($>0.25\text{M}$ KCl or metal chelators), and is stabilized by low salt and molybdate ions. Antibodies prepared against the charged domain of Hsp 90 did not precipitate the Hsp 90 complexed to steroid receptor as was found for soluble Hsp 90 from cell extracts. This indicates that the antibody binding site was hidden through binding with receptor. In the case of the glucocorticoid receptor, the Hsp 90 is thought to inactivate the receptor by keeping it unfolded.

Hsp 90 contains a region of highly negative charge in its amino terminus which resembles the region of DNA containing negatively charged phosphates. Therefore, the receptor binds to Hsp 90 instead of DNA until the presence of hormone activates the release. Once hormone binds, Hsp 90 is released and the receptor possibly with the help of Hsp 70 and Hsp 56, undergoes a conformational change leading to its dimerization and acquisition of a specific DNA-binding domain (Pratt, 1990; Welch, 1992). The Hsp 70 may function to mediate or stabilize those conformational changes accompanying receptor dimerization. The role of Hsp 56 is unclear, but cloning of its gene reveals that it encodes a protein belonging to a family of proteins called peptidyl-proline isomerases (PPIase) or immunophilins (Pratt, 1993; Wynn et

al., 1994). Immunophilins are proteins which interact with protein phosphatases of the signal transducing pathways of the cell. This interaction occurs only in the presence of immunosuppressive drugs and Hsp 56 binds to the immunosuppressant FK506. Immunophilins can catalyze cis-trans isomerization of peptidyl-prolyl bonds in polypeptide chains. Immunophilin-Hsp 90 bound complex may be involved in affecting immunosuppression activity of drugs by changing conformation of chaperone-bound protein complex (Jakob and Buchner, 1994).

Endoplasmic Reticulum Hsp 90

Hsp 90 homologue has been identified only in the endoplasmic reticulum (ER) of mammals. This protein is referred to as Grp 94 (glucose regulated protein) since it is induced by glucose starvation. It is slightly larger than the cytosolic protein with an apparent molecular weight of 94-108 kDa based on SDS gels. It contains N-linked oligosaccharides and is phosphorylated (Olden et al., 1978). Mazzarella and Green (1987) revealed that the amino acid sequence contains a 20-amino acid hydrophobic domain within the first 1/3 of the molecule. Booth and Koch (1989) found a ER retention signal sequence (KDEL) at its extreme C-terminus.

Grp 94 is induced by heat, steroids, and other agents. It has calcium binding properties like the cytosolic proteins. It appears to play a role in the secretory pathway of protein based on its increase in synthesis in the presence of agents which perturb protein synthesis (Welch, 1992). More information is not yet available. This Hsp is under further study.

3. Heat Shock Protein 70

The Hsp 70 family is comprised of proteins with molecular weights of 70 kDa and are found in many different organisms including yeast, mammals, plants, trypanosomes, and *E. coli*. Homologs have been found in many compartments including cytoplasm, nucleus, endoplasmic reticulum, mitochondria, and chloroplasts. The members include Dna K in *E. coli*; Ssa1p, Ssa2p, Kar2p, and Ssc1p in yeast; and Hsp 70, Hsc 70 (p73, clathrin uncoating ATPase) and BiP (grp 78) in mammalian cells. These proteins are both stress induced and present in normal cells at lower concentrations. The Hsp 70 genes among organisms show a great degree of conservation with at least 50% homology.

Hsp 70 from different organisms have very similar biochemical properties. They can bind ATP with high affinity and possess weak ATPase activity, which is stimulated by binding to unfolded proteins and synthetic peptides. Flaherty et al. (1990) through structural analysis of a proteolytic digestion of the bovine Hsc 70 protein by X-ray crystallography revealed two domains in the Hsp 70 protein; a nucleotide binding site and a polypeptide binding site. The amino terminal (N-terminal) fragment contains the nucleotide binding site whereas the carboxy-terminal (C-terminal) fragment contains the polypeptide binding site.

Chappell et al. (1987) referred to the N-terminal fragment of Hsp 70 proteins with molecular weight of 44 kDa as the ATPase fragment. They found that mutation studies involving the deletion of N-terminal residues in Dna K resulted in the loss of both ATPase activity and ATP binding ability. The three

dimensional crystal structure of the N-terminal fragment of bovine heat shock cognate (Hsc 70) protein has been deduced to 2.2Å (Rippmann et al., 1991).

Flaherty et al. (1990) studied the three dimensional structure of the ATPase fragment in detail. They found that the ATPase fragment contains two structural lobes forming an ATP binding cleft similar to that seen with G-actin and hexokinase. Crystallography studies also revealed the presence of two K⁺ ions in the ATP binding cleft (Palleros et al., 1993). Flaherty et al. (1990) also described each lobe as containing two separate biological domains, an upper domain consisting of helices and antiparallel beta-strands, and a lower domain of five strand beta-sheets flanked by helices. The topology of the Hsc 70 ATPase fragment is shown in Figure 3 (a). Right and left lobes are denoted I and II and the lower and upper domains are designated A & B, respectively. Domain IA denotes residues 1-39 and 116-188; IB denotes residues 40-115; IIA denotes residues 189-228 and 307-end; and IIB denotes residues 229-306. The ATPase domain is highly conserved in its amino acid sequence. Some of the regions are more conserved than others. Figure 3 (a) shows the most highly conserved elements (shaded in dark grey) and the more variable regions (not shaded). Domains IA and IIB are the most conserved; this is the region of the protein that appears to interact directly with the nucleotide (Flaherty et al., 1990). Figure 3 (b) shows the topology of hexokinase. Domains IA and IIA of the Hsc 70 fragment have identical folding topology to segment of hexokinase but the other domains IB and IIB show no similarity to the corresponding region of hexokinase. Hsc70 fragment contains domain IB and a pair of antiparallel beta strands in domain IIB which are not present in hexokinase. Hexokinase

also has a segment of 80 residues before the beginning of domain IA. This is not present in the Hsc 70 fragment.

Figure 3: (a) Topology of the Hsc 70 ATPase fragment. Alpha-helices (cylinders) and beta-strands (arrows) are shaded according to the degree of amino-acid sequence conservation: Sequences of 14 representative eukaryotic Hsp 70-related proteins available in the Genbank sequence database were aligned by eye. (b) Topology of hexokinase. Permission was requested (Flaherty et al., 1990).

Another similarity between hexokinase, actin, and Hsp 70 involves their mechanism of action. ATP binding facilitates global changes in the tertiary structure of hexokinase. ATP binding changes the conformation of actin and allows its polymerization into a filament. In the same way, Hsp 70 upon ATP hydrolysis elicits a conformational change that is transferred to the carboxy-terminal domain (Flaherty et al., 1990). The Dna K protein has been shown to undergo a conformation change upon binding to ATP (Landry et al., 1992). Mammalian Hsp 70 binds unfolded proteins less tightly in the presence of hydrolyzable ATP. ADP, however, inhibits the ATP-dependent release of Hsp 70 from an unfolded protein (Craig et al., 1994).

The carboxy-terminal domain with a molecular weight of 25 kDa contains the polypeptide binding capacity and, therefore, is also referred to as the peptide binding domain. There is very low sequence homology $\leq 30\%$ in the carboxy terminal domain among different Hsp proteins (Langer and Neuport, 1991). The carboxy-terminal domain is more variable in sequence with the greatest variation in sequence occurring in the last 30-50 amino acid sequence (Figure 4). It has been suggested that this domain is the site for peptide binding, containing activities specific to individual Hsp 70s, for example, the role in the disassembly of coated vesicles in the case of uncoating ATPase.

Rippmann et al., 1991, analyzed the primary and secondary structure of the carboxy terminal region of Hsp 70 and found similarities to the peptide-binding domain of the histocompatibility antigen proteins (MHC class I HLA presenting molecule). The proteins share amino acid homology of 24% and their predicted secondary structure was found to be similar. Both proteins bind peptides in an extended conformation.

Figure 4: Schematic drawing of the functional organization of stress-70 proteins within their primary structure. Numbers correlate with the amino acid sequence of bovine Hsc 70. This was copied with permission (McKay, 1993).

All Hsp 70s bind to unfolded proteins. The Hsp 70 of the endoplasmic reticulum, BiP, binds to immunoglobulin heavy chains which have not completely assembled with light chains (Munro and Pelham, 1986). The constitutive Hsp 70 of mammalian cells can bind to and uncoat clathrin-coated vesicles (Schlossman et al., 1984). Mitochondrial Hsp 70 bind proteins as they enter the matrix from the cytosol (Stuart et al., 1994). Hsp 70 has been found to be associated with many other proteins such as cellular protooncogene p53, certain transactivating proteins such as E1a of adenovirus, incompletely assembled or malformed protein in the endoplasmic reticulum, and in phage lambda replication (Langer and Newport, 1991).

Not much is known about the mechanism of action of Hsp 70 proteins. It is well known that ADP-ATP play a significant role in the reaction cycle. The ADP form of Hsp 70 exhibits higher affinity for peptides, forming a more stable complex. A nucleotide exchange results in the formation of ATP bound Hsp 70 protein which has low affinity for substrate. Therefore this results in the release of the protein. Nucleotide hydrolysis then results in the formation of ADP bound protein leading to more binding. A model for Hsp 70 function suggests that Hsp 70 prevents wrong protein-protein interactions by binding to hydrophobic sequences which become exposed as a result of heat shock or during cellular processes such as translocation across membranes. By binding to exposed areas, aggregation or misfolding is prevented.

Craig et al. (1994) studied the structure of a polypeptide during and immediately after release from Dna K using circular dichroism and fluorescence measurements. They found that the protein while bound to Hsp 70 and after ATP driven release is in an unfolded conformation. This suggests the role of

Hsp 70 in maintaining nonnative states. They also play a role in many cellular processes such as synthesis, transport, folding, and assembly of proteins. They have been found to bind to nascent chains on ribosomes, maintain translocation-competent conformations of ER and mitochondrial precursors in the cytosol, facilitate translocation from inside the target compartment, block nonproductive folding interactions, and modulate the oligomeric state of macromolecular assemblies.

Cytosolic Hsp 70

Mammalian Cells

Mammalian cells contain two cytoplasmic Hsp 70s, one that is induced, Hsp 72, and one that is constitutive, Hsp 73. After heat shock, Hsp 72/73 is found in the nucleolar region of the nucleus (Pelham, 1984; Velazquez et al., 1984; Welch and Feramisco, 1985). Welch and Feramisco (1985) showed that the nuclei is severely altered by heat shock and that there is an inhibition of ribosomal biogenesis events. Using high resolution electron microscopy, they revealed after heat shock that there was significant amounts of denatured and aggregated preribosomal components. They suggested that after heat shock treatment, the maturing preribosomal components associate with Hsp 72/73 in order to facilitate their folding or assembly. Finley et al. (1984) and Pelham et al. (1986) suggested that Hsp 72/73 may function to help repair the damaged nucleolus by either helping refold unfolded ribosomal proteins or by solubilizing the denatured ribosomal proteins to facilitate their turnover. Once the nucleoli recovers, the Hsp 72/73 returns to the cytoplasm and colocalized with the translation machinery (Lewis et al., 1985).

Synthesis of Hsp 72/73 is regulated within the cell. Hsp 72/73 exists in the cell in equilibrium between its free state and substrate bound state. When the substrate bound state is in excess, the amount of free Hsp 72/73 is reduced which in turn activates new Hsp 72/73 synthesis. Once a critical level of free Hsp 72/73 is reached, the response is turned off and the cells return to their normal pattern of protein synthesis. This theory correlates with observations seen with Hsp 72/73. Hsp 72/73 appeared to be autophosphorylated and its synthesis rate proportional to the severity of the stress (DiDomenico et al., 1982; Mizzen and Welch, 1988). The concentrations of Hsp 72/73 present in preexisting cells influenced the amount of new Hsp 72/73 synthesis following second induction of stress. Microinjection of denatured protein into frog oocytes activated synthesis of Hsp 72 (Ananthan et al., 1986). Microinjection of antibodies specific to Hsp 72/73 also resulted in Hsp 72/73 synthesis (Welch et al., 1991). This may also hold true for the regulation of the endoplasmic reticulum Hsp 70, BiP (Welch et al., 1991).

Cytosolic Hsp 70 proteins play a role in membrane translocation of proteins. Eilers and Schatz (1986) studied the posttranslational translocation of a fusion protein containing presequence of subunit IV of cytochrome oxidase and mouse dihydrofolate reductase (DHFR). They found that methotrexate, a substrate analog, inhibited import. Methotrexate is known to stabilize the tertiary structure of DHFR. If the protein was first denatured with urea or point mutations, import of mitochondrial precursor proteins was more efficient. This suggested that proteins need to be in an unfolded state prior to import. ATP is also needed for translocation across membranes. It is required for import of proteins into ER, peroxisomes, chloroplasts, mitochondria, and nucleus.

Zimmermann et al. (1988) found that mammalian Hsp 70s with the help of a N-ethylmaleimide-sensitive factor can stimulate the translocation of M13 procoat protein into mammalian microsomes and the import of pre-ornithine carbamoyl carboxylase fused to dihydrofolate reductase into mitochondria (Langer and Newport, 1991; Welch et al., 1987). This suggests that Hsp 70 proteins play a role in folding and assembly of proteins *in vivo* in an ATP dependent fashion.

Beckmann et al. (1990) studied the interaction of Hsp 70 with intracellular proteins by immunoprecipitation with monoclonal antibodies specific for Hsp 72/73. HeLa cells grown at 37 °C were labeled with [35S]Met for 20 minutes. One group of cells was immediately harvested and another was incubated two hours without label and then harvested (pulse chased). After the cells were lysed, ATP was added to half the lysates and apyrase to the other half to degrade endogenous ATP. In the pulse-labeled cell lysates depleted of endogenous ATP, immunoprecipitation with antibodies to Hsp 72/73 resulted in the coprecipitation of many newly synthesized polypeptides ranging in size from 20 to 200 kDa. In the presence of exogenous ATP, very few proteins coprecipitated. Even fewer proteins coprecipitated with Hsp 72/73 in lysates treated with apyrase and when ATP was added to the cell lysate.

They then treated the HeLa cells with a proline analog, L-azetidine 2-carboxylic acid (AZC) to induce stress. Cells were treated with AZC for four hours, labeled for fifteen minutes with [35S]Met while still in the presence of the analog and immediately harvested or pulsed or further incubated in absence of label and AZC for two hours. A significant number of proteins coprecipitated with anti-Hsp 72/73 in apyrase treated lysates. Addition of ATP resulted in

coprecipitation of fewer proteins. In contrast to normal cells, the proteins were still complexed with Hsp 72/73 after a two hour chase period.

These studies suggest a dual function for Hsp 70. One function is to transiently bind to newly synthesized proteins, maintain them in a translocation-competent form, and guide them to their intended destination where another chaperone can facilitate their folding into their biologically active conformation. This has been found to occur with proteins which can fold normally. However, in the case of proteins such as AZC-containing polypeptides which cannot fold properly and fail to move properly along the secretory pathway, the Hsp 72/73 chaperones bind to them in a stable, longer lasting complex. The Hsp 72/73 may facilitate the slow folding of these proteins and prevent them from aggregating or protect the cell from a blockage in the cellular processes due to these abnormal proteins.

Figure 5 gives a model of Hsp 70 interaction with newly synthesized proteins. Hsp 72/73 binds to nascent polypeptide chains still associated with ribosomes. These precursor proteins are in an extended conformation with little secondary or tertiary structure. Hsp 72/73 prevents aggregation of precursors and stabilizes them in a translocation-competent conformation until transfer to another heat shock protein, Hsp 60. Hsp 60, with the hydrolysis of ATP, can guide the protein to fold properly into its functional three-dimensional structure (pathway 1). Studies by Beckmann et al. (1990) and Prouty et al. (1975) also revealed that interaction of Hsp 70 with newly synthesized proteins in some cases remained in a complex for a longer time after their synthesis suggesting that the Hsp 72/73 may also stabilize complete proteins requiring additional time to reach their final conformation. (pathway 2 and 3). These proteins

include those that are assembled into multimeric structures, those that are posttranslationally translocated into organelles, or proteins that exist in equilibrium between monomeric and polymeric states (clathrin, cytoskeletal proteins).

Figure 5: A model by which Hsp 70 interacts with and facilitates maturation of newly synthesized proteins. Pathways for monomeric proteins (1), proteins that are to be assembled into an oligomeric structure (2), and proteins that are translocated from the cytosol into organelles (3). Subsequent folding of the protein occurs within the organelle, perhaps facilitated by chaperonin-like molecules. Permission was requested (Beckmann et al., 1990).

Hsp 70 has also been found to bind to and uncoat clathrin-coated vesicles (Chappell et al., 1986). These specialized coated vesicles secrete proteins from the Trans-golgi to the plasma membrane. Clathrin triskelions coat the membrane vesicles (Pearse, 1976, 1980; Goldstein et al., 1979). They consist of three clathrin molecule forming a three-legged structure called a triskelion (Ungewickell and Banton, 1981; Kirchhausen and Harrison, 1981). Each clathrin molecule contains one heavy chain (180 kDa) and one light chain (35 kDa). Anderson et al. (1977) found that the uncoating of the vesicle was found to occur in the cytosol before attachment of vesicles to target membranes. Patzer et al. (1982) showed that the coated vesicles rapidly uncoat when incubated with a cytosolic fraction of bovine brain and ATP. Both heavy and light chains of clathrin were released and no indication of proteolysis was seen. Chappell et al. (1986) later found that a protein, referred to as "uncoating ATPase," played a role in releasing the clathrin triskelions from coated vesicles. They also found that this uncoating ATPase was a member of the Hsp 70 family based on the following biochemical studies: The uncoating ATPase copurified with a Hsc 70 from HeLa cells. Two dimensional gel analysis and peptide mapping revealed that the two proteins were identical. The uncoating ATPase also reacted to antibodies raised specifically against a synthetic peptide, VGIDLGTTYSC, derived from the highly conserved N-terminal domain of Hsp 70. Antibodies raised against uncoating ATPase reacted with several ATP-binding proteins (Hsp 70) present in yeast cytosol.

Rothmann and Schmid (1986) proposed a mechanism for the uncoating reaction. This is shown in Figure 6. First, Hsc 70 binds to a site on the clathrin that contains light chains, LCa and LCb. Schmidt and Rothman (1985), through

gel filtration studies separating bound from free uncoating protein, revealed that Hsc 70 proteins were bound to unassembled triskelions. They found that ATP was required for binding but not for hydrolysis. Nonhydrolysable analogues of ATP promoted binding. Rothman and Schmidt (1986) studied direct interactions of purified LCa and LCb with Hsc 70 and revealed that LCa stimulates the binding more than LCb. LCa was also found to inhibit the uncoating reaction by Hsc 70. Through studies with antibodies raised against specific sequence peptides, a region of the clathrin light chain consisting of residues 47-71 was identified as the binding site for Hsc 70. Once Hsc 70 binds to the LCa binding site, ATP hydrolysis causes a conformational change of a portion of triskelion; which exposes a previously buried site. This is called "displacement." The Hsc 70 can then bind to the newly exposed site on the triskelion and capture or stabilize the conformation. ATP increases the rate at which the Hsc binds to the triskelions. In doing so, it minimizes or blocks the reassembly of the leg of triskelion into the clathrin lattice. The Hsc 70 displaces and captures one leg at a time until the triskelion has been released. Three molecules each of ATP and Hsc 70 are required for the uncoating reaction. Once the clathrin coat is disrupted, the vesicle can then fuse into the membrane bilayers (Rothman and Schmidt, 1986).

Yeast Cells (S. cerevisiae)

S. cerevisiae expresses two groups of cytoplasmic Hsp 70s; SSA and SSB. The genes for these proteins function differently from each other and the individual genes do not complement each other (Craig et al., 1994). During heat shock, SSA genes are induced whereas SSB genes are turned off. The

SSA gene encodes four proteins (Ssa1p, Ssa2p, Ssa3p, Ssa4p) which have 80-97% sequence similarity. The SSA subfamily is needed for cell viability. One or more of the proteins must be present at high levels for cell viability (Werner-Washburne et al., 1987).

Ssa1p and Ssa2p, the two constitutively expressed proteins, play a role in the protein translocation into both the ER and mitochondria. Chirico et al. (1988) found that an accumulation of unimported precursor mitochondrial F1-

Figure 6: Uncoating is a Two-Stage Process. The first stage, "displacement" of a clathrin triskelion from the cage, is driven by ATP hydrolysis. The second stage, "capture" of the displaced triskelion, requires ATP as a catalyst but does not require ATP hydrolysis. Presumably, when this process occurs three times to the same triskelion, the triskelion is released as a complex bound to three molecules of uncoating protein. The clathrin cage is depicted as a hexagon; uncoating protein is depicted as a stippled sphere; LC signifies the clathrin light chain. This was copied with permission (Rothman and Schmid, 1986).

ATPase Beta subunit (F1 β) and secreted protein prepro-alpha-factor (pp[F]) occurred in yeast cells depleted of SSA proteins (Langer and Newport, 1991). Their *in vitro* studies showed that SSA Hsp 70s with N-ethylmaleinimide (NEM) sensitive activity and ATP are needed to stimulate translocation of proteins into mitochondria and endoplasmic reticulum. Chirico et al. (1988) showed that Ssa1p and NEM-sensitive activity are not needed for transport if the pp[F] is already denatured by urea and in its unfolded state before addition to the microsomes. This suggests that Hsp 70 may play a role in either unfolding the precursor or maintaining it in an unfolded, translocation-competent conformation.

Ssa1p/Ssa2p may salvage precursors that prematurely fold into translocation-incompetent conformations during translation. The model proposed is similar to that proposed for Hsc 70 in uncoating clathrin (Chappell et al., 1986; Chirico et al., 1988). Ssaps hide their "active" hydrophobic site by a conformational change. This can be exposed by rebinding to ATP. The exposed hydrophobic site of Ssaps can then interact with another molecule of Ssaps or to another protein.

The SSB subfamily contains two genes which encode proteins (Ssb1/2) that are 99% identical. Inactivation of both genes slows the growth of cells at all temperatures (Craig, 1985). Nelson et al. (1992) examined their association with the synthesis of proteins, in particular with translating ribosomes. Crude extracts of yeast cells were prepared, centrifuged through a sucrose density gradient and fractions containing ribosomes run on SDS-polyacrylamide gel and immunoblotted using the Ssb1/2p antiserum. Results revealed that Ssb1/2p and polysome profiles were similar representing up to 73% of the total

cellular content of Ssbp. This complex was also found to be sensitive to amino acyl tRNA analog puromycin. Puromycin is an amino acyl-tRNA analog which causes release of nascent chains from preribosomes. This suggests that SSB proteins bind to newly synthesized polypeptides. Their role in translation was implicated through studies with *ssb1 ssb2* double mutants. Craig et al. (1993) and Nelson et al. (1992) found the double mutants to be sensitive to translation inhibitors such as aminoglycosidase and verucerrin A. These results are consistent with that seen with mammalian cell cytosolic Hsp 70 (Beckmann et al., 1990; Zimmermann et al., 1988).

Bacteria Cells (E. coli)

The *E. coli* Hsp 70 is located in the cytosol and is referred to as Dna K. It is the only prokaryotic heat shock protein, encoded on the heat shock regulon of *E.coli*, that is homologous to eukaryotic Hsp 70 protein (Bardwell and Craig, 1984). It is 48% identical in sequence to *Drosophila* (Bardwell and Craig, 1984) and 49.8% identical in sequence to the yeast Ssa1 protein (Craig et al., 1989). The name reflects the fact that it is necessary for bacteriophage lambda DNA replication and because DNA synthesis is shut off when mutants are shifted to high temperatures (Georgopoulos, 1977; Sunshine et al., 1977). It, like other Hsp 70 proteins, possesses a weak ATPase activity with a turnover rate of 1 ATP per minute (Zylicz et al., 1983). It has been found to be autophosphorylated on threonine residues (Zylicz et al., 1983). The ATPase is DNA independent and modulated by lambda O and lambda P protein *in vitro* and *in vivo* (Zylicz et al., 1983). ATP hydrolysis and autophosphorylating activity is dependent on divalent cations such as calcium ions (Cegielska and

Georgopoulos, 1989; Dalie et al., 1990). Calcium ions inhibit ATPase and stimulate autophosphorylating activity (Cegielska and Georgopoulos, 1989).

Dna K protein has been found associated with purified *E. coli* RNA polymerase (RNAP) protein (Skelly et al., 1988). Skowrya et al. (1990) studied the role of Dna K in protecting and restoring RNAP's activity. They found that the Dna K protein protects RNAP from heat inactivation. Incubation of RNAP at 45 °C and under low salt conditions of 50 mM NaCl in the absence of Dna K resulted in the loss of RNAP's enzymatic activity (90-95% within 5 minutes). In the presence of Dna K, RNAP activity was recovered. Increasing amounts of Dna K resulted in an increased activity with maximal protection occurring when approximately 60 Dna K monomers per RNAP holoenzyme were used. They also studied the effect of Dna K on already heat-inactivated RNAP enzyme. They found that it can reactivate heat-inactivated RNAP enzyme in an ATP-dependent reaction. Another biological function of Dna K revealed in this study was its ability to dissociate protein aggregates as they form. Heat treatment of RNAP at 45 °C in 50mM NaCl for 10 min. resulted in the formation of a protein aggregate with no detectable enzymatic activity. In the presence of Dna K, aggregate formation was still seen. However, addition of Dna K and ATP resulted in the formation of dimers possessing normal polymerizing activity. Skowrya et al. (1990) referred to these two biological properties as the healing powers of Dna K. They proposed that during incubation at nonpermissive temperatures, hydrophobic groups may be exposed on the surface of the RNA polymerase holoenzyme. If these are not protected, inter and intra molecular interactions could lead to aggregation. Dna K is needed to shield the exposed sites to prevent further interactions.

Dna K is regulated by two other heat shock proteins, the 44 kDa chaperone Dna J and 20 kDa GrpE protein. Dna J is encoded by the same operon as Dna K and it stimulates ATP hydrolysis by Dna K (Liberek et al., 1991; Saito et al., 1978; Zylicz et al., 1985). The resulting increase in the concentration of ADP-Dna K which binds substrate with a high affinity (Palleros et al., 1991). Grp E is a constitutively expressed stress protein detected only in prokaryotic organisms. It is the groP-like class E protein which blocks *E.coli* DNA replication at high temperatures; its name was never changed to reflect its molecular chaperone role. It acts specifically to accelerate the release of all bound nucleotides. It stimulates ATPase activity of Dna K when Dna J is present (Georgopoulos, 1992) and it forms a tight complex with Dna K. Georgopoulos et al. (1990) found that this Grp E-Dna K complex is resistant to 2M KCl but is disrupted following ATP hydrolysis.

Dna J, unlike GrpE, has many homologs in eukaryotes. All Dna J homologs contain a conserved cysteine-rich motif as well as a J-domain which interacts with Hsp 70 regulating ATPase activity. The carboxy terminus is less conserved and contains the polypeptide binding region (Cyr et al., 1994).

The Dna J-like proteins interact with two different regions of Dna K. They could stimulate the ATPase activity through the interaction of the J-domain with the polypeptide binding groove (Gething and Sambrook, 1992). Studies have showed that this substrate binding to the polypeptide binding groove stimulates hydrolysis of ATP. They also interact with another site on the Hsp 70 molecule; the region located in the carboxy terminus consisting of a 10 kDa fragment (deMurcia et al., 1983). There is a 18 kDa region between the ATPase and carboxy-terminal which has properties of the polypeptide binding domain. It is

thought that the Dna J-like protein facilitates binding of the partially folded proteins to the peptide-binding groove through interactions with the 10 kDa fragment of Hsp 70.

In *E. coli*, Dna K, Dna J, and Grp E cooperatively play a role in cellular processes such as the assembly of the initiation complex during phage lambda DNA replication, in the replication of p1 Rep A initiator protein, and in mediating the folding pathway of newly synthesized proteins. The role of these proteins in bacteriophage lambda replication has been studied extensively (Georgopoulos, 1990; Liberek et al., 1988; Alfano and McMacken, 1989; Zylicz et al., 1989; Dodson et al., 1989; Ang et al., 1991). The assembly of the initiation complex during phage lambda DNA replication involves cooperation from Dna K, Dna J, Grp E, and lambda O and lambda P proteins. First, the dimeric lambda O proteins bind specifically to ori lambda. Liberek et al. (1988) found that it required about 60 molecules of lambda O monomers per ori lambda. LeBowitz et al. (1986) and Yamamoto et al. (1987) then revealed the interaction of the lambda O-ori lambda complex with complexes of lambda P protein and *E. coli* Dna B. Once Dna J binds to the lambda O-ori lambda-Dna B-lambda P complex, then Dna K can complete the initiation complex. Liberek et al. (1988) found that lambda O and lambda P proteins retained well on Dna K affinity columns. This strongly suggested an interaction between Dna K and these proteins. Yamamoto et al. (1987) suggested that in the presence of ATP, Dna K, and Dna J cause a partial dissociation of the initiation complex. This loosens the association between Dna B and lambda P protein. Dna B helicase is then activated in the presence of *E. coli* single stranded binding protein and initiates localized unwinding of the DNA template.

The three proteins are also involved in the replication of p1 Rep A initiator protein. Zylitz et al. (1989) observed that RepA-Dna J binds to five 19 base pair direct repeats in the P1 origin. One hundred fold less Rep A was needed for ori1 DNA binding with addition of Dna K and ATP. GrpE was not required for Rep A activation. DnaJ-Rep A protein complexes could be activated by Dna K and ATP. Nonhydrolysable ATP analogue ATP-gamma-S was found to be a competitive inhibitor of the reaction. The binding was dependent on time of incubation and temperature, occurring at 24 °C for twenty minutes but not at 0 °C. Dna J binds to inactive form of Rep A at 0 °C forming a 2:2 tetramer. This is activated by ATP dependent Dna K. The ATP can activate and release Rep A to bind to DNA.

The *in vitro* cooperation of the three proteins was seen in the folding of newly synthesized rhodanase from bovine mitochondria. Rhodanase, once denatured, usually aggregates without the presence of other factors. However, in the presence of Dna K this aggregation is inhibited. Addition of Dna J increased the potency of Dna K to interact with denatured rhodanase. ATP and Mg^{2+} were needed to stabilize the complex. This however did not result in the folding of the rhodanase protein to its active conformation. With the addition of Grp E, the refolding of rhodanase occurred at a slow rate, revealing a yield of 30% folded functionally active rhodanase after eight hours. The addition of GroEL, a Hsp 60 protein increased the yield of a folded functional rhodanase protein (Langer et al., 1992).

Hartl et al. (1994) proposed a model to describe the cooperation of the three proteins-Dna K, Dna J, and Grp E (Figure 7). First, the unfolded polypeptide interacts with Dna J. This aids in the targeting of the protein to

Figure 7: Model of the reaction cycle of Dna K, Dna J, and Grp E in protein folding. (a) Unfolded protein interacts with Dna J. (b) Dna K is recruited by Dna J and hydrolyses bound ATP to ADP. This results in the formation of a tight ternary complex. (c) Grp E causes the dissociation of ADP from Dna K. This weakens the interaction between Dna K and the unfolded protein, allowing protein transfer to GroEL in the case of rhodanase. (d) Upon binding of ATP to Dna K, the ternary complex dissociates. U may fold to the native state (N), rebind to Dna J-Dna K, or be transferred to GroEL, depending on the folding properties of the substrate protein. This was copied with permission (Hartl et al., 1994).

Dna K forming a stable ternary complex. The formation of the complex requires ATP hydrolysis by Dna K. Dna K in its ADP binding site is stabilized by Dna J. The ADP-bound state of Dna K has high affinity for unfolded protein. The protein Grp E regulates Dna K and allows release of bound polypeptide for either folding to the native state or transfer to a chaperone located downstream. Grp E acts as a nucleotide exchange factor inducing dissociation of ADP from Dna K. Binding of Dna K to ATP dissociates the ternary complex, since ATP-bound Dna K has low affinity for peptide. The polypeptide can then fold to native state or it could rebind to a Dna K-Dna J complex. For luciferase, several cycles of Dna K-Dna J interactions are needed for efficient folding (Hartl et al., 1994).

Mitochondrial Hsp 70

Mitochondrial Hsp 70 is found in yeast, mammalian cells, and trypanosomes (*Trypanosoma cruzi*). The yeast Hsp 70 (Ssc1p) has been well studied. It is encoded by the gene SSC1. Ssc1p contains a N terminal leader sequence (28 amino acid) typical for mitochondrial proteins. This leader sequence is proteolytically cleaved upon entry into the mitochondrial matrix. Ssc1p is most homologous to *E. coli* Hsp 70 Dna K protein.

Ssc1p plays a role in the translocation of proteins from the cytosol into the matrix of the mitochondria. This was revealed through studies with temperature-sensitive yeast mutants carrying mutations in SSC1 (Craig et al., 1987). It was found that shifting mutant ssc1-2 cells to nonpermissive temperatures resulted within minutes in an accumulation of precursor proteins which normally are transported to the mitochondria. Kang et al. (1990) also

found that mitochondria isolated from *ssc1-2* cells grown at the permissive temperatures were successful in import, but if moved to nonpermissive temperatures of 37 °C, they were defective in import. At the nonpermissive temperature, a minimal number of proteins were transported, but the binding and cleavage of targeting sequences were unaffected.

The model proposed for this process by Craig et al. (1993) consist of two steps and is described in Figure 8. First, the targeting sequence of the precursor protein is recognized by specific receptor proteins on the cytosolic surface of the outer mitochondrial membrane. It is then partially inserted across the outer and inner membrane in a way that the N-terminus reaches the matrix. The membrane potential drives the positively charged presequence through the contact site across the outer and inner membrane. The targeting sequence of the precursor protein is then proteolytically removed. The remainder of the protein remains on the cytosolic side of the outer membrane. The exact mechanism by which the mitochondrial import machinery transports the precursor across both the outer and inner membranes is not known. The Ssc1p protein is found to be important for the completion of translocation. The transport of proteins into mitochondria requires the protein to be in an unfolded or loose conformation. The second step involves the interaction of Ssc1p with the precursor as revealed through immunoprecipitation and crosslinking experiments. Ssc1p was found associated with polypeptides before they were transported to the matrix (Craig et al., 1993).

Ssc1p binds to the unfolded precursor proteins as the N-terminus enters the matrix. The binding of Ssc1p to the protein sterically prevents it from moving backwards toward the cytosol due to its large globular size. Ssc1p maintains

Figure 8: Model of the role of Ssc1p and Hsp 60 in protein translocation into mitochondria. See text for discussion. This was copied with permission (Craig et al., 1993).

the imported precursor in an unfolded state until it is released for subsequent folding by other components in the mitochondrial matrix. The precursor can be pulled towards the inside of the mitochondria by interaction with one or more Ssc1p molecules. The energy required for complete unfolding of a protein is 5-10kcal/mol. The free energy released by binding of Ssc1p to polypeptide chain may be larger than that needed for protein unfolding (Kang et al., 1990). Therefore, the Ssc1p Hsp 70 plays a role in stabilizing the protein in a partially folded state until it is released. Upon ATP hydrolysis, the Hsp 70 is released and the protein can then fold and the chaperones can be recycled for further transport of proteins (Craig et al., 1993).

Endoplasmic Reticulum Hsp 70

Endoplasmic reticulum of eukaryotic cells contain a Hsp 70 member called Kar2p in yeast and BiP in mammals. Kar2p was named because the mutation named Kar2-1 resulted in defect in karogamy, nuclear fusion (Conde and Fink, 1976; Polaine and Conde, 1982). Kar2p is synthesized with a 42 amino acid presequence on the amino terminus and a C-terminal HDEL sequence. The amino terminus sequence is responsible for targetting to the ER and the carboxy terminus sequence acts as a retention signal. The Kar2 is essential for cell viability (Munro & Pelham, 1986). This chaperone has not been studied as thoroughly as BiP.

BiP was discovered as a protein bound to unassembled immunoglobulin heavy chains and, therefore, called immunoglobulin heavy chain binding protein (Morrison et al., 1975; Haas et al., 1983). It was later found to be identical to the glucose-regulated protein Grp 78 (Carlsson & Lazarodes, 1983).

It has 60% amino acid identity to cytoplasmic Hsp 70 and binds similarly to ATP. Under normal growth, it is synthesized constitutively and abundantly. It comprises 5% of lumenal content of ER of mammalian cells (Welch et al., 1991). Accumulation of secretory precursors or mutant proteins in the ER or different stress conditions that serve to increase the concentration of unfolded polypeptides can induce BiP. It is synthesized with a cleavable hydrophobic signal sequence directing the protein to the ER and a carboxy terminal sequence KDEL as a retention signal (Munro & Pelham, 1987).

BiP binds to immunoglobulin heavy chains in preB cells, nonglycosylated yeast invertase and prolactin containing incorrect disulfide bonds (Kassenbrock et al., 1988), malfolded and mutant viral glycoproteins (Machamer and Rose, 1988; Hurtley et al., 1989), and hydrophilic peptides in an ATP-reversible manner (Flynn et al., 1989). BiP can discriminate accurately between folded and unfolded proteins, with a preference for unassembled or incorrectly folded proteins (Langar et al., 1991). BiP possesses a peptide-dependent ATPase activity with a low turnover number and high affinity for ATP (Kassenbrock and Kelly, 1989; Flynn et al., 1989). The presence of ATP or ADP showed decreased sensitivity of BiP to proteolytic degradation (Kassenbrock and Kelly, 1989). This suggests that possibly adenine nucleotides may stabilize special conformations of BiP (Langer, 1991).

BiP is proposed to have dual functions. It has been found to form a stable complex with some proteins (Gething et al., 1986; Bole et al., 1986; Hurtley et al., 1989) and a transient complex with other proteins (Gething et al., 1986; Bole et al., 1986; Ng et al., 1989; Dorner et al., 1987; Biorient et al., 1991). It was found to form a stable complex with unassembled immunoglobulin heavy

chain and mutated forms of influenza virus. Inhibition of N-glycosylation for immunoglobulin heavy chains and human serum glycoproteins increased the associations with BiP (Bole et al., 1986). Underglycosylated IgG heavy chains were unable to properly associate with the light chains. They remained bound to BiP (Haas and Wabl, 1983; Bole et al., 1986). Similarly, mutated forms of influenza virus hemagglutinin which are unable to fold correctly were found associated with BiP (Hendershot et al., 1987). BiP is thought to recognize abnormal proteins that are unable to correctly mature or require longer times for folding and fail to move properly along the secretory pathway. In doing so, it could either help the protein maintain solubility until presentation to the appropriate proteolytic system for degradation or facilitate their slow folding (Hartl, 1991; Langer et al., 1991).

Blond-Elguindi et al. (1993) tested various peptide substrates for stimulation of ATP hydrolysis by ER Hsp 70 BiP. Their results revealed that optimal stimulation occurred with a minimal peptide length of seven residues. Further studies using synthetic heptapeptides of random sequence revealed the preference of BiP for certain amino acid residues. BiP was found to bind to peptides containing a subset of aromatic and hydrophobic amino acids in alternating peptides. This suggests that the peptides bind in an extended conformation with the side chain of alternating residues pointing into a cleft on the BiP molecule.

Flynn et al. (1991) proposed that the peptide-binding pocket of BiP is filled with a heptameric motif of Hy(w/x)HyXHyXHy where Hy is a large hydrophobic amino acid (Trp, Leu, or Phe), W is Trp, and X is any amino acid (Flynn et al., 1991). The peptide-binding site on BiP contains four pockets

(Figure 9). A stable interaction between peptides and BiP molecule requires occupancy of any two of the pockets. This model is similar to that for the binding of peptides to major histocompatibility antigens (Rippmann et al., 1991; Flajnik et al., 1991). It has already been shown that the three dimensional C-terminal peptide binding domain of Hsp 70 protein resembles that of the peptide binding superdomain of the human major histocompatibility leukocyte antigen. The motif allows for the two features of BiP to occur; its ability to recognize variety of nascent polypeptides that have no sequence similarity and its ability to discriminate between properly folded and unfolded structures. The motif has a high degree of redundancy and it contains residues whose side chains would normally be buried in the interior of the folded protein (Blond-Elguindi, 1993). Synthetic octapeptides containing the motif were found to bind to BiP with apparent affinities of 10-60 μ M (Flynn et al., 1989).

Figure 9: Schematic Model of the Peptide-Binding Site of BiP. The peptide backbone is shown as an extended chain. The side chains of the even-numbered residues extend into four deep pockets in the peptide-binding site that have similar overall preferences for large hydrophobic or aromatic side chains. Permission was requested (Blond-Elguindi, 1993).

Using genetic techniques, Kozutsumi et al. (1988) revealed that the temperature-sensitive BiP mutant failed to import normal proteins which are secreted into the ER. This suggests that BiP may play a role in the translocation of proteins into the ER. Similar to other Hsp 70 family members, BiP is thought to maintain precursor proteins in a translocation-competent conformation and facilitate their transport into their intended destination. Hydrolysis of ATP results in the release of the protein allowing it to complete its folding pathway and carry out its biological function.

4. Heat Shock Protein 60

Hsp 60 proteins, also collectively referred to as chaperonins, are found in the cytosol of all bacteria (Eubacteria, archaebacteria, cyanobacteria, rickettsiae), yeast cells, and plastids of eukaryotic cells. In *E. coli*, mitochondria, and chloroplasts, Hsp 60 is referred to as GroEL, Hsp 60, and Rubisco subunit binding protein, respectively. These proteins are abundant and constitutive proteins. They are induced by stress such as heat shock, bacterial infections of macrophages, and an increase in the cellular content of unfolded proteins. They can act as protein antigens in a variety of infections. There is high degree of sequence identity within the chaperonin family. The yeast mitochondrial chaperonin 60 protein shares 45% identical residues with the wheat plastid chaperonin 60, and 54% identical residues with the *E. coli* chaperonin 60 (Hartl, 1991; Hemmingsen et al., 1988; Reading et al., 1989).

There are two types of chaperonin that are sequence related to each other; cpn 60 (molecular weight of 60 kDa) called GroEL and cpn 10 (molecular weight of 10 kDa) called GroES. Wynn et al. (1994) compared chaperonin 10 from various bacterial species to cpn 60 from different species. Results revealed that the two proteins have similar sequences in region extending from residue 101 of the cpn 60 sequence. They also found that all cpn 60 proteins interact with the smaller cpn 10.

Cpn 60 chaperonins consist of fourteen subunits of 60 kDa arranged in two stacked heptameric rings, forming a symmetrical double toroid with a central cavity. The substrate protein binds within that space. Proteins of up to 90 kDa can bind (Wynn et al., 1994).

Cpn 60 chaperonins have several properties in common. They all are constitutively expressed, but can be induced by heat shock. Their molecular weights and isoelectric points are identical. All members also possess a weak ATPase activity. Studies have implicated them in many cellular processes such as morphogenesis of bacteria (Georgopoulos et al., 1972; Cheng et al., 1989; Horwich et al., 1990), DNA replication (Fayet et al., 1986; Jenkins et al., 1986; Wada et al., 1987), protein folding (Ostermann et al., 1989), translocation of proteins across membranes (Randall and Hardy, 1986; Bochkareva et al., 1988; Lecker et al., 1989), and assembly of oligomeric proteins (Goloubinoff et al., 1989).

Bacterial Chaperonin 60 (GroE Family)

GroE proteins consisting of GroEL and GroES are encoded in the groE operon, part of the *E. coli* heat shock regulon. The molecular weight based on denaturing gel electrophoresis is 60 kDa for GroEL and 10 kDa for GroES. GroEL is induced 4 to 5 fold upon heat shock, with an increase in temperature from 37 °C to 46 °C (Georgopoulos and Welch, 1993). GroEL protein has a weak ATPase activity that is inhibited by GroES (Chandrasekhar et al., 1986; Hendrix et al., 1979). Many studies have revealed interactions between the two proteins, in the presence of ATP and MgCl₂ (Langer and Newport, 1991).

GroEL proteins are cylindrical structures, with a sevenfold symmetrical axis, a diameter of 15 nm and a longitudinal axis of 15 nm. The structure appears star-shaped with central hole of 6nm in diameter when the top view is observed with an electron microscope. The side view of the protein appears

rectangular in shape, with four equally spaced striations. This may represent the tail-to-tail arrangement of two heptameric GroEL rings (Hartl et al., 1994).

Native GroES protein has a heptameric ring with a donut-like shape, with a diameter of 8nm. It is not known to bind to any other proteins except GroEL. A GroES homologue was found in bovine mitochondria. It was identified based on its interaction with *E. coli*-GroEL (Lubben et al., 1990). This may be identical to a mitochondrial Cpn 10 from rats which also interacts with *E.coli*-GroEL (Hartman et al., 1992). GroES from peas and spinach chloroplasts with two distinct GroES domains arranged head to tail have also been isolated (Bertsch et al., 1992).

GroE proteins have been implicated in many cellular processes- morphogenesis of bacteriophages, DNA replication, translocation of proteins across membranes, and protein folding and assembly of oligomeric proteins. Mutations in either of the two gro genes (GroEL or GroES) renders bacteria unable to support assembly of infecting bacteriophage (Sternberg et al., 1973). The involvement of GroEL and GroES proteins in the morphogenesis of bacteriophages has been well established (Georgopoulos et al., 1973; 1978). They both play a role in head assembly of the phage lambda and tail assembly of T5 phages. They are also necessary for T4 head assembly. The assembly of bacteriophage lambda first involves the formation of an oligomeric structure of 12 identical phage-encoded polypeptides. This preconnector is the basic structure on which head phage proteins assemble. The correct formation involves the intermediate formation of 1:1 noncovalent complex between a phage protein monomer and GroEL oligomer. Without GroEL, the head proteins of phage T4 form large insoluble aggregates that associate with cell

membranes. GroES is also needed for correct phage head formation playing a role in the release of the phage protein from GroEL. Neither protein forms part of the assembled phage (Becker and Craig, 1994).

GroE proteins are required for viability under normal growth conditions (Fayet et al., 1989). Georgopoulos et al. (1993) found that mutants of GroEL show similar properties as that seen with the Hsp 70 homologue, Dna K. Mutants lacking GroE proteins have reduced DNA and RNA synthesis, are blocked in cell division at nonpermissive temperatures, and show reduced protease activity.

The GroEL mutants are also implicated in the maintenance of secretory precursors in a transport-competent state. GroEL binds to partially unfolded polypeptide forming stable binary complexes. This binding keeps the protein in a conformation that prevents it from misfolding, assists it in correct folding, and allows it to be more readily transported. They have also been shown to mediate the refolding and assembly of a variety of enzymes denatured in vitro using urea, guanidinium, or heat as the denaturant. The enzymes examined include ribulose biphosphate carboxylase (Goloubinoff et al., 1989), dihydrofolate reductase (Martin et al., 1991; Viitanen et al., 1991), rhodanase (Martin et al., 1991; Mendoza et al., 1991, 1992), citrate synthase (Buchner et al., 1991), and alpha-glucosidase (Holl-Neugebauer et al., 1991). GroEL was not found to bind to the native structure of these proteins (Hendrick et al., 1993).

Many other denatured *E. coli* proteins have been found associated with purified GroEL and are released with addition of hydrolyzable Mg-ATP and GroES (Viitanen et al., 1992). Labeled pre- β -lactamase and chloramphenicol acetyl transferase with [35S] were found complexed with GroEL immediately

following synthesis in a bacterial translation system (Bochkareva et al., 1988). Heat-denatured myoglobin, but not native protein, can displace the labeled protein suggesting that the specificity of GroEL protein is for unfolded forms (Hendrick et al., 1993).

Martin et al. (1991) and Bochkareva et al. (1992) examined the interaction of rhodanase and DHFR with GroEL. They found that the denatured forms of both proteins were bound to GroEL. Rhodanase in the presence of GroEL bound to this protein, but in the absence, it formed aggregates. DHFR also was found to bind to GroEL, but in the absence of GroEL, it rapidly refolded instead of aggregating. Both protein complexes were sensitive to protease, and bound to 1,8-anilino-naphthalene sulfonate (ANS, a fluorophore with high affinity for hydrophobic protein surfaces). These properties are consistent with those observed for the molten globule state of proteins indicating that the chaperonins maintain the proteins in a translocation-competent conformation (Martin et al., 1992; Mendoza et al., 1992).

GroE proteins have been found to bind specifically to the molten globule state of various polypeptides and not their native forms. The proteins bind within the central hole of the GroEL structure, thus minimizing possible inter-polypeptide aggregation. It can bind protein up to 90 kDa (Christensen et al., 1991). Studies revealed that one or two substrate molecules are bound to GroEL multimer (Langer et al., 1992; Saibil et al., 1992; Braig et al., 1993). The affinity and strength of GroEL binding to different polypeptides varied (Langer et al., 1992; Naneyx & Gatenby, 1992). Mg-ATP and GroES were needed for release of the protein from the complex. GroES may serve many functions. It was shown that GroES causes a conformational change throughout the length

of the GroEL structure (Langer, 1992; Baneyx & Gatenby, 1992). This may guide polypeptide folding in the GroEL cavity or guide polypeptide release (Becker et al., 1994). GroES also affects GroEL ATPase activity in two ways. It inhibits overall ATPase activity (Chandrasekhar et al., 1983; Viitanen et al., 1990) and increases the cooperativity of ATP hydrolysis by GroEL subunits (Gray & Fersht, 1991; Bochkareva and Gershovich, 1992). GroES may interact directly with the bound polypeptide substrate. Crosslinking experiments by Bochkareva & Gershovich, (1992) revealed the interaction between GroES and GroEL bound pre β -lactamase substrate. This is seen only in the presence of all three proteins indicating that the interaction must occur on GroEL.

The role of GroE proteins in assembly of oligomeric proteins was implicated from experiments on rubisco purified from *Rhodospirillum rubrum*. Rubisco is a dimeric protein whose active site involves both subunits. Goloubinoff et al. (1991) found that the the dimer can be denatured with high concentrations of guanidine HCl or urea into monomers containing little secondary structure. If the inactive protein solution was diluted 200 fold into buffer lacking denaturant at 25 °C, less than 2% of the original rubisco activity appeared after incubation for 2 hours and less than 10% after 24 hours. At high protein concentration, insoluble aggregates formed. If the temperature of incubation was reduced below 25 °C, the recovery of active enzyme increased until all activity was recovered at 10 °C. If the buffer at 25 °C contained a molar excess of *E. coli* cpn 60 oligomer to rubisco monomer, a stable 1:1 complex formed between the rubisco monomer and the oligomeric form of cpn 60. MgATP, potassium ions, and cpn 10 caused the release of the cpn 60 chaperone. Eighty percent of the original rubisco activity was in the form of

dimers. GroE proteins act as chaperones conveying no steric information for protein assembly but act kinetically to assist self assembly.

A model, described in Figure 10, has been proposed by Hartl et al. (1994) for the mechanism of the GroEL-GroES-mediated protein folding. First, a partially folded precursor protein binds within the central cavity of the GroEL double ring; a single heptameric ring can bind unfolded protein. The initial interaction with the substrate probably occurs with the seven-subunit ring distal to GroES. The GroES heptameric ring binds asymmetrically to one end surface of the GroEL cylinder. ATP hydrolysis is enhanced by GroES and results in simultaneous release of protein. The protein is prevented from being released immediately by GroES-induced conformational changes in GroEL. Therefore, the protein is allowed to begin its folding pathway within the GroEL cavity. Dependent on the protein and its folding pathway, many cycles of binding and ATP hydrolysis may be necessary. If a protein is released as an intermediate exposing interactive or hydrophobic sites, it can rebind to GroEL and begin the reaction again. Small proteins may fold immediately and require only one cycle whereas larger proteins may bind several times before they are folded properly.

Chloroplast Chaperonin 60

Ribulose-1,5-bisphosphate carboxylase/oxygenase (rubisco) is a protein found in chloroplasts which catalyzes CO₂ fixation, the rate limiting step in photosynthesis. The structure consists of a hexadecamer composed of eight large subunits each with a molecular mass of 52 kDa and eight small subunits with molecular mass of 15 kDa (Gatenby & Ellis, 1990). They found that newly synthesized rubisco large subunits are associated with a large oligomeric

Figure 10: Reaction cycle of GroEL and GroES in protein folding. D* represents the high affinity ADP-binding state of a heptameric GroEL ring; D, the low affinity ADP-binding state; T, the ATP-binding state; U and N are the unfolded and native protein, respectively. This was copied with permission (Hartl et al., 1994).

protein prior to their assembly into rubisco holoenzyme. This protein is known as the rubisco subunit binding protein and the cDNA sequence reveals that it is closely related to GroEL protein of *E. coli* and cpn 60 of the mitochondria.

Rubisco subunit binding protein is an abundant protein found in stroma of chloroplasts. It contains two types of subunits with molecular weight of 61 kDa (alpha) and 60 kDa (beta) that have a 50% sequence identity. Rubisco subunit binding protein possesses a weak ATPase activity. Chaudhari et al. (1987) found that Mg-ATP in equimolar concentration caused a reversible dissociation of binding protein into monomeric subunits. They are encoded by nuclear genes and are imported into chloroplasts after synthesis in the cytosol. Without the binding protein, rubisco aggregates into inactive lumps. Ellis et al. (1991) showed that one hundred percent of active Rubisco may be recovered after a long incubation at 10 °C but less than 1% can be recovered at 25 °C. They found the remaining 99% of the protein in the form of insoluble aggregates (Ellis et al., 1991).

Goloubinoff et al. (1991) studied *in vitro* reconstitution of active rubisco with *Rhodospirillum rubrum* rubisco as the substrate and purified *E. coli* chaperonins, cpn 60 and cpn 10. They denatured rubisco in 8M urea, 6M guanidine HCL or by acid treatment. Their results revealed that at 25 °C, Rubisco formed an inactive aggregate that was unable to enter a nondenaturing gel upon removal of denaturant. If cpn 60 was present with the denatured rubisco, a complex between cpn 60 and denatured rubisco formed. Cpn 60 and not cpn 10 was found to be necessary for the formation of an active rubisco dimer (Table 2, lanes 1-4, vs 5). Pretreatment of cpn 60 with Mg-ATP reduced formation of an active rubisco dimer (Table 2, lanes 2 & 3). The presence of cpn

10 in the pretreatment with cpn 60 and Mg-ATP increased the yield (Table 2, lanes 1 & 2). Addition of cpn 10 and Mg-ATP as a pretreatment or after addition of denatured rubisco did not significantly affect the yield. Both procedures resulted in the formation of active rubisco (Table 2, lanes 1 & 3). This suggests that ATP, in the absence of cpn 10, may induce conformational changes in cpn 60 preventing binding to the denatured protein. Cpn 10 may function to help maintain the active conformation of cpn 60 which can bind to denatured protein acting as a coupling factor between hydrolysis of ATP and the protein folding reaction. Addition of hexokinase and glucose to deplete ATP resulted in a very low yield of active rubisco (Table 2, lanes 1 & 4). Hydrolysis of Mg-ATP was required for the release of the protein from the cpn 60-cpn 10 complex. Non-hydrolyzable analogs of ATP inhibited the release.

Model for the chaperonin action on reconstitution of dimeric rubisco is expressed in Figure 11. First, denatured rubisco protein forms an unstable folding intermediate, rubisco I (4a). At low nonphysiological temperatures (10 °C), rubisco I slowly and spontaneously renatures. At 25 °C, rubisco-I aggregates if cpn 60 is not present. A stable binary complex of cpn 60-rubisco-I forms in the presence of cpn 60. *In vivo*, rubisco-I probably binds to a cpn 60-cpn 10 complex. Cpn 10 probably binds to cpn 60 to prevent possible conformational changes of cpn 60 induced by endogenous ATP (4b). Potassium ions are required along with hydrolysis of ATP for the release of folded rubisco protein (4c) (Goloubinoff et al., 1991).

	1	2	3	4	5
Time of Addition					
0'	cpn 60 cpn 10 MgATP	cpn 60	cpn 60	cpn 60 cpn 10	cpn 10
20'				Glucose/ Hexokinase	
23'	rubisco	rubisco	rubisco	rubisco	rubisco
33'		cpn 10	cpn 10		cpn 60
34'			MgATP		MgATP
94'	assay	assay	assay	assay	assay
Reconstitution	45.5%	24.9%	48.2%	0.5%	0%

Table 2: The sequential order of the chaperonin assisted refolding of rubisco. *In vitro* reconstitution of urea-denatured rubisco was performed as specified; however, [cpn 60]₁₄, [cpn 10]₇, MgATP were applied in different orders to the unfolded rubisco substrate. Permission was requested (Goloubinoff et al., 1991).

Figure 11: Model for chaperonin-dependent reconstitution of Rubisco. Native Rubisco (Rubisco-N) is denatured by urea, guanidium-HCl, or acid (pH 3.0), to form after removal of the denaturing agent; Rubisco-I (a) an unstable folding intermediate. Rubisco-I aggregates, unless (b) the chaperonin [cpn60]₁₄ or [cpn60-cpn10]₁₄ associate with it. The complex [cpn60-cpn10]₁₄-Rubisco requires Mg-ATP and K⁺ ions to catalyze the refolding reaction (c) and the formation of a monomeric Rubisco-N that spontaneously dimerizes to form active Rubisco. Permission was requested (Goloubinoff et al., 1991).

Mitochondrial Chaperonin 60

Mitochondrial cpn 60 has been well studied in yeast, *S. cerevisiae*, and is encoded by the essential nuclear gene MIF4. This protein was found to be similar to GroE proteins in supramolecular structure, amino acid sequence, and function. The protein is synthesized with an amino terminal targeting sequence which is cleaved when cpn 60 enters the matrix. Its structure is similar to other chaperonins, consisting of two stacked rings of seven 60 kDa subunits each. It shows a 54% sequence identity to GroEL and a 43% identity to Rubisco subunit binding protein (Becker et al., 1994).

The yeast cpn 60 is also similar to GroEL in its function. Mutations in the MIF4 gene allow the transport and processing of mitochondrial proteins but these proteins failed to fold properly or assemble (Cheng et al., 1989). In *Neurospora* mitochondrion, they showed that newly imported proteins were associated with cpn 60 in the absence of ATP. The complex was sensitive to protease indicating that the proteins were probably in a loosely folded state. Addition of ATP released the protein precursor and it was found to be resistant to proteases indicating that it is in its fully folded state. Like GroEL protein, mt-cpn 60 facilitates protein folding and assembly in an ATP-dependent manner. It interacts with loosely folded protein and not the native state.

Mitochondrial Hsp 60 plays a role in mitochondrial protein export for the formation of oligomeric protein complexes. Cheng et al. (1989) and Horwich et al. (1990) studied the mitochondrial import function of beta-subunit of F1ATPase and the matrix enzyme ornithine trans-carbamylase in a temperature sensitive cpn 60 mutant, *mif4*, which is defective in the gene coding for cpn 60. The two proteins imported from the cytosol to the mitochondria were unable to assemble

into their functional oligomeric complexes. Hartl et al. (1987) found that cytochrome b₂, a protein of the intermembrane space, also failed to assemble into its oligomeric structure in the hsp 60 mutant.

5. Tailless Complex Polypeptide (TCP1)

Tailless complex polypeptide, TCP1, was first identified in the testes of mice and later was found to be in all cell types. It is a 57 kDa protein whose homologs have been found in fruit flies, humans, and *S. cerevisiae* (Silver, et al. 1979). Gupta et al. (1990) compared sequences of cpn 60 genes with databases of known sequences and found a weak similarity between cpn 60s and TCP1. Further studies revealed that although sequence homology is weak, the two proteins have some functional similarities. TCP1 has high homology to the heat inducible protein of thermophilic archaebacterium *Sulfolobus shibatae*, TF55 (Trent et al., 1991). TF55 is a large complex of a single 55 kDa polypeptide and can bind unfolded proteins and has ATPase activity suggesting its role as a chaperone. Studies with the folding of actin (Gao et al., 1992) and folding of tubulin (Yaffe et al., 1992) revealed a function of TCP1 in protein biogenesis. In both studies, the proteins were radiolabelled during synthesis in the cytosol. The proteins were monitored by electrophoresis in nondenaturing polyacrylamide gels to probe associations to proteins. Results revealed that newly synthesized actin and tubulin were found associated with a 60 kDa protein which reacted with antibody raised against TCP1. They also found that ATP hydrolysis resulted in the release of a folded, protease resistant protein but was not required for the formation of the complex.

TCP1 from human cells exists as part of a heteromeric complex with a molecular weight of 950 kDa. This complex is referred to as TRiC (TCP1 ring complex) since it contains TCP1 as one of the subunits. TRiC also contains five to seven proteins of 50 to 68 kDa (Frydman et al., 1992; Gao et al., 1992; Lewis

et al., 1992; Yaffe et al., 1992). TRiC appears as a cylindrical particle consisting of two stacked rings with eightfold symmetry, 17nm in diameter (Frydman, et al., 1992).

TRiC binds to unfolded proteins, prevents aggregation and helps ATP dependent refolding of proteins *in vitro*. The refolding of guanidinium-chloride denatured alpha and beta-tubulin, and firefly luciferase mediated by both TRiC and GroEL/ES was studied by Lewis et al. (1992). TRiC was found to refold tubulin efficiently in the presence of ATP and GTP, but without any addition of a co-chaperone such as GroES analog. GroEL mediated folding of tubulin required ATP and the co-chaperone GroES. Only TRiC and not GroEL/ES was able to facilitate the folding of luciferase. Further studies are needed to understand the chaperone-like function of this protein.

6. Small Hsps

Small heat shock proteins (sHsps) have molecular masses between 15 and 30 kDa. They are ubiquitous and conserved. They have been found in the cytosol of both stressed and normal cells of many organisms. All small Hsps are related and have many similarities. Figure 12 shows the amino acid sequences of small Hsps from different organisms. They all share a similar hydropathy profiles, but only a small area of amino acid identity. The sequence aa1-aa2-glycine-aa3-leucine-aa5-aa6-aa7-proline-aa8 is found near the carboxy-terminus in a similar position in this class of proteins. The three amino acids, glycine, leucine, and proline, are invariant whereas aa1-8 are more conserved. Amino acid 4 is threonine in all species and amino acid 5 is always isoleucine, valine, or leucine (Lindquist et al., 1988). Small Hsps have greater homology within a organism than between organisms. The soybean small Hsps have 90% homology with each other but only 20% homology with proteins of *D. melanogaster*, *C. elegans*, and *X. laevis* (Nagao et al., 1986).

The small Hsps share similar structural properties. They can form highly polymeric structures called heat shock granules with sedimentation coefficients of 15-20S (Arrigo et al., 1987; Nover et al., 1988; Schuldt et al., 1985). Mammalian proteins (Arrigo et al., 1987; Kim et al., 1984) and the *Drosophila* proteins (Rollet et al., 1986) are both phosphorylated under different conditions and have been found to contain RNAs. The small Hsps are induced at certain stages in development at normal temperatures. Seven small Hsps have been identified in *D. melanogaster* and are present during the late larval and early pupal stages, in nursing cells, and in developing oocytes (Ayme et al., 1985;

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1  GYISRCFTTRKYTLFPFGVDPTQVSSSLSPGTLTVEAPHPKLATQSNEIT--IPV...
2  GHINGMFVRRYKVPDGYKAEQVVSQSSDGVLTVSIPKPE-AVEDKSKERI IQIQ..
3  ERSSGKFTTRFALPENAKVNEVKASH-ENGVLTVTVPKEE--VKKPDVKA-IEISG
4  RKSYSCYDTRLKLPDNCEKEKVKAEL-KDGVLYITIPKTK--IERTVID--VQIQ
5  ERPRGVFNRLVLGENLDTERILASY-QEGVLKLSIPVAERAKPRK-ISVDRGNN...
6  GYISREFHRRYRLPSNVDSALSCLSLADGMLTFSGPKIPSGVDAGHSERAI PV...
      A  AA  AA  A      A  AA  *AA  *A

```

Figure 12: Comparison of small Hsps. Top: the amino acid sequences of the most conserved domain, located at or near the carboxy terminus of several small hsps. 1. Human Hsp 27 (aa# 132-186). 2. Drosophila Hsp 26 (aa# 127-180). 3. Soybean Hsp 17.5E (aa# 103-154). 4. Pea chloroplast Hsp 21 (aa# 183-232). 5. Mycobacterium 18kDa antigen (aa# 81-133). 6. Bovine alpha-crystallin A chain (aa# 108-161). (*) Invariant amino acids; (^) identical amino acids in at least 4 of the 6 proteins. Bottom: hydropathy profiles of members of the small Hsp protein family. The profiles are aligned by the most conserved domain, which is indicated in black. Permission was requested (Lindquist et al., 1988).

Ingolia et al., 1982).

The functional role of small Hsps is still unknown. It has been suggested that they may act as chaperones assisting the formation or maintenance of the native conformation of cytosolic proteins. They are induced under heat shock conditions and at elevated temperatures they have a protective effect on cell viability (Rossmann et al., 1974). Interestingly, Bossemeyer et al. (1993) found that the C-terminal region of the eye lens protein alpha- β -crystallin was found to be homologous to small Hsps. Heat shock induces the expression of both small Hsps and alpha- β -crystallin reaching 1% of the total cellular protein. Small Hsps were found to be in high concentrations in stationary phase tumor cells, during differentiation, and in growth-arrested B-lymphocytes (Nover et al., 1991; Merck et al., 1993). The cells with high concentration of sHsps also had increased thermotolerance and also specifically inhibited cell proliferation (Jakob et al., 1994).

Small Hsps along with alpha-B-crystallin have been suggested to act as molecular chaperones playing a role in preventing aggregation and assisting protein folding. Jakob et al. (1993) experimented with citrate synthase and alpha-glucosidase to study the influence of small Hsps and alpha-crystallin on protein folding and unfolding reactions. At elevated temperatures, both proteins were denatured and in aggregated form. In the presence of small Hsps and alpha-crystallin individually, aggregation of both proteins was inhibited in the presence and absence of ATP. This suggests that small Hsps bind to proteins that are thermally denatured and protects them from premature interactions that can result in aggregate formation. Both small Hsps and alpha-crystallin seemed to be as effective in preventing aggregation.

The role of sHsps and alpha-crystallin in the refolding of proteins was also studied by Jakob et al. (1993). In the absence of sHsps or alpha-crystallin, 8% of citrate synthase and 20% of alpha-glucosidase folded correctly. In the presence of either sHsps or alpha-crystallin, an increase in the yield of folded proteins was observed; the citrate synthase had a 25% yield and the alpha-glucosidase had a 45% yield. Similar results were obtained with alpha-crystallin suggesting that they both aid in the refolding of some proteins (Jakob et al., 1993).

More information on the function of sHsps is not available yet. Like other molecular chaperones, they may play a role in assisting protein folding, preventing aggregation, and possibly in transport of proteins as well.

D. SEC B CHAPERONE MACHINE

Sec B is a cytosolic protein that is necessary for efficient export of some proteins in *E. coli*. It is an acidic, 64 kDa protein consisting of four identical 16 kDa subunits. It comprises 0.08% of the cytosolic proteins (Watanabe et al., 1989). It is encoded by the *secB* gene which is not essential. Both the nucleotide sequence of the *secB* gene and its predicted amino acid sequence of 155 residues has been determined and is presented in Figure 13.

Comparison of the sequence with other protein sequences revealed that there is 22.9% identity with the human fibroblast growth factor receptor 4 within a 108 amino acid overlap (Partanen et al., 1991) and 22% identity over 123 amino acids with the *S. cerevisiae* trans-activating factor B (Rep 1) within a 123 amino acid overlap (Hindley & Phear, 1979), and 23% identity with an estrogen regulated human 27 kDa heat shock protein (Hsp 27) within a 61 amino acid overlap (Fuqua et al., 1989; Hickey et al., 1986). It is interesting that the region in Hsp 27 which is homologous to Sec B is the most conserved region in small HSPs. Sec B does not contain an ATP-binding domain and hence is not affected by 5mM ATP (Collier et al., 1993).

Sec B has been found to play a role in translocation of certain proteins across the *E. coli* plasma membrane. It is necessary for the export of maltose binding protein (MBP), LamB, outer membrane protein A (OmpA), and outer membrane protein F (OmpF) but not ribose binding protein (RBP), alkaline phosphatase, beta-lactamase, or M13 coat protein suggesting a Sec B dependent and independent translocation pathway (Collier et al., 1988; Kumamoto and Gannon, 1988; Liu et al., 1989; Altman et al., 1990).

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1/1                               31/11
ATG TCA GAA CAA AAC AAC ACT GAA ATG ACT TTC CAG ATC CAA CGT ATT
Met ser glu gln asn asn thr glu met thr phe gln ile gln arg ile

                               61/21                               91/31
TAT ACC AAG GAT ATC TCT TTC GAA GCG CCG AAC GCG CCG CAC GTT TTC
tyr thr lys asp ile ser phe glu ala pro asn ala pro his val phe

                               121/41
CAG AAA GAT TGG CAA CCA GAA GTT AAA CTT GAT CTG GAT ACG GCA TCT
gln lys asp trp gln pro glu val lys leu asp leu asp thr ala ser

151/51                               181/61
TCC CAA CTG GCA GAT GAC GTA TAC GAA GTG GTA CTG CGT GTT ACC GTA
ser gln leu ala asp asp val tyr glu val val leu arg val thr val

                               211/71
ACG GCC TCT TTG GGC GAA GAA ACC GCG TTC CTG TGT GAA GTT CAG CAG
thr ala ser leu gly glu glu thr ala phe leu cys glu val gln gln

241/81                               271/91
GGC GGT ATT TTC TCC ATC GCG GGT ATC GAA GGC ACC CAG ATG GCG CAT
gly gly ile phe ser ile ala gly ile glu gly thr gln met ala his

                               301/101                               331/111
TGC CTG GGA GCA TAC TGC CCG AAC ATT CTG TTC CCG TAT GCT CGT GAG
cys leu gly ala tyr cys pro asn ile leu phe pro tyr ala arg glu

                               361/121
TGC ATC ACC AGC ATG GTA TCC CGC GGT ACA TTC CCG CAA CTG AAC CTT
cys ile thr ser met val ser arg gly thr phe pro gln leu asn leu

391/131                               421/141
GCG CCG GTT AAC TTC GAT GCG CTG TTC ATG AAC TAT TTG CAG CAG CAG
ala pro val asn phe asp ala leu phe met asn tyr leu gln gln gln

                               451/151
GCT GGC GAA GGT ACT GAA GAA CAT CAG GAT GCC TGA
ala gly glu gly thr glu glu his gln asp ala OPA

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Figure 13: Predicted amino acid sequence of SecB. The nucleotide sequence of secB is shown above the predicted amino acid sequence for SecB. Permission was requested (Collier et al., 1993).

The export of periplasmic maltose-binding protein of *E.coli* has been studied extensively (Bankaitis et al., 1985; Randall and Hardy, 1987). Randall and Hardy (1987) found that there was a correlation between the folding of the precursor MBP (pMBP) in the cytoplasm and a loss in its ability to be posttranslationally exported. They suggested that translocation of pMBP must occur before the protein is able to fold into its stable, tertiary structure.

Bankaitis et al. (1984) reported that synthesis of MBP with an export defective signal peptide interfered with normal export of several envelope proteins. Collier et al. (1988) studied various export defective proteins containing various regions of MBP for their ability to interfere with normal protein export. Mutations at various regions of MBP were studied. They found that residues 151 through 186 of mMBP was necessary to mediate interference in normal protein export. It was the only region common to all of the interfering species and absent in each of the noninterfering species. It was suggested that this region may represent the precursor binding region playing an active role in the export process.

Mutations in three MBP species which were defective in their ability to fold into a stable, tertiary conformation were evaluated in sec B⁻, sec B⁺, and in sec B overproducing cells by Collier et al. (1988) in order to understand the role of Sec B in facilitating pMBP export. They revealed that the three proteins were less dependent on Sec B for export than the wild-type. The folding of pMBP into its stable, tertiary structure was found to be rapid in sec B⁻ cells, slower in sec B⁺ cells, and undetectable in sec B-overproducing cells. Addition of excess Sec B could not unfold the folded pMBP. These results suggests that Sec B prevents the premature folding of cytoplasmic pMBP into export-incompetent

state. Purified Sec B also was found to enhance the activity of in vitro secretion systems and inhibit folding of precursor proteins. Sec B acts as an antifolding agent playing a role in controlling the rate of pMBP folding before it interacts with membrane components.

Altman et al. (1991) found that the inhibition of normal export by Sec B binding to export defective mutant pMBP was reversed upon induction of the heat shock response. Heat shock response was induced by overproduction of the transcription factor, σ^{32} which regulates the heat shock response. Three proteins, LamB, MBP, and OmpA were studied. An increase in export kinetics of all three proteins was seen with the heat shock response-50%, 100%, and 100% for MBP, LamB, and OmpA, respectively. This result can be explained if Sec B was a heat shock protein. Induction of the heat shock response would increase the synthesis of Sec B which would then overcome the limitation of Sec B due to its binding to the mutant pMBP. However, induction of the heat shock response resulted in a decrease in the synthesis of Sec B thereby indicating that Sec B is not a heat shock protein. The heat shock response must then induce other proteins which can substitute for the Sec B function. The likely proteins that substitute for the chaperone function of Sec B is the Hsp 60 proteins GroES/EL and the HSP 70 protein Dna K. However, overproduction of these proteins resulted in an increase in precursor accumulation and hence an inhibition of the export pathway suggesting that they do not substitute for Sec B function (Altman et al., 1991).

A model for the interaction of nonnative polypeptides with Sec B was proposed by Randall et al. (1992) (Figure 14). At low ionic strength, the Sec B is thought to be in an open conformation exposing a region of the carboxy

terminus. Binding of positively charged peptides or an increase in the ionic strength such as physiological ionic strength results in a change in the conformation of the Sec B. The Sec B in this conformation is resistant to protease. A hydrophobic site is also exposed in order to be accessible for the binding of polypeptide ligands. Upon binding to the preprotein, it can then maintain the preprotein in an export-competent conformation and target the preprotein to the translocation machinery at the membrane. Precursor binding to Sec B increases the affinity of Sec B binding to another factor called Sec A and facilitates the docking of Sec B-precursor complex at the export site. The polypeptide can then be released and it can form properly into its three dimensional structure and begin to fulfill its biological function (Randall et al., 1992).

Figure 14: A model of interaction of nonnative polypeptides and SecB. See text for discussion. This was copied with permission (Randall et al., 1992).

E. PapD CHAPERONE

Chaperones are present in the periplasm of Gram-negative bacteria. They are required for the assembly of many virulence-associated adhesive structure called pili. Pili are heteropolymeric fibers that mediate attachment to eukaryotic cells. Formation of pili is an important step for bacterial infections. Bacteria cells must fold protein subunits and assemble them into adhesive surface fibers. This involves domain-domain interactions within and between proteins after their secretion across the cytoplasmic membrane. Exposed hydrophobic sites on the protein may lead to premature interactions causing unproductive aggregates. The proper formation of these domains in the periplasm requires the function of a periplasmic chaperone. They protect interactive sites on the protein subunits and prevent aggregation. Chaperones also help secretion of the pilus subunits across the cytoplasmic membrane. Once the protein subunits complexed with the chaperone reach their destination, the chaperone is released and the correct subunit-subunit interactions can occur leading to assembly of ordered structures.

The P pilus system of *E. coli* has been studied to the greatest extent. *E. coli* is responsible for many bacterial infections in humans such as urinary tract infection and bacteremia. The infection process first involves the presentation of adhesin molecules. These are components of long, thin filamentous protein appendages called pili or fimbriae. Common diameters are 5-10 nm and length of up to 2 μm . These have been cloned, sequenced, and characterized by Hultgren et al. (1993). There are 11 genes identified in the biosynthesis and expression of functional P pili. These are represented as clusters at different

sites on the *E.coli* chromosome. The pap gene clusters from the human urinary tract *E.coli* isolate J96 have been cloned and is represented in Figure 15.

Figure 15: The pap operon and the assigned role in genetic regulation and pilus biogenesis of 10 of the 11 gene products. Permission was requested (Hultgren et al., 1993).

Jacob-Dubuissin et al. (1993) describes P pili as composite fibers consisting of thin heteropolymeric tip fibrillum joined end to end to a thick pilus rod. Hultgren (1993) found that the tip fibrillum is composed of four subunits, PapG, PapF, PapE, and PapK. PapG is an adhesin that mediates the binding to the Gal α (1-4)Gal receptor present in the glycolipids in the kidney. This subunit is encoded by the papG gene located at the distal end of the pap operon. PapF is an adapter protein that joins PapG to PapE (Jakob-Dubuissin et al., 1993). Repeating units of PapE make up the bulk of the tip fibrillum. The tip fibrillum is joined to the helical rod by PapK adapter protein. The rod or the stalk of the pilus fiber consists of repeating subunits of PapA arranged in a right-handed helix (Gong et al., 1994). PapA subunits (18 kDa) are encoded by the papA

gene located at the promoter proximal end of the pap operon. Inactivation of the papA gene inhibits pilus expression with no effect on the receptor binding and hemagglutinating ability of the bacterium. The fibrillum attaches to the bacterial cell by PapH. PapD acts as a chaperone and binds to each of the pilus subunit types as they are translocated across the cytoplasmic membrane. It maintains the subunit proteins in an assembly-competent, nativelylike conformation until it reaches the outer membrane containing PapC. PapC is called a molecular usher because it 'ushers' the subunits from the PapD chaperone and assembles them into the growing pilus in specific domain-domain interactions (Jones et al., 1992).

The three-dimensional structure of PapD periplasmic chaperones has been elucidated to 2.0 angstrom resolution by Holmgren and Branden (1989). Figure 16 gives a ribbon model of the structure. PapD consist of two globular domains, and is shaped like a boomerang. Each domain is a beta-barrel structure formed by two antiparallel beta-pleated sheets. The topology is similar to an immunoglobulin fold. Significant homology has been found between PapD and eleven periplasmic proteins involved in the assembly of cell surface structures in pathogenic bacteria. These proteins are 30-60% identical in sequence. The most conserved regions play a role in maintaining the overall structure and orientation of the domains toward one another (Kuehn et al., 1993).

PapD was found to bind and protect an interactive surface of PapG. This was tested in several ways. First, the stability of the PapD-PapG complex in 2M urea in the presence of dithiothreitol (DTT) was studied by Hultgren (1993). Results indicated that 80% of the complex was destroyed. The renaturation of

Figure 16: Ribbon model of the three-dimensional structure of the PapD chaperone. The consensus sequence for twelve members of the family was superimposed on the tertiary structure of PapD. The position of the invariant amino acid residues is shown in black, and that of the residues conserved in at least eight of the sequences, in gray. Permission was requested (Kuehn et al., 1993).

the denatured complex was attempted by subjecting it to conditions known to stabilize the complex. However, instead of the native form, aggregates had formed. This suggested that the site on PapG that PapD bound to was an interactive site. Denatured conditions inhibited the complex revealing the interactive surface which then participated in incorrect interactions leading to aggregation. An *in vitro* assay for denatured complex of PapG-PapD under optimal conditions for renaturing and in the presence of increasing

concentrations of PapD revealed that the complex was reformed in proportion to the concentration of the PapD (Kuehn, 1991). This further substantiates the hypothesis that PapD stabilizes the interactive surface of PapG. PapD binds to the domain of PapG not incorporating the region binding to Gal α (1-4)Gal. In particular, it binds to the carboxy terminus of PapG. This binding is required for the incorporation of the adhesin into the pilus. It also protects PapG from proteolysis, possibly suggesting the role of PapD in guiding correct folding of polypeptides. PapD may also cap and uncapped other pilus subunits that are imported into the periplasmic space. PapD acts as a chaperone by binding to interactive sites and preventing them from forming incorrect interactions with the environment or with other domains. Once it guides the subunit to its required location, it is released by some conformation or chemical mechanism so that the subunit is now free to be polymerized into the pilus rod. This process continues in distinct sites within the cell and in an ordered fashion (Hultgren et al., 1993).

The process is not complete until the pilus subunits are ushered into the pili from the chaperone complex by PapC. PapC is an 88kDa outer membrane protein. Mutations in the PapC gene results in a blockage of the assembly pathway (Norgren et al., 1987). Accumulation of the chaperone-subunit complex were seen in the periplasm. Therefore, PapC somehow functions to target the complex to the outer membrane. *In vitro* results for different PapD-subunit complexes in the presence of PapC revealed that PapD recognizes and binds to PapC only when already complexed to another subunit (Dodson et al., 1993). If PapD is free in solution, it will not bind to PapC. Complex of PapD-PapG, PapD-PapE, and PapD-PapF all bound to the PapC verifying that PapC functions to target the complex to the outer membrane where it can be

assembled into the pili. PapC is thought to act as an "usher" or molecular doorkeeper. It modulates the ordered targeting of chaperone-subunit complexes to the outer membrane assembly sites. The model of the ordered assembly of P pilus is described in Figure 17 (Hultgren et al., 1993; Jones et al., 1992).

Figure 17: Model of pilus subunit transport through the periplasm and pilus assembly. In the absence of PapD, nascent subunits aggregate and are subjected to proteolytic degradation. Association with PapD to form preassembly complexes caps interactive surfaces, preventing aggregation, and stabilizes the subunits. The bimolecular complexes are targeted to the membrane assembly site, PapC (C), where uncapping PapD occurs and pilus assembly is directed. As a result of the differing affinities of the chaperone subunit complexes for PapC (C), the adhesin resides at the terminus of the fibrillum, which is joined end to end to the pilus rod. Permission was requested (Jones et al., 1992).

III. MOLECULAR CHAPERONES: ROLE IN CELLULAR PROCESSES

Molecular chaperones cooperatively play a role in protein folding, assembly, and transport in the cell. They form stable complexes with precursor proteins as they are synthesized to prevent them from folding into mature structures prematurely or from misfolding or aggregating. They help maintain them in a "translocation-competent" conformation by binding to exposed hydrophobic and charged sites. Cooperation from the various family of molecular chaperones are implicated in protein folding, protein import into the mitochondrion, bacterial protein translocation, protein assembly, and in antigen presentation and processing. An example for the cooperative role of chaperones in each of these processes is described below.

A. PROTEIN IMPORT INTO MITOCHONDRIA

The model for translocation of proteins into mitochondria consist of three stages involving cooperation of mt-Hsp 70 and Hsp 60. First, mt-Hsp 70 plays a role in the passage of the targeting presequence across the inner membrane in response to membrane potential. The transport apparatus of the outer membrane guides the amino terminal presequence of the precursor into the intermembrane space. Proteinaceous channels are formed helping the passage of an unfolded polypeptide chain. Mt-Hsp 70 then enters the process by binding to segments of the incoming polypeptide serving to hold the protein precursor in the matrix. Mt-Hsp 70 stabilizes the unfolded conformation of the

polypeptide. Further binding of mt-Hsp 70 allows more and more structure to be drawn into the inner membrane. (Figure 18) (Stuart et al., 1994).

Figure 18: Model of mt-Hsp 70 mediated unfolding of preproteins outside the mitochondrion during the translocation process. This was copied with permission (Stuart and Craig, 1994).

Once the protein is translocated into the mitochondria, it is then transferred to Hsp 60. The exact mechanism of the transfer is unclear. Hsp 60 along with Hsp 10 allows for the folding in an ATP dependent reaction. The protein is released from Hsp 60 in conjunction with ATP hydrolysis. The protein can then fold into its correct three dimensional native state. It can then carry out its function in the destined compartment, the mitochondria (Stuart et al., 1994).

B. BACTERIAL TRANSLOCATION

Bacterial protein translocation involves transport of proteins synthesized in the cytoplasm into the inner membrane by translocation machinery localized at the inner membrane. There are two pathways, Sec B dependent and Sec B independent. The Sec B dependent pathway, which will only be discussed here, is used for the transport of certain proteins such as maltose binding protein (MBP), outer membrane protein A (OmpA), and outer membrane protein F (OmpF). It requires the cooperation of the molecular chaperone Sec B with other factors such as Sec A, and Sec Y/E. Sec A is a peripheral membrane protein of 102 kDa (Schmidt et al., 1988). It hydrolyzes ATP and couples the energy to translocation (Lill et al., 1989; Swidersky et al., 1990). Sec Y/E is the integral membrane protein that supports translocation (Brundage et al., 1990; Bilker and Silhavy, 1990). It constitutes about 0.6% of the protein of the plasma membrane and comprises of three subunits, the Sec Y polypeptide (Ito et al., 1983;1984), the Sec E polypeptide (Riggs et al., 1988; Schatz et al., 1989,1990) and a polypeptide of unknown gene. The polypeptide of unknown gene is

termed band 1 and copurifies with the Sec Y and Sec E subunits during either column chromatography or immunoprecipitation.

The Sec B-dependent pathway can be described with an example using outer membrane protein A (pro-OMPA). A two step process is involved for the translocation of this protein in the bacteria (Figure 19). Sec B binds to the mature region of the preprotein (preOMPA) in the cytosol. It forms a 1:1 complex and maintains the protein in a stable and translocation-competent conformation. It serves two functions, a chaperone function stabilizing preprotein in cytosol before binding to the membrane, and a targetting function after binding to the membrane guiding the preprotein to the membrane. The Sec B precursor complex has higher affinity for Sec A, peripheral membrane ATPase, than Sec B alone. Sec A binds to both the signal sequence and mature domain of the preprotein. Sec A requires both Sec Y/E protein and acidic lipids for high affinity binding to the membrane. When Sec A protein is in contact with a preprotein with an authentic leader and mature domain, the Sec Y/E protein, and acidic phospholipids, it hydrolyzes ATP. This activated ATPase activity is termed the "translocation ATPase", since it depends on each of the elements needed for successful translocation. ATP hydrolysis results in the release and translocation of the precursor. Other genes recently identified as sec genes, sec C, sec D, sec E, and sec F fulfill other roles in the prokaryote export. Their exact role has not been elucidated yet (Hartl, 1990; Lecker, 1989).

Figure 19: A model for the export of protein to the periplasmic space of *E. coli*. This was copied with permission (Randall, 1992).

C. ASSEMBLY OF PROTEINS

Two examples reflecting the role of molecular chaperones in the assembly of proteins can be described with the formation of microtubules and pilus structures. Microtubules are filamentous protein structure of eukaryotic cells consisting of tubulin, a heterodimer made up of two related protein subunits, alpha and beta. Studies with several Chinese hamster ovary (CHO) cell mutants revealed the identification of two cellular proteins, p1 and p2 as chaperones (Gupta et al., 1984; 1985). P1 is a 60 kDa protein showing 42-60% homology with GroEL protein of *E. coli*, ribulose-1,5-bisphosphate carboxylase (rubisco) subunit-binding protein of plant chloroplasts and Hsp 60 protein of yeast (Hemmingsen et al., 1988; Reading et al., 1989). The structure consists of either seven or fourteen subunits. It also has a weak ATPase activity (Picketts et al., 1989). Gupta et al. (1985) showed that the P2 protein is identical to the constitutive form of Hsp 70 (Hsc 70). It was found to migrate at the same position as Hsc 70 on two-dimensional polyacrylamide gels. P2 also reacted to a monoclonal antibody specific for Hsc/Hsp 70. Cloning of Hsc 70 cDNA from CHO cells released a product which comigrates with P2 in two dimensional gels (Gupta, 1984; 1985).

Gupta et al. (1985) examined mutant p1 and p2 and showed reduced binding to antimitotic drugs, colchicine and podophyllotoxin. Since antimitotic drugs bind to free tubulin with high affinity, these results suggest that the P1 and P2 may interact with free tubulin before it is incorporated into microtubules. Their studies also showed that P1 and P2 were associated with tubulin/microtubules in vivo under various conditions. P1 was found bound to

structures identified as mitochondria through immunofluorescence studies with P1 antibody. Microtubules were also found to be associated with mitochondria as revealed through double immunofluorescence labeling of cells with anti-P1 and anti-tubulin antibodies.

The roles that P1 and P2 play in the assembly of microtubules is not understood completely. However, Gupta et al. (1990) proposed a model speculating the p1 and p2 role based on known observations (Figure 20). First, nascent alpha and beta polypeptide chains bind to P2 as they are being translated. This facilitates their transport to the mitochondria as described for Hsp 70 proteins. Once in the mitochondria, the tubulin subunits bind to P1; P1 is the chaperone responsible for assembly of tubulin into functional tubulin dimers. It allows the tubulin to correctly fold into its functional three dimensional structure. The tubulin dimers are transferred from P1 through ATP hydrolysis to the growth sites in microtubules. (Gupta, 1990).

In *E.coli*, the molecular chaperone family of PapD has been implicated to participate in P pilus assembly. Jones et al. (1992) describes the model for the ordered assembly of P pilus in Figure 17 (refer to p. 81). First, the various subunits are translated as they are encoded by the genes on the pap operon. This occurs in the periplasm where PapD then binds to each subunit maintaining them in a translocation-competent conformation. PapD serves to assist translocation, prevent premature interactions from occurring, and preventing aggregation (Norgren et al., 1987). It also targets the subunits to PapC. PapC then binds to the complex. It was found that PapD-PapG complex binds to PapC first due to its high affinity (Dodson et al., 1993). Pap C then ushers the subunit to orient properly into the pilus structure. The PapG

becomes localized at the distal ends of the fibrillae. Next, the tip of the fibrillum is assembled so the PapF, PapE, and PapK complexes bind to PapC. PapA-PapD bind only once the tip fibrillae is assembled. This cycle continues with cooperation from PapD and PapC assuring that correct interactions for the production of ordered adhesive structure is taking place (Jones, 1992).

FIGURE 20: A model for in vivo assembly of microtubules. See text for details. This was copied with permission (Gupta, 1990).

D. ANTIGEN PRESENTATION AND PROCESSING

Molecular chaperones, in particular the heat shock proteins of the Hsp 70 family, play a role in the immune system by participating in the processing of foreign antigens. They have been implicated in both antigen presentation and processing. Antigenic peptides require an association with an MHC class II molecule on the surface of an antigen presenting cell (APC) to activate antigen-specific T cells. First, the antigen is internalized by the APC (macrophage, B cells, dendritic cells) either by phagocytosis or pinocytosis. The antigen is then degraded by proteolysis and the fragments are then associated with MHC class II molecules. They can then be recognized by the T cell receptor resulting in production of more T cells containing receptors for the particular antigen. Two proteins with molecular weight 72 and 74 kDa called peptide binding proteins (PBP72/74) were purified from B cells, T cells, macrophages, fibroblasts, and tissues (Lakey et al., 1987). These proteins were found to be specific for certain features of the peptide. PBP 72/74 was found to competitively elute from a pigeon cytochrome c (Pc) 81-104 peptide but not to native Pc protein. It can also bind to other peptide fragments of mouse cytochrome c, Pc 66-80 residue, and lactate dehydrogenase C4 (LDH-C4).

Nagel and Pierce (1991) studied the effect of antibodies raised against PBP 72/74 on APC function. They revealed that the PBP 72/74 specific antisera inhibited the processing and presentation of pc to a Pc-specific T cell hybrid. If T cells were incubated with the antisera, antigen presentation was successful while it is not successful with APC incubated with antisera.

Nagel and Pierce (1991) revealed that PBP 72/74 is a Hsp 70 protein. It was able to bind to a monoclonal antibody which recognizes a number of Hsp 70 proteins. PBP 72/74 also recognized an antibody specific for a constitutive Hsp 70 and not one for a stress inducible Hsp 70 protein. N-terminal amino acid sequence analysis confirmed that it is a heat shock protein by virtue of its N-terminal region sequence VGIDLG (shared by Hsp 70 proteins). The sequence of PBP 74, however, was not identical to any of the Hsp 70 proteins identified thus far, indicating that it is a new member of the Hsp 70 family. PBP 72 has not been characterized yet.

PBP 72/74 also has the ability to bind ATP like Hsp 70s. ATP causes release of PBP 72/74 from a peptide column just like ATP causes release of Hsp 70 proteins from their substrates (Munro et al., 1986).

Based on the above observation, Nagel and Pierce (1991) proposed that PBP 72/74 may serve to scavenge peptides in degradative compartments and prevent them from complete proteolysis. It maintains the peptide in a partially fragmented conformation so that it can be targetted to a MHC class II molecule. MHC class II molecules consist of alpha and beta chains. The alpha and beta chains are transported from the ER to the Golgi-body. Perhaps these chaperone proteins may serve to assemble MHC structure as seen with other chaperone role in assembly of microtubule. More information is needed to understand the mechanism of action of these proteins.

IV. CONCLUSION

Molecular chaperones play a major role in ensuring that cellular processes occur efficiently in normal as well as in stressed cells. Many chaperone proteins have been characterized and implicated in the transport, folding, and assembly of newly synthesized polypeptides, providing an insight into the regulation of protein folding in the cell. The challenge lies in understanding the specific molecular mechanism by which chaperones recognize target proteins and maintain protein conformations in the cell.

Understanding molecular chaperone function in the cell can provide information useful in medicine. Chaperones have been found to be dominant immunogens in human bacterial infections and are involved in certain autoimmune diseases. Vaccines can be developed against these foreign invaders to help fight these infections (Gaston et al., 1991; Kaufmann et al., 1991). They may also play a role in the progress of cardiac hypertrophy and ischemic diseases. Cells induced to produce high levels of chaperone proteins were found to be more resistant to sudden ischemic insults than control cells. A possible therapy could be to induce a stress response to increase the synthesis of chaperones hoping they aid the cells to recovery (William et al., 1991).

Knowledge of molecular chaperones can provide insight into the marvelous mechanisms by which cells regulate and protect important cellular processes in normal and stressed situations. With a better understanding, these chaperones may be useful as diagnostic and therapeutic agents.

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Telephone: (905) 525-9140, Ext.
Fax (905) 522-9033

June 26, 1996

Ms. B. McGowan
14673 S. Stitt Court
Oregon City, OR
97045

Dear Ms. McGowan:

I give you permission to site Fig. 1 from my article in Trends in Biochemical Sciences (1990) 15, 415-417 in your thesis, as requested.

Yours sincerely,

Radhey S. Gupta
Professor of Biochemistry

RSG/bs

Bhavna McGowan
14673 S Stitt Ct
Oregon city, OR 97045
(503) 657-8570
June 15, 1996

Elizabeth A. Craig
Department of Biomolecular Chemistry
University of Wisconsin-Madison
1300 University Avenue
Madison, Wisconsin 53706

Dear Dr. Craig,

I would like to request permission to use figures 1 and 2 found in Microbiological Reviews (June 1993, Vol 57 No 1, p. 402-414), figure 4 found in Annu. Rev. Genet. (1988, Vol 22, p.631-677) and figure 4 found in Cell (Aug. 12, 1994, Vol 78, p. 365-372) as illustrations in a thesis report. I am completing a MA in Chemistry at San Jose State University in San Jose, CA. My thesis report is an overview of molecular chaperones describing their role in protein folding, translocation, and assembly in the cell. Your articles were very helpful during the research phase of my thesis. The use of the figures would greatly enhance my ability to present the information on molecular chaperones.

I would appreciate a letter granting permission to be mailed to the address listed above or faxed to (503) 657-8571. Thank you very much for your help.

Sincerely yours,

Bhavna McGowan
Bhavna McGowan

7/2/96.
permission to use figures
mentioned above is
granted.
Elizabeth Craig

STANFORD UNIVERSITY SCHOOL OF MEDICINE

FAIRCHILD CENTER • STANFORD, CALIFORNIA 94305-5400
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22 January 1996

Ms. Bhavna McGowan
18111 SW Deerbrush Avenue
Lake Oswego
Oregon 97035

Dear Ms. McGowan:

You have my permission to use figure 5 in *Advances in Protein Chemistry* (1993, Vol. 44, p.67-93) and figure 3 found in *Nature* (Aug. 16, 1990, Vol. 346, p.623-628), as illustrations in your thesis report.

Sincerely,

A handwritten signature in black ink that reads "David McKay". The signature is fluid and cursive, with the first name "David" and last name "McKay" clearly distinguishable.

David B. McKay
Professor of Structural Biology

THE UNIVERSITY OF TEXAS
SOUTHWESTERN MEDICAL CENTER
AT DALLAS

Department of Biochemistry

1-22-96

Bhavna McGowan
18111SW Deerbrush Ave.
Lake Oswego, OR 97035

Dear Mr. McGowan:

I want to thank you for your letter and the kind words you wrote concerning our chaperonin paper in J. Lab. Clin. Med. We have received many compliments concerning this publication. At last count, we had sent out just over 300 reprint request!! I'm very pleased that you want to include Table 1 in your thesis. You certainly have my permission to use this table and any of the other figures in the manuscript.

Thank you for writing to seek permission to use this table. I'm pleased that this article helped you with your thesis. Remember, science flourishes best in a setting where cooperation is maintained between all parties. Much success in your endeavors.

Sincerely yours,

R. Max Wynn, Ph.D.
Assistant Professor Internal Medicine

Dr. Ursula Jakob
Department of Biology
The University of Michigan Ann Arbor, Michigan 48109-1048

Telephone (313)
Fax (313) 747-0884

TO: Bhavna McGowan
San Jose State University
San Jose, CA
FAX: 503 624 7424

Dear Bhavna,

Thank you for your letter. Please feel free to use the figures of our TIBS review. I am happy to hear that they are of use for you. Best luck for your thesis report.

Sincerely yours

Dr. Ursula Jakob

UPPSALA UNIVERSITET

January 27, 1996

Dear Bharna McGowan,

Forgive me for my delay in responding to your letter of January 15, 1996. I am presently on sabbatical leave in Uppsala Sweden and the letter only reached me yesterday.

I am pleased that you found my articles helpful and am happy to grant you permission to use figure 7 from the article in Science Vol 257, pp 241-245. I modified that figure and published a slightly different version in Trends in Biochemical Science Vol 20 pp 65-69. If you should prefer to use any of the figures in that review I also grant permission. I am in the process of packing my office here to return to Pullman next Friday and I found I have two reprints here which you might find useful, one of the review in TIBS and another from a book. I shall send both to you by mail.

I thank you for your interest in our work.

Regards-

Air Randall

Bhavna McGowan
18111 SW Deerbrush Ave.
Lake Oswego, OR 97035
(503) 598-0986
Jan. 15, 1996

James E. Rothman
Department of Biochemistry
Stanford University
Stanford, CA 94305

Dear Dr. Rothman,

I would like to request permission to use figure 2 found in Cell (July 4, 1986, Vol 46, p. 5-9) as an illustration in a thesis report. I am completing a MA in Chemistry at San Jose State University in San Jose, CA. My thesis report is an overview of molecular chaperones describing their role in protein folding, translocation, and degradation in the cell. Your articles were very helpful in the early phase of my thesis. The use of the figure would greatly enhance my ability to present the information on molecular chaperones.

I would appreciate a letter granting permission to be mailed to the address listed above or faxed to (503) 624-7424. Thank you very much for your help.

Sincerely yours,

Bhavna McGowan
Bhavna McGowan

*You have my
permission as
indicated by my
signature.*

James E. Rothman
2/6/96

January 23, 1996

F. Ulrich Hartl
Cellular Biochemistry and Biophysics Program
Rockefeller Research Laboratories
Sloan-Kettering Institute
1275 York Avenue - Box 519
New York, NY 10021

Bhavna McGowan
18111 SW Deerbrush Ave.
Lake Oswego, OR 97035

Dear Bhavna McGowan,

Please feel free to use the figures indicated in your letter for your thesis report.

Sincerely,

F. Ulrich Hartl
F. Ulrich Hartl

*Memorial Sloan-Kettering Cancer Center
1275 York Avenue, New York, New York 10021
NCI-designated Comprehensive Cancer Center*

Bhavna McGowan
14673 S Stitt Ct
Oregon City, OR 97045
(503) 657-8570
June 15, 1996

Richard Beckmann
Departments of Medicine and Physiology
University of California
San Francisco, CA 94143

Dear Dr. Beckmann,

I would like to request permission to use figure 5 found in Science (May 18, 1990, Vol 248, p. 850-854) as an illustration in a thesis report. I am completing a MA in Chemistry at San Jose State University in San Jose, CA. My thesis report is an overview of molecular chaperones describing their role in protein folding, translocation, and assembly in the cell. Your articles were very helpful during the research phase of my thesis. The use of the figures would greatly enhance my ability to present the information on molecular chaperones.

I would appreciate a letter granting permission to be mailed to the address listed above or faxed to (503) 657-8571. Thank you very much for your help.

Sincerely yours,

A handwritten signature in cursive script that reads "Bhavna McGowan".

Bhavna McGowan

Bhavna McGowan
14673 S Stitt Ct
Oregon City, OR 97045
(503) 657-8570
June 15, 1996

Sylvie Blond-Elguindi
Department of Biochemistry
Howard Hughes Medical Institute
University of Texas Southwestern Medical Center
Dallas, Texas 75235

Dear Dr. Blond-Elguindi,

I would like to request permission to use figure 15 found in Cell as an illustration in a thesis report. I am completing a MA in Chemistry at San Jose State University in San Jose, CA. My thesis report is an overview of molecular chaperones describing their role in protein folding, translocation, and assembly in the cell. Your articles were very helpful during the research phase of my thesis. The use of the figures would greatly enhance my ability to present the information on molecular chaperones.

I would appreciate a letter granting permission to be mailed to the address listed above or faxed to (503) 657-8571. Thank you very much for your help.

Sincerely yours,

Bhavna McGowan
Bhavna McGowan

Bhavna McGowan
14673 S Stitt Ct
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June 15, 1996

S.J. Hultgren
Department of Molecular Microbiology
Washington University School of Medicine
Box 8230
660 S. Euclid Avenue
St. Louis, Missouri 63110

Dear Dr. Hultgren,

I would like to request permission to use figure 2 and 3 found in Infection and Immunity (Nov. 1992, p. 4445-4451) and figure 2 and Table 1 found in Science (Nov. 19, 1993, Vol 262, p.1234-1241) as illustrations in a thesis report. I am completing a MA in Chemistry at San Jose State University in San Jose, CA. My thesis report is an overview of molecular chaperones describing their role in protein folding, translocation, and assembly in the cell. Your articles were very helpful during the research phase of my thesis. The use of the figures would greatly enhance my ability to present the information on molecular chaperones.

I would appreciate a letter granting permission to be mailed to the address listed above or faxed to (503) 657-8571. Thank you very much for your help.

Sincerely yours,

Bhavna McGowan