HEAT SHOCK (STRESS) PROTEINS IN BIOLOGY AND MEDICINE

Organizers: William J. Welch and Larry Hightower

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Plenary Sessions *Page*

Poster Sessions

Structure/Function in Eukaryotes

B6-001 STRESS PROTEINS FUNCTIONING AS MOLECULAR CHAPERONES, William J. Welch, C. Randell Brown, Ly Q. Hong-Brown,Daryl Eggers, Ian Fitch, William J. Hansen, Panda Hershey, Robert Martin, George Minowada, Hirsohi Nagata, Depts. of Medicine and Physiology, University of Calif., San Francisco.

Members of both the hsp 70 and hsp 60 proteins families of stress proteins function as molecular chaperones within different intracellular compartments of eukaryotic cells. Using both animal cells, as well as in vitro translation systems, we continue to examine the role of these chaperones in facilitating the early stages of proteins synthesis and maturation. In order to define those components which might be interacting with nascent polypeptides we prepared an antibody to the antibiotic puromycin. Addition of puromycin to translating polysomes results in the covalent attachment of puromycin to the nascent chain and an accompanying release of the nascent chains from the translation machinery. Released nascent chains can then be captured via the anti-puromycin antibody. Our results characterizing the properties of the puromycin released chains along with the identification and characterization of relevant nascent chain binding proteins will be presented. We have also been examining the possible role of molecular chaperones as it relates to the assembly and/or disassembly of mature proteins in the cell. In this regard we have found that a portion of both the hsp 73 and TCP-1 cytosolic chaperones are present within centrosomes, the organelle involved in establishing the microtubule based spindles needed for proper chromosome segregation during meiosis/mitosis. At least for the TCP-1 component we will present data implicating a role for this molecular chaperone in facilitating the growth of the microtubules off the centrosome. In addition the adverse consequences of heat shock treatment on microtubule growth processes will be discussed. Finally, time permitting, we will present some new information regarding the possible role of the low mw hsp (hsp 28) as it relates to its interaction with cytoskeletal components.

Structure/Function in Prokaryotes

B6-002 THE ADVENTURES OF THE THREE MUSKETEERS OR THE BIOLOGY OF THE DNAK CHAPERONE MACHINE, Costa Georgopoulos[1], Dan Wall[1], Bin Wu[1], Krzysztof Liberek[1,2], Olivier Deloche[1], Debbie Ang[1], Alicja Wawrzynow[2], Bogdan Banecki[2], and Maciej Zylicz[2], [1]Département de Biochimie Médicale, Centre Médical Universitaire, 1 rue Michel-Servet, 1211 Genève 4, Switzerland, [2]Division of Biophysics, Department of Molecular Biology, University of Gdansk, 24 Kladki, Gdansk 80-822, Poland.

Over the past few years our laboratories have been investigating the biological properties of the so-called "DnaK chaperone machine". The DnaK chaperone machine of *Escherichia coli* is composed of three members (or musketeers), DnaK, DnaJ and GrpE. Their corresponding genes were originally discovered because mutations in them blocked bacteriophage λ DNA replication. Subsequent studies established that the DnaK chaperone machine participates in important intracellular processes, such as prevention of polypeptide aggregation and disaggregation of certain protein complexes or aggregates. Two of these musketeers, DnaK and DnaJ, have been shown to be bona fide chaperone proteins, capable of promiscuously binding to certain unfolded forms of many polypeptides.

The 70,000-Mr DnaK polypeptide exhibits an extremely weak ATPase activity (1 ATP molecule hydrolyzed/5 min/monomer), which is accelerated 50-fold in the presence of the two other cohorts. DnaJ acts like a "GAP" protein, since it catalytically accelerates the rate of hydrolysis of DnaK-bound ATP. By deletion and point mutant analyses we demonstrated that the N-terminal, so-called "J" domain, of the DnaJ protein is responsible for the acceleration of DnaK's ATPase. The structure of the "J" domain was solved by 2D NMR analysis in collaboration with Dr. Kurt Wüthrich's laboratory. The structure contains certain interesting features including 4 α-helices, three of which interact intimately, through the formation of a hydrophobic core. GrpE protein acts like "GNRP", since it releases all DnaK-bound nucleotide, thus accelerating the rate of the ATP/ADP exchange reaction of DnaK.

The DnaK and DnaJ chaperone proteins can bind synergistically to certain polypeptide substrates, in an ATP-dependent reaction. Specifically, the presence of DnaJ enables DnaK to bind better to all of its polypeptide substrates in the presence of ATP. Surprisingly, a 108 amino-acid N-terminal DnaJ fragment, DnaJ108, that contains only the "J" and "G/F" domains, can also catalytically "activate" DnaK to bind to its polypeptide substrates in an ATP-dependent reaction. The DnaJ108 fragment itself does not bind to any polypeptide substrates tested. Further details of the DnaK/DnaJ interaction will be discussed.

Mutations in the *grpE* gene were isolated, sequenced, their corresponding mutant protein products purified, and tested for their ability to (a) oligomerize (b) physically interact with DnaK and (c) accelerate DnaK's ATPase activity. These *grpE* mutations identified functionally important and highly conserved amino acid residues, since their introduction in the Yeast GRPE homologue inactivated its functionas well.

B6-003 DO PRION PROTEINS AND HEAT SHOCK PROTEINS INTERACT? Stanley B. Prusiner[1], Jörg Tatzelt[1], Jianru Zuo[2], Richard Voellmy[2] and William J. Welch[1], [1]University of California, San Francisco, CA 94143-0518, [2]University of Miami School of Medicine, Miami, FL

The concept that prions are novel pathogens which are different from both viroids and viruses has received increasing support from many avenues of investigation over the past decade. Enriching fractions from Syrian hamster (SHa) brain for scrapie prion infectivity led to the discovery of the prion protein (PrP). Prion diseases of animals include scrapie and mad cow disease; those of humans present as inherited, sporadic and infectious neurodegenerative disorders. The inherited human prion diseases are genetically linked to mutations in the PrP gene that result in non-conservative amino acid substitutions. The fundamental event underlying scrapie infection seems to be a conformational change in the prion protein (PrP). Transgenic (Tg) mice expressing both SHa and mouse (Mo) PrP genes were used to demonstrate that the "species barrier" for scrapie prions resides in the primary structure of PrP. This concept was strengthened by the results of studies with constructed chimeric Mo/human (Hu) PrP transgenes which differ from MoPrP by 9 amino acids between residues 96 and 167. All of the Tg(MHu2M) mice developed neurologic disease ~200 days after inoculation with brain homogenates from three patients who died of Creutzfeldt-Jakob disease (CJD). Inoculation of Tg(MHu2M) mice with CJD prions produced MHu2MPrP^{Sc}; inoculation with Mo prions produced MoPrP^{Sc}. About 10% of Tg(HuPrP) mice expressing HuPrP and non-Tg mice developed neurologic disease >500 days after inoculation with CJD prions. The different susceptibilities of Tg(HuPrP) and Tg(MHu2M) mice to human prions indicate that additional species specific factors such as chaperone proteins are involved in prion replication. To investigate proteins that might feature in the conversion of the cellular prion protein (PrP^{C}) into the infectious scrapie prion protein (PrP^{Sc}) we examined the expression and cellular distribution of molecular chaperones in scrapie infected neuroblastoma (ScN2a) cells. In contrast to uninfected cells, heat shocked ScN2a cells did not respond with increased expression of Hsp 72 and Hsp 28 as determined by immunoblotting. Transcription of the Hsp 72 gene in ScN2a cells was also unchanged by heat shock. In contrast, the induction of Grp 94 and Grp 78 (BIP) after exposure to azetidine was not impaired, thereby arguing for a selective suppression of Hsp 72 and Hsp 28. Upon heat shock in the uninfected cells the constitutively expressed Hsp 73 was translocated from the cytoplasm into the nucleus and nucleolus. Although the levels of Hsp 73 were unchanged in ScN2a cells, Hsp 73 failed to redistribute after heat shock. Furthermore, in ScN2a cells Hsp 73 was found to be associated with Triton X-100 insoluble structures. The alterations in the expression and cellular distribution of specific heat shock proteins in scrapie infected neuroblastoma cells may reflect cellular consequences due to the formation of PrP^{Sc} and contribute to the pathogenesis of prion diseases. How alterations in the stress response lead to vacuolation of neurons and attendant astrocytic gliosis which are the morphologic hallmarks of scrapie remains to be established.

B6-004 PROTEIN REMODELING CATALYZED BY MOLECULAR CHAPERONES: ACTIVATION OF P1 RepA DNA BINDING BY ClpA AND BY THE COMBINATION OF DnaK, DnaJ AND GrpE, Sue Wickner[1], Michael Maurizi[1], Susan Gottesman[1], Keith McKenney[2] and Dorota Skowyra[1], [1]National Cancer Institute, National Institutes of Health, Bethesda, MD 20892 and [2]National Institute of Standards and Technology, Gaithersburg, MD 20899.

We have used the P1 RepA activation system to study the mechanisms of action of molecular chaperones. RepA activation results in the conversion of dimers to monomers. The monomer form of RepA binds with high affinity to specific DNA sequences. We previously discovered that the Hsp70 chaperone system of *E. coli*, consisting of DnaK and DnaJ, mediates ATP-dependent RepA activation. DnaJ dimers form tetrameric complexes with RepA and the DnaJ-RepA complexes target RepA for DnaK action. *In vivo* GrpE acts with DnaJ and DnaK in RepA activation. We have found that it is required for RepA activation *in vitro* as well, when the free Mg^{2+} concentration is in the μM range. It facilitates the utilization of Mg^{2+} for RepA activation, specifically at the step of ATP hydrolysis by DnaK. We tested other proteins in the RepA activation system and found that *E. coli* ClpA functions like the combination of DnaK and DnaJ, demonstrating that ClpA is a new molecular chaperone. Clp proteins are a large family of highly conserved proteins. They are present in all organisms tested and contain binding sites for ATP and polypeptides. ClpA is an ATPase with no intrinsic proteolytic activity but associates with ClpP forming an ATP-dependent protease. We found that ClpAP degrades RepA. By characterizing the intermediates and products of the ClpA-dependent activation and degradation of RepA, we have demonstrated the mechanisms of these reactions. Our results suggest that the protein unfolding function of molecular chaperones is essential for both activation and degradation of proteins.

Stress Proteins Functioning As Molecular Chaperones I

B6-005 **MOLECULAR MECHANISM OF HSP70 CHAPERONES: BOUND SUBSTRATE CONFORMATION AND REGULATION BY NUCLEOTIDES AND COFACTORS,** Anthony L. Fink, Daniel R. Palleros, Katherine L. Reid, Micheline Markey and Li Shi, Department of Chemistry and Biochemistry, University of California, Santa Cruz, CA 95064.

We propose a model for the hsp70 cycle of interaction with unfolded proteins in which nucleotides, as well as protein cofactors such as DnaJ and GrpE, play a major role in regulating the cycle, which is critically dependent on the presence of K^+. The kinetics of unfolded substrate protein binding and dissociation were measured under various conditions, and are quite sensitive to the concentrations of nucleotide and cofactor. Dissociation of substrate protein occurs prior to ATP hydrolysis, as shown by comparison of rate of dissociation versus rate of ATP hydrolysis. Incubation of hsp70 with unfolded NCA staphylococcal nuclease, which is thermally unstable (it is native at 20°C and unfolded at 37°C), results in formation of a complex at 37°C. No complex is formed at 10°C. Comparison of the circular dichroism and fluorescence properties of the complex (cooled to 10°C) with those of free DnaK and folded substrate indicates that the bound substrate protein is predominantly unfolded in the complex at 10°C. The addition of ATP dissociated the complex releasing the substrate protein also in an unfolded conformation.

B6-006 PATHWAYS OF CHAPERONE-MEDIATED PROTEIN FOLDING,
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Protein folding in the cell depends on helper proteins, so-called molecular chaperones and folding catalysts. Of specific interest are the functions of members of the hsp70 and hsp60 (chaperonin) families of molecular chaperones in *de novo* protein folding. Hsp70 and hsp60 act sequentially in this process, determined by their differential specificity for structural elements exposed by a polypeptide chain at different stages of the folding pathway. Hsp70 recognizes extended polypeptide segments emerging from ribosomes. The fully synthesized polypeptides, adopting the conformation of partially folded intermediates, are then transferred to the central cavity of the hsp60 double-toroid for folding to the native state. In the eukaryotic cytosol, polypeptide transfer from Hsp70 to the chaperonin TRiC can ocuur co-translationally.

Using the isolated heat-shock proteins of *E. coli*, we have reconstituted the process of chaperone-mediated protein folding *in vitro*. The function of the Hsp60 GroEL is known to be dependent on the single-ring co-factor GroES. Under most conditions GroES binds to one end of the GroEL cylinder forming an asymmetrical GroEL:GroES complex. Stimulated by the recent obeservation of symmetrical GroES:GroEL:GroES complexes, we have reinvestigated the stoichiometry of GroEL and GroES in the functional chaperonin complex. Our kinetic analysis indicated that formation of symmetrical GroES:GroEL:GroES particles is not required for the normal function of the chaperonin reaction cycle.Moreover, biochemical binding assays and structural analysis by electron micoscopy (collaboration with W. Baumeister, Munich, and A. Engel, Basel) revealed that the symmetrical complex is only produced at high concentrations of Mg2+ and at elevated pH. We conclude that, in agreement with previous observations, the asymmetrical GroEL:GroES complex is sufficient to mediate the process of chaperonin-mediated protein folding.

B6-007 QUALITY CONTROL IN THE ENDOPLASMIC RETICULUM. A. Helenius, I. Braakman, U. Tatu, W. Chen, J. Helenius, J. Peterson, D. Hebert, J. Simons, and C. Hammond, Dept. of Cell Biology, Yale School of Medicine, New Haven, CT 06510.

Our studies in live cells and in isolated ER vesicles have focused on the conformational maturation process that glycoproteins undergo in the ER lumen and the interactions that they have with unique folding factors present in the ER. The rate and efficiency of folding and oligomeric assembly determines how efficiently, how fast, and in which form they are secreted or expressed on the plasma membrane. Thus, the ER possesses a system to distinguish fully mature conformations from misfolded and incomplete ones. It is a sorting system that is very selective, which is why it often proves to be a true stumbling block for the mass production of heterologous glycoproteins and for the health of patients with hereditary protein defects.

Calnexin, a membrane-bound ER chaperone, is one of the key factors in this quality control. It binds only to glycoproteins, and unless they remain permanently misfolded or unassembled, binding is transient. Using viral and cellular proteins, we have demonstrated that binding occurs to the N-linked oligosaccharides when these have reached a partially trimmed $Glc_1,Man_{9\text{-}7}GlcNAc_2$ form. Calnexin is part of the sophisticated machinery shown schematically in the figure. The system includes: i) **calnexin** as a lectin-like retention molecule, ii) **glucosidases I and II** as signal modifiers responsible for removing glucoses from the original core oligosaccharide that is added cotranslationally to glycoproteins, and iii) **UDP-glucose:glycoprotein glucosyltransferase** that serves as a folding sensor.

BiP/GRP78, a hsp70 homologue, is another protein that we have found to have a direct role in glycoprotein folding, maturation and quality control. By following the folding of carboxypeptidase Y, a vacuolar enzyme, in temperature sensitive BiP mutants of *S. Cerevisiae*, we were able to show that BiP/GRP78, indeed, plays a crucial role in the folding process, and not only in the translocation of polypeptides in to the ER. The mutant studies clearly show, however, that chaperones are redundant in the ER, i.e. proteins can make use of more than one chaperone system.

B6-008 CHAPERONIN CYCLES, Paul V. Viitanen, Matthew J. Todd, and George H. Lorimer, Central Research and Development Department, Molecular Biology Division, E. I. DuPont de Nemours and Co., Experimental Station, Wilmington, DE 19880-0402.

The bulk of our mechanistic knowledge on the chaperonins is derived from *in vitro* studies with the purified groEL and groES proteins of *E. coli*. GroEL binds nonnative proteins with high affinity, a process that suppresses both aggregation and "on path" folding events. Under conditions suboptimal for spontaneous folding, the release of groEL-bound target proteins, in a state committed to the native state, requires ATP hydrolysis and groES. For Rubisco, multiple iterative cycles of complete release and rebinding are also necessary for successful refolding. The critical role of groES in this process is to coordinate the ATPase activity of the individual subunits within the two groEL toroids, and hence systematically alternate their relative affinities for nonnative proteins. This is accomplished through the formation of an asymmetric, bullet-shaped complex ($groEL_7 \cdot groEL_7 \cdot ADP_7 \cdot groES_7$), in which only the unoccupied groEL toroid is free to hydrolyze ATP. In contrast to groEL alone, ATP hydrolysis by the asymmetric complex is "quantized". Single turnover experiments reveal that the system is extremely dynamic: one round of ATP hydrolysis by the asymmetric complex leads to the complete release of the groES and ADP that were tightly bound to the other end of the groEL cylinder. Concomitantly, the asymmetric complex reforms-perhaps via transient, symmetrical, football-shaped intermediates (e.g. $groES_7 \cdot ATP_7 \cdot groEL_7 \cdot groEL_7 \cdot ADP_7 \cdot groES_7$). Our results suggest that the unfolded target protein plays only a passive role in the chaperonin catalytic cycle.

Stress Proteins Functioning As Molecular Chaperones II

B6-009 FUNCTIONAL DIFFERENCES AMONG CHAPERONINS, Guoling Tian, Irina Vainberg, William Tap, Nicholas J. Cowan, New York University Medical Center, New York NY 10016.

When urea-denatured actin is presented to eukaryotic cytosolic chaperonin in the presence of Mg-ATP, the target protein forms a binary complex with the chaperonin and is converted to the native state with an efficiency of about 50%. Presentation of the same labelled target protein to GroEL also results in the generation of binary complex, but no native actin is produced upon incubation with nucleotide, whether or not the cochaperonin GroES is included in the reaction. Nevertheless, when cytosolic chaperonin is incubated with Mg-ATP and actin/GroEL binary complex as the only potential source of target protein, an actin/cytoplasmic chaperonin binary complex is formed which discharges native actin with about the same efficiency as reactions in which the target protein is presented directly to cytoplasmic chaperonin from urea. Similar results are obtained in parallel reactions in which GroES is included, except that, as might be expected, there is a more rapid discharge of target protein from the GroEL binary complex. From these data, we conclude that 1) Unfolded actin is capable of forming a binary complex with GroEL; 2) Incubation of the GroEL/actin binary complex with nucleotide does not lead to the production of native actin, with or without the addition of GroES; 3) Incubation of actin/GroEL binary complex with nucleotide leads to the discharge of the target protein in a configuration that, while incapable of commitment to a productive folding pathway, is nonetheless capable of forming a binary complex with cytosolic chaperonin; 4) This binary complex is competent for the production of native actin upon incubation in the presence of Mg-ATP. These observations are consistent with a concept of chaperonin-mediated protein folding by GroEL in which target proteins are discharged upon incubation with nucleotide in an unfolded conformation. However, it is clear that a profound difference exists between the nature of unfolded intermediates discharged by GroEL (which, in the case of actin, are unable to partition to the native state) and those discharged by cytosolic chaperonin.

B6-010 STRUCTURE AND FUNCTION STUDIES OF THE CHAPERONIN GROEL, Arthur L. Horwich, David C. Boisvert, Kerstin Braig, Wayne A. Fenton, Rashmi Hegde, Corinne Hohl, Oleg Kovalenko, Zbyszek Otwinowski, Jonathan S. Weissman, and Paul B. Sigler. Howard Hughes Medical Institute and Yale University School of Medicine, New Haven, CT.

The chaperonin GroEL facilitates folding of a large number of unrelated proteins through cycles of binding and ATP-mediated release of non-native forms - with each cycle of release, polypeptide partitions between folding to the native state or rebinding to another molecule of chaperonin. Our recent determination of the crystal structure of GroEL at 2.8 Å and assignment of function in polypeptide binding, GroES binding, ATP hydrolysis, and polypeptide release, to specific regions via examination of mutants, has provided a framework in which to pursue a detailed understanding of the mechanics of polypeptide binding and release. Important issues to resolve concern the stoichiometry, topology, and order of polypeptide and GroES binding to GroEL during productive folding reactions. To examine these issues we are taking both structural and biochemical approaches. Concerning the latter, we have carried out a series of crosslinking and order-of-addition experiments using wild-type GroEL, and have prepared a variety of mutant single ring forms of GroEL whose functions have been examined both in vivo and in vitro. The question of whether and how GroEL functions as a one or two ring engine will be addressed.

Regulation of Stress Protein Expression in Eukaryotes

B6-011 REGULATION OF THE TRANSCRIPTIONAL ACTIVATION DOMAINS OF HUMAN HSF1. Marie Green, Elizabeth Newton, and Robert E. Kingston. Department of Molecular Biology. Massachusetts General Hospital. Boston, Massachusetts 02114

Our goal is to understand the mechanism that activates human HSF1 in response to heat shock. We have transfected mammalian cells with fusion proteins containing the carboxy-terminal portions of human HSF1 fused to the DNA binding/oligomerization domain of GAL4. We deleted the amino terminal 200 amino acids of HSF, consisting of the DNA binding and trimerization domains to avoid potential heterotrimerization with the endogenous factor. We identified two separable and potent activation domains in the C-terminal half of the protein and a separate domain that regulated the activation domains in a heat inducible manner. We used deletion mutagenesis to attempt to define the minimal number of amino acids necessary for activation by the activation domains. Activation domain I can be reduced to a small, conserved 20 amino acid region. Point mutagenesis revealed the importance of hydrophobic and acidic residues in activation by this domain. Activation domain II can be reduced to approximately 40 amino acids, although it does not appear to contain as defined borders as activation domain I. Both of these activation domains (including the 20 amino acid segment of activation domain I) are regulated by a central domain that is located between the trimerization domain and activation domain I and is conserved in human, chicken, and mouse HSF1s, but not in HSF2 or HSF3. Fusion proteins containing HSF1 activation domains and the regulatory domain are repressed at control temperature and heat inducible following heat shock. Deletion mutagenesis revealed that this domain can be reduced to 90 amino acids and point mutagenesis has identified specific amino acids necessary for regulation. In addition, we have found through the use of chimeras that the conserved sequence in chicken HSF1 shows conserved function; it both represses the human activation domains and renders them heat inducible We have identified, therefore, a regulatory domain in human HSF1 that is conserved among the heat shock factors that respond primarily to heat and that can regulate the HSF1 activation domains in a heat-inducible manner. Thus, the regulation of HSF1 transcriptional activation uses a different mechanism than the regulation of DNA binding.

B6-012 STRESS REGULATION OF HUMAN HEAT SHOCK TRANSCRIPTION FACTOR HHSF1, Richard Voellmy[1], Jianru Zuo[1], Wenle Xia[1], Duri Rungger[2], Ruben Baler[3], Gerhard Dahl[1] and Jianying Zou[1], [1]University of Miami, Miami, FL 33101, [2]University of Geneva, Switzerland and [3]National Institutes of Health, Washington.

Human heat shock transcription factor 1 (hHSF1) is responsible for the stress regulation of heat shock protein (hsp) genes in human cells. hHSF1 is a 529-amino acid polypeptide containing three 4-3 hydrophobic repeats referred to as leucine zipper regions 1, 2 and 3 (LZ1-3). In extract from unstressed cells hHSF1 is found either as a monomer (the predominant fraction) or as a hHSF1-hsp70 heterodimer (a smaller, somewhat variable fraction). Neither monomers nor heterodimers have DNA-binding ability. Heat stress induces trimerization of hHSF1. Concomitantly, hHSF1 acquires specific DNA-binding ability, is hyperphosphorylated and appears in the nuclear fraction. hHSF1 expressed in microinjected Xenopus oocytes is subject to the same kind of heat stress regulation of trimerization, DNA-binding ability and hyperphosphorylation except that regulatory changes occur at 33-37°C rather than at 39-43°C. A systematic analysis of hHSF1 deletion and substitution mutants using the Xenopus expression system demonstrated that LZ1 is minimally required for factor trimerization as well as uncovered three discrete regions that play an essential role in preventing hHSF1 trimerization and acquisition of DNA-binding ability at non-stress temperatures. These regions correspond to the beginning of LZ1, to LZ2 and to LZ3. These findings that were confirmed in experiments with human cells suggest that the core of monomeric hHSF1 may be a triple-stranded coiled-coil composed of regions from all three LZs. This structure may be stabilized by interactions with hsp70 or other proteins. In human cells made to overexpress hHSF1, a predominant fraction of hHSF1 is found in the trimeric form and has DNA-binding ability, suggesting titration of a cellular factor that interacts with and stabilizes the hHSF1 monomer. Surprisingly, this trimeric hHSF1 is incapable of transactivating hsp genes but can be activated by heat stress treatment. We conclude that the transcription-enhancing ability of hHSF1 is regulated by a mechanism that is separate from the one controling DNA binding and operates after the factor has undergone trimerization and has acquired DNA-binding ability. Mutational analyses defined several regions participating in this control that are well separated from the transcription activation domain. A potential role for protein kinases in this regulation will be discussed.

B6-013 TRANSCRIPTIONAL REGULATION OF HEAT SHOCK GENES, Carl Wu, Michael Fritsch, Nobuko Hosokawa, Paul Jedlicka, Soon-Jong Kim, Andras Orosz, Jan Wisniewski, Laboratory of Biochemistry, National Cancer Institute, Bethesda, MD 20892.

Organisms respond to heat stress and to a variety of chemical and physiological agents by rapidly increasing the synthesis of the heat shock proteins, which act as molecular chaperones to alleviate the deleterious effects of cellular stress. The transcriptional induction of heat shock genes in eukaryotes is mediated by the transcription factor Heat Shock Factor (HSF). Considerable progress in our understanding of the structure of HSF and how its activity is regulated by heat shock has been achieved since the recent cloning of *HSF* genes. All HSF proteins have a conserved DNA-binding and trimerization domain. The structure of the DNA binding domain has been solved by multi-dimensional NMR spectroscopy, and found to be similar to the helix-turn-helix family of DNA binding motifs. Analytical ultracentrifugation studies indicate that the DNA binding domain of HSF binds as a 1:1 complex to a single NGAAN repeat of the Heat Shock Element with low affinity. *Drosophila* HSF and human HSF1 both possess trimerization and transactivator domains with intrinsic activity, which are suppressed under normal conditions involving separate regions of HSF. Activation of HSF during heat shock occurs via the relief of negative regulation. Unlike the several copies of vertebrate and plant HSF genes, the *Drosophila HSF* gene is unique. We have recently isolated mutants of the *Drosophila* HSF gene; the loss of HSF function in *Drosophila* leads to lethality under normal growth conditions. Thus, the *Drosophila* HSF protein, like *S. cerevisiae* HSF, appears to perform an essential function in the absence of heat stress.

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Stress Proteins in Medicine I

B6-014 THE HEAT SHOCK RESPONSE AND MYOCARDIAL PROTECTION

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Following hyperthermic treatment (42°C for 15 min) of rats or rabbits, the most obvious change is the synthesis of HSP71, the highly inducible member of the HSP70 family of heat shock proteins. Transcripts for HSP71 are undetectable in control hearts, but are evident immediately after the hyperthermic treatment at 0 hr of recovery, and at 1.5 and 3 hr of recovery. Peak accumulation is at 1.5 hr recovery and the transcripts decay to control levels at 6 hr recovery. While there is little HSP71 in control hearts, it is a major cellular constituent after hyperthermic treatment. Associated with the increase in HSP71 after the hyperthermic treatment, is the acquisition of improved post-ischemic cardiac recovery. Following 30 min of ischemia, isolated and perfused hearts, from rats 24 and 48 hr after hyperthermic treatment, have stronger ventricular contractile recovery, decreased release of creatine kinase, and increased activity of the anti-oxidative enzyme catalase. At 24 hr (but not 48 hr) post-heat shock in rabbits, cardiac infarct size is decreased, after a 30 min occlusion of the left anterior decending artery and 3 hr of reflow. Reduction of infarct size was not present when the ischemic period was extended to 45 min suggesting that induction of the heat shock response extends the time (during ischemia) before irreversible injury occurs and that this protection is transient, decaying before 48 hr post-hyperthermia in rabbits and after 48 hr post-hyperthermia in rats.

Specific and persistant over-expression of the inducible HSP70 is also associated with myocardial protection. Transgenic mice, containing the human HSP70 gene under the control of the ß-actin promoter have a high level of the inducible HSP70 in several organs including the heart. Following 30 min of low-flow ischemia, reperfused hearts from transgenic mice had stronger recovery of contractile force and ±dF/dt than litter mates not containing the human HSP70 gene. Control hearts had greater release of creatine kinase, indicating greater cellular injury upon reperfusion, than that released from the transgenic hearts. Whether induced by metabolic stress, such as hyperthermic treatment, or genetically engineered, high levels of the inducible HSP70 (HSP71) appears to have a role in protection of the myocardium from ischemic injury.

B6-015 HEAT SHOCK PROTEINS AND ISCHEMIC INJURY, Wolfgang H. Dillmann, Michael S. Marber, Frank Giordano, and Ruben Mestril, Department of Medicine, University of California, San Diego, CA 92103.

Protective effects of heat shock proteins (HSPs) against ischemic myocardial damage have been postulated, however, have until recently not been directly demonstrated. In order to determine if the increased expression of specific isoforms of HSPs in cardiac myocytic cells would lead to a protection against ischemic damage, we pursued the following studies. A cardiac myogenic cell line, H9c2 cells, which are derived from the cardiac ventricle of a 13 day old rat embryo were used to produce stable lines with a neomycin selection protocol. A clonal stable line showing marked increased expression of the human inducible HSP70 (HSP70i) transgene was obtained. This stably transfected cell line was found to be significantly more resistant to an ischemic-like stress than control myogenic cells only expressing the selectable marker. These findings implicated for the first time that inducible HSP70 protein is playing a role in protecting cardiac cells against ischemic injury. In order to determine if a similar protective effect could be elicited under *in vivo* conditions, we produced lines of transgenic mice in which a CMV enhancer *β*-actin promoter drove expression of the rat inducible HSP. In heterozygous animals, significant expression of the HSP70 transgene occurred in the heart, skeletal muscle, and brain. Hearts were harvested from transgene positive and negative mice. Hearts were submitted to Langendorf perfusion and subjected to 20 mins of ischemia and up to 120 mins of reflow while coronary recovery and creatine kinase efflux was measured. Myocardial infarction was demarcate by triphenyltetrazolium. In transgene positive compared to transgene negative hearts, the zone of infarction was reduced by 40%, contractile function at 30 mins of reflow was doubled and efflux of creatine kinase was reduced by approximately 50%. These findings suggest, therefore, that increased myocardial HSP70i expression results in protection in the hearts against ischemic injury and that anti-ischemic properties of HSP70 has possible therapeutic relevance. In order to determine if increased HSP expression by a gene therapy approach would lead to cardiac protection, we constructed a replication deficient adenovirus in which the EIA and EIB region was deleted. In the adenovirus, a CMV enhancer drives expression of the rat HSP70i. This adenovirus will be used in rabbits in gene therapy-type studies in order to determine if myocardial protection can be obtained. CONCLUSION: 1) high levels of HSP70 transgene products can be expressed in heart derived myocytes without any detectable deleterious effect; 2) the protein expressed off the HSP70i transgene is not sequestered and is fully functional and protects against ischemic injury.

Stress Proteins in Medicine II

B6-016 STRESS PROTEINS AND RENAL TRANSEPITHELIAL TRANSPORT, J. Larry Renfro[1], Caroline Sussman-Turner[1], Lawrence E. Hightower[2], Mary A. Brown[2], Raghuveerender P. Upender[1], and Sonda L. Parker[1], [1]Department of Physiology and Neurobiology, [2]Department of Molecular and Cell Biology, The University of Connecticut, Storrs, CT 06269.

Integrated tissue functions may respond homeostatically to physicochemical stress. Mild heat stress sufficient to induce synthesis of several heat shock proteins (hsps) can provide subsequent protection of physiological function from the damaging effects of more severe stress. Hsps have been generally associated with the development of thermotolerance, i.e., a capacity to survive a temperature increase that might otherwise be lethal. We have examined the effects of heat and chemical stresses on tissue level function. Primary monolayer cultures of flounder renal proximal tubule epithelium mounted in Ussing chambers were used to characterize transepithelial transport. Mild thermal stress decreased the inhibitory actions of certain toxic agents on a normal active renal transepithelial transport process, sulfate secretion. Exposure to mild chemical stress, in the form of $ZnCl_2$ sufficient to induce hsps, protected this tissue function from the effects of severe thermal stress. This stress-induced "protection" actually resulted from enhancement of normal secretion and thus offset, rather than prevented, the damaging effects of more severe stress. The latter phenomenon was distinguished from another type of inducible transport which probably promotes both tissue survival and the excretion of xenobiotics. Unstressed tissues performed active net transepithelial secretion of the cytotoxin, daunomycin (DAU), but the transport rate was slow and inconsistent. Mildly heat shocked tissues (5°C elevation for 6-8 h followed by return to normal temperature) consistently secreted DAU at almost double the control rate. This response was inhibited approximately 40% if the protein synthesis inhibitor, cycloheximide, was present during the time of heat shock . DAU secretion was inhibited by verapamil, vinblastine, cyclosporin A, and to a lesser degree by the organic cation, tetraethylammonium. The transepithelial reabsorptive flux of DAU and the electrical characteristics of the tissues, including rheogenic glucose transport, were unaffected by any of the above treatments. Reaction of tissues with a monoclonal antibody to P-glycoprotein (C219) revealed the presence of this transporter on only apical microvilli. The data indicate that flounder possess an active mechanism for the renal excretion of DAU that is stimulated by mild heat shock. This mechanism is distinct from organic anion, but not organic cation, transport and has characteristics consistent with transport by an apical P-glycoprotein. Thus, a clear protection phenomenon was produced by the heat shock induction of a P-glycoprotein-like multi-drug transporter which produced net secretion of DAU by these cultured epithelial sheets.

B6-017 EXPRESSION OF HEAT SHOCK GENES IN THE NERVOUS SYSTEM, Ian R. Brown, Sheila Rush, Pat Manzerra and Jane Foster, Department of Zoology, University of Toronto, Scarborough Campus, West Hill, Ontario, Canada M1C 1A4.

Our laboratory investigates the expression of constitutive and hyperthermia-inducible members of the hsp70 multigene family in the nervous system of the New Zealand white rabbit (1). Recently we have employed non-radioactive in situ hybridization using digoxigenin (DIG)-UTP labelled riboprobes and antibody detection to identify neural cell types expressing hsc70 and hsp70 mRNA. This DIG procedure is rapid and offers improved signal localization compared to radioactive methods. In 1 hr hyperthermic animals, hsp70 mRNA was detected at the cytoplasmic cap areas of individual oligodendrocytes and also in microglia and astrocytes whereas adjacent neuronal cell types showed no detectable signal. Conversely, hsc70 mRNA is highly localized to the cytoplasm of individual neurons. High constitutive levels of hsc70 in certain neurons may dampen hsp70 induction after hyperthermia in these cell populations. Two dimensional Western blotting facilitated the resolution of hsc70 protein from hsp70 isoforms in body tissues of the adult rabbit. Tissue-specific differences in the magnitude of hsp70 induction were apparent. Induction of hsp70 was greatest in kidney, liver and heart compared to regions of the nervous system such as cerebellum and retina. Interestingly, these neural regions showed abundant levels of hsc70 protein whereas lower hsc70 levels were detected in the non-neural tissues. Western blotting also revealed the presence of hsp70 isoforms in unstressed (control) rabbit brain and lower levels in non-neural tissue. Whole cell extracts from various brain regions of control and hyperthermic animals were prepared for gel shift assays. Activation of heat shock factor 1 (HSF1) was apparent after whole body hyperthermia with cerebellum and retina showing the greatest signal. Supershifts using an HSF1 antibody indicated that the results which were obtained in the gel shift assays reflected activation of HSF1. Western blotting revealed that the cerebellum was the brain region which exhibited the highest levels of HSF1. An increase in the apparent molecular weight of HSF1 was observed after hyperthermia, consistent with stress-induced phosphorylation of the factor.

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B6-018 HSP27 GENE EXPRESSION IN HUMAN BREAST TUMOR CELLS, Suzanne A. W. Fuqua[1], Eileen Hickey[2], Lee Weber[2], Pierre Lemieux[1], Craig Allred[1], Dan Ciocca[3], and Steffi Oesterreich[1], [1]University of Texas Heath Science Center, San Antonio, TX 78284, [2]University of Nevada, Reno, NV. 89557, [3]Laboratorio de Reproducción, Mendoza, Argentina.

Breast cancer cells express high levels of several hsp's that may serve to augment tumor aggressiveness. Hsp27 is found at high levels in a large percentage of human breast tumors. We and others have shown that elevated hsp27 expression correlates with steroid receptors, which are known predictors of disease-free survival and response to endocrine therapy in breast cancer patients. We thus performed an immunohistochemical analysis of hsp27 levels in 788 node-negative breast cancer patients which demonstrated that hsp27 was prognostic of disease-free survival, but not overall survival, and only in untreated, estrogen receptor-positive patients. These results suggest that analysis of hsp27 expression in treated patients may be complicated by the fact that certain chemotherapeutic agents may actually modulate hsp27 levels, and that hsp27 itself may be involved in drug resistance.

Landry, et al. originally reported that hsp27 overexpression in CHO cells resulted in an atypical multidrug-resistant phenotype. Our first evidence that hsp's may be involved in clinical resistance came from an observation that heat shock treatment of human breast cancer cell lines increased their resistance to doxorubicin killing. However, these cells were not cross-resistant to other commonly used chemotherapeutic agents. We have used MDA-MB-231 breast cancer cells which normally express low levels of hsp27 and have transfected them with the hsp27 gene, resulting in an increase in resistance to doxorubicin. Conversely, MCF-7 breast cancer cells which express high constitutive levels of hsp27 were transfected with hsp27 in the antisense orientation, and this was found to increase drug sensitivity. We have taken two approaches to modulate hsp27 expression for therapeutic benefit. First, we have examined the regulatory sequences which control hsp27 transcription. We hope to identify either regulatory transcription factors, or specific promoter regions which could be clinically targeted to interfere with hsp27 expression. Sequencing 1.1 kb of the hsp27 promoter revealed multiple consensus TATA, overlapping Sp1/AP2 sites, and CAAT boxes, in addition to the known heat shock response element. Basal hsp27 transcription appears to be driven mainly by elements within the most proximal 210 bp promoter region. The region surrounding the distal TATA box appears to bind a novel protein in gel-retardation analyses which is not TATA-binding protein. We have also identified a negative regulatory element in the hsp27 promoter. This promoter region is able to down-regulate transcription in heterologous promoter systems, such as an SV40 and an hsp70 promoter-driven construct. Our second approach to down-regulate hsp27 expression is to use different pharmacological modulators of hsp expression, such as the flavones. We have determined that certain flavones inhibit doxorubicin resistance associated with hsp induction by mechanisms independent of flavone's known effect on heat shock transcription factors. In conclusion, we have targeted hsp27 for therapeutic intervention of clinical drug resistance in breast cancer patients.

B6-019 REGULATION OF HEAT SHOCK FACTOR ACTIVITY BY ANTI-INFLAMMATORY DRUGS : BETTER LIFE THROUGH CHEMISTRY, Richard I. Morimoto, Jie Chen, Jose Cotto, Donald Jurivich, Michael Kline, Paul Kroeger, Betty Lee, Sanjeev Satyal, and Yanhong Shi. Dept. of Biochem., Molec. and Cell Biol. Northwestern University, Evanston, Il. 60208.

The heat shock response is a highly conserved and essential response exhibited by all organisms to a wide range of environmental and physiological conditions. Exposure to heat shock, oxidative stress, heavy metals, various toxic chemicals, and anti-inflammatory drugs results in the activation of heat shock factor (HSF), the initial step in the inducible transcription of heat shock genes. Vertebrate cells ubiquitously express a family of HSF genes, however for each HSF, DNA binding activity is negatively regulated. HSF1, the predominant heat shock and stress-responsive factor responds to stress by the reversible transition from a monomer in the control cells to a trimer in the DNA binding state. The process by which HSF1 detects and involves multiple steps including oligomerization, acquisition of DNA binding activity, translocation, and phosphorylation. Domains of HSF1 required for intramolecular negative regulation, intermolecular oligomerization, transcriptional activation, and stress-responsiveness have been identified. Trimerization and acquisition of DNA binding can occur without inducible phosphorylation which suggests that phosphorylation of HSF1 may have a role in modulation or attenuation of the transcriptional response. During attenuation of the heat shock response, HSF1 associates with specific heat shock proteins providing a possible mechanism by which HSF1 activity is autoregulated during the recovery from stress. An important aspect of the heat shock response has been revealed by the numbers and diversity of disease states including ischemia, oxidant injury, cardiac hypertrophy, neoplasia, fever, infection, and inflammation in which a role for heat shock proteins has been implicated. We have found that non-steroidal anti-inflammatory drugs (salicylate, diflusinal, indomethacin, ibuprofen) and the fatty acid arachidonate regulate HSF1 activity at multiple steps. Pretreatment with indomethacin, for example, acts in synergy with elevated temperature to alter the profile of HSF1 activation primarily by reducing the temperature threshold of HSF1 activation to lower temperatures which by themselves do not lead to activation of a heat shock response. Treatment with anti-inflammatory drugs leads to the protection of cells against cytotoxic conditions thus revealing that certain pharmacologically active drugs may have protective effects through the activation of genes encoding molecular chaperones.

Stress Proteins, Role in Immunology

B6-020 IMMUNE RESPONSES TO THE MYCOBACTERIAL 65KD HEAT-SHOCK PROTEIN; A ROLE IN ANTITUMOR AND ANTIBACTERIAL IMMUNITY, Douglas B. Lowrie, Katalin V. Lukacs, Celio Silva, Ricardo Tascon and M. Joseph Colston, The National Institute for Medical Research, The Ridgeway, Mill Hill, London, U.K.

Heat shock proteins are highly immunogenic molecules. They are commonly recognised antigens following infection or immunisation with a range of different bacteria or parasites. This high degree of immunogenicity has been attributed to their high degree of conservation, resulting in constant priming of the immune system. In addition to their role as antigens during infection, they have been implicated in a range of autoimmune and immunopathological conditions; in such situations, high levels of expression of 'self' heat shock proteins are frequently found at the site of tissue damage. These findings suggest that either the heat shock proteins themselves could be the target of autoimmune recognition or that their presence at high levels could be enhancing the immune recognition of other auto antigens. In order to test the latter hypothesis, a weakly immunogenic murine tumor cell line was transfected with the mycobacterial 65kD heat shock protein gene. When mice were immunised with this hsp-expressing cell line and then challenged with the untransfected cell line, they were protected against tumor formation. These results indicate that expression of the heat shock protein increases the recognition of weak tumor-associated antigens.

We are now investigating the potential of using immunisation with hsp-expressing DNA to generate immune responses. Using plasmids which utilise either viral or murine sequences to drive expression of mycobacterial genes, we have generated immune responses to the mycobacterial 65kD heat shock protein following intramuscular injection of plasmid DNA. We find that mice which are immunised in this way are significantly protected against infection with *Mycobacterium tuberculosis*. Since immunisation with the protein itself does not generate significant protection, it appears that endogenously expressed antigen is more effective in generating protective immunity. These findings should provide new insights into the relationship between immune recognition and protective immunity.

B6-021 IMMUNE RESPONSES TO HEAT SHOCK PROTEINS OF BACTERIA AND PARASITES, Thomas M. Shinnick, Centers for Disease Control and Prevention, Atlanta, GA 30333.

Stress response proteins play an important role in the humoral and cellular immune responses to many bacterial and parasitic pathogens including the etiologic agents of tuberculosis, leprosy, Q fever, malaria, filariasis, and schistosomiasis. Immunoreactive protein antigens have been identified that have homology with members of five families of heat shock proteins: the hsp90, hsp70, hsp60, low molecular weight, and hsp10 families. The heat shock proteins of the pathogenic microbes display 50-60% amino acid sequence identity with the sequences of the homologous human proteins. The antibodies and T cells elicited during an infection are directed against nonconserved as well as conserved sequences of the heat shock proteins. In addition, certain heat shock proteins also contain immunoreactive regions that cross-react with other host proteins, such as MHC molecules and cartilage proteins. The immune response to such shared sequences may have important consequences for the host with respect to the recognition of self and non-self and may play an important role in the pathogenicity and autoimmune consequences of infections.

B6-022 BACTERIAL HSP60 INDUCES SELF-HSP60 AUTOREACTIVE T CELLS WITH DISEASE SUPPRESSIVE POTENTIAL IN AUTOIMMUNITY, Willem van Eden, Stephen M. Anderton, Ruurd van der Zee, Berent A.B.J. Prakken, Ellie A. Toebes, Alida Noordzij and Ger T. Rijkers, Utrecht Institute for Infection and Immunity, Utrecht University, Yalelaan 1, 3584 CL Utrecht, The Netherlands

The relationships between bacterial hsp's and autoimmunity were first uncovered by the observation in mycobacteria induced arthritis (adjuvant arthritis) that transfer of a T cell clone responsive to mycobacterial hsp60 caused disease in naive recipient rats. However, subsequent experiments have shown that immunization with mycobacterial hsp60 itself did not produce arthritis, but unexpectedly, protection against arthritis. Similar protection was found in experimental models of arthritis which did not use (myco)bacteria for induction of disease. This rather general arthritis protective potency of bacterial hsp60 seems to result from the capacity of conserved areas in the protein to activate T cells that recognize the mammalian (self)homologues expressed on (stressed?) cells at the site of inflammation. It is possible that immunological recognition of bacterial hsp's is part of a general strategy, the immune system uses for the regulatory control of the potentially harmful recognition of self-antigens. If so, bacterial hsp's could be of use for the therapeutical intervention in autoimmune diseases. The analysis of proliferative responses of primed lymphnode cells and short term hsp60-specific T cell lines revealed nine hsp60 epitopes. Differential epitope recognition following immunisation with either hsp60 (AA-protective protocol) or whole *M.Tuberculosis* (AA-inducing protocol) was observed. T cell lines were generated against eight of the identified epitopes. Cross-reactive T cell recognition of mycobacterial and rat hsp60 was limited to a single epitope, 256-265. Administration of the T cell line recognizing the 256-265 epitope clearly reduced AA severity. Lines recognizing mycobacterial hsp60-unique epitopes had no effects. Moreover, preimmunisation with synthetic peptides containing individual hsp65 epitopes showed that also the cross-reactive 256-265 epitope could induce effective protection not only against AA, but also against arthritis induced with the lipoidal amine CP20961. Therefore, in contrast to the concept that cross-reactive T cell recognition of foreign and self antigens might induce aggressive autoimmune disease, cross-reactivity between bacterial and self hsp60 may also be exploited to maintain a protective, self-reactive T cell population. The data as obtained sofar in the rat, seem to be compatible with findings made in children suffering form Juvenile Chronic Arthritis (JCA). In these patients T cell proliferative responses to hsp60 were seen in the oligoarticular (remitting) forms of the disease, and not in (non-remitting) polyarticular or systemic disease. In a longitudinal follow-up it was seen that clinical remission was preceeded by responses to human hsp60. Therefore, it is possible that also in patients with chronic arthritis, responses to self hsp60 are associated with disease suppressive T cell regulatory events.

Novel Aspects of Stress Proteins

B6-023 ROLE OF HSP27 IN THE MITOGENIC AND STRESS RESPONSE OF ACTIN MICROFILAMENT, Jacques Landry, Josée N. Lavoie, Johane Guay, Herman Lambert, François Houle and Jacques Huot, Centre de recherche en cancérologie de l'Université Laval, L'Hôtel-Dieu de Québec, Québec, Canada G1R 2J6

In cells that constitutively overexpress HSP27 following gene transfection, cell thermoresistance is positively correlated with the concentration of HSP27. HSP27 is rapidly phosphorylated during heat shock and this phosphorylation appears to be required for activating the protective function of HSP27, since overexpression of a non-phosphorylatable mutant of HSP27 fails to provide protection. HSP27 is also phosphorylated following cell exposure to oxiradical-generating agents, cytokines and mitogens suggesting that the phosphorylation-activated function of the protein that is useful during stress may also be important in more "normal" physiological conditions. By comparing the phenotype of Chinese hamster cells expressing constitutively either the wild type human HSP27 or a non-phosphorylatable mutant form of HSP27, we demonstrated that HSP27 can regulate growth factor-inducted F-actin polymerization. HSP27 enhances the microfilament responses to mitogenic stimuli, whereas mutant HSP27 exerts a dominant negative effect and inhibits this response. Protection during stress may result from a similar function at the level of the actin microfilament. In cells treated with cytochalasin D, a specific actin filament disrupting agent, wild type HSP27 accelerates the reappearance of actin filaments and increases cell survival, whereas the non-phosphorylatable HSP27 fails to induce protection. Similarly, HSP27 but not mutant HSP27 stabilizes the actin filaments during heat shock, which may explain the enhanced survival. Analyses of the signal transduction pathways responsible for HSP27 phosphorylation suggest that HSP27 may be part of a common feedback protective response mechanism activated by a variety of chemical and physical stressors. Characterization of the HSP27 kinase activity in cells stimulated by various agents identified HSP27 kinase as MAPKAP kinase-2, a kinase which in vitro can be activated by the mitogen-activated protein (MAP) kinase isoforms p42 (ERK 1) and p44 (ERK 2). However, although ERK1 and ERK2 are co-induced with MAPKAP kinase-2 by most inducers of HSP27 phosphorylation, they do not appear to be essential upstream elements in the signalling cascade leading to HSP27 phosphorylation. Instead, recent results suggest that activation of p38 MAP kinase, the mammalian homologue of the yeast HOG-1 kinase, may be more closely related to induction of HSP27 phosphorylation by growth factors or stress, suggesting that an alternative signal transduction pathway mediates phosphorylation of HSP27 and the induction of the protective response at the level of microfilaments. The phosphorylation of HSP27 in response to hyperthermia could thus be considered as part of a homeostatic feedback mechanism aimed at protecting actin fibers from the disrupting action of heat. The 10-fold increase in the concentration of the protein found in thermotolerant cells then provides a more efficient protective mechanism resulting into an enhanced microfilament thermostability and global thermoresistance. *Supported by the Medical Research Council of Canada.*

B6-024 HEAT SHOCK PROTEINS AND THE ASSEMBLY OF STEROID RECEPTOR COMPLEXES, David Toft, Jill Johnson, Edward Diehl, William Sullivan, Robert Schumacher, and Ronald Corbisier, Mayo Medical School, Rochester, MN 55905.

When in its inactive form in cytosol extracts, the avian progesterone receptor, like most steroid receptors, is complexed with hsp90. This complex does not form through a simple association but through a process that requires ATP hydrolysis and several additional proteins. Other proteins in the receptor complex are hsp70 and three peptidylprolyl isomerases or immunophilins. Two of these, FKBP52 and FKBP54, bind the immunosuppressant drug FK506 and the third, CyP-40, binds cyclosporin A. A final protein in the receptor complex is p23 which is a unique phosphoprotein. The complex of receptor with these proteins can be formed in vitro by incubating free receptor in rabbit reticulocyte lysate at 30° in the presence of ATP and Mg^{++}. Further analysis of p23 reveals it to be a ubiquitous protein which is bound to hsp90. Isolation of p23:hsp90 complexes from rabbit reticulocyte lysate shows the presence of hsp70 and the three immunophilins as well. Thus, all of the proteins in steroid receptor complexes appear to exist in a complex prior to receptor assembly. P23 complexes can be disrupted by high salt treatment and then re-assembled in reticulocyte lysate by a process that requires ATP/Mg^{++}. Unlike the assembly of receptor complexes, the assembly of p23 complexes is supported by the ATP analog AMPPNP indicating that ATP hydrolysis is not required. We propose that receptor complexes are formed through association with p23 complexes by a process requiring ATP hydrolysis.

B6-025 ROLE OF MORTALIN, A NOVEL MEMBER OF MOUSE HSP70 FAMILY IN CELLULAR MORTALITY AND IMMORTALIZATION, Renu Wadhwa, National Institute of Bioscience and Human-Technology, AIST, 1-1 Higashi, Tsukuba Science City, Ibaraki 305, Japan

Normal cells in culture have limited proliferation potential in contrast to the cancerous cells which acquire immortal phenotype *in vitro*. A genetic program implicated in these mechanisms has lead to the quest for genes that limit cellular potential on the one hand and permit transformed cells to divide indefinitely on the other. Our strategy on the search for protein markers associated with mortal and immortal phenotypes in mouse fibroblasts led to the identification of a 66-kDa cytosolic protein (mortalin) associated with mortal phenotype. Molecular cloning characterized it as a novel member of murine hsp70 family of proteins (1). Subsequently this protein was found in both normal and immortal cells but with different intracellular distribution (2). All immortal human and mouse cells, tested so far, are devoid of the uniformly distributed cytosolic form of the protein which is characteristic of normal cells (3). Senescent mouse embryonic fibroblasts (doubling time >5 days) were transiently induced to divide by the microinjection of anti-mortalin antibody (1). Overexpression of the cytosolic form ($p66^{mot\text{-}1}$) on the other hand, induced senescence in NIH 3T3 cells that normally harbor the perinuclear form ($p66^{mot\text{-}2}$) which differs from $p66^{mot\text{-}1}$ by two amino acids (4). The cytogenetic analysis has assigned these genes to chromosome 18 in mouse and 5q31.1 in human, the locus of a putative tumor suppressor gene involved in myeloid malignancies. The genetic analyses and the functional aspects of the differentially distributed forms will be discussed.

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Novel Aspects of Stress Response

B6-026 VISUALIZATION AND QUANTIFICATION OF ENVIRONMENTALLY-INDUCED STRESS IN A WHOLE MULTICELLULAR ORGANISM. E. Peter M. Candido[1], Don Jones[1] and Eve G. Stringham[2]. [1]Department of Biochemistry and Molecular Biology, University of British Columbia, Vancouver V6T 1Z3. [2]Laboratory of Physiological Chemistry, Ledeganckstraat 35, 9000 Gent, Belgium.

Caenorhabditis elegans is a small, transparent, microbivorous nematode which is very amenable to genetic manipulation and easily cultured. Its transparency and small size have allowed the complete description of its developmental cell lineage as well as definition of the wiring diagram of its nervous system. The small heat shock proteins of this organism (hsp16s) are16 kilodalton in size, and are undetectable in animals under normal growth conditions, but are synthesized in large amounts following a heat shock. Like the small heat shock proteins of other organisms, the hsp16s are evolutionarily related to mammalian α-crystallins. In order to determine the tissue specificity of hsp16 induction, fusions between the control regions of *hsp16* genes and the *lacZ* reporter gene from *E. coli* were created and inserted into the *C. elegans* genome to produce transgenic strains (1, 2). In response to a heat shock these strains produce β-galactosidase, along with normal stress proteins. The β-galactosidase is readily detected histochemically in intact animals, or by various spectrophotometric assays. The availability of these strains allows one to easily and rapidly screen chemicals for their ability to induce the stress response. As has been seen in other organisms, heavy metals are effective inducers of the stress response in *C. elegans*, cadmium ions being the most potent. Dose response curves have been obtained for Cd^{2+}, Cu^{2+}, Hg^{2+}, Pb^{2+}, Zn^{2+} and arsenite ions. Unexpectedly, histochemical staining revealed that different metal ions yield different patterns of induction. For instance, Pb^{2+} induces the reporter gene in the posterior pharynx, Cd^{2+} throughout the pharynx, and Hg^{2+} in the intestine. Stress reporter gene induction occurs below the LC50 of these substances. Other substances tested which induce the stress response include the herbicide, paraquat, and a number of insecticides and fungicides. The simplicity of the stress assay using transgenic nematode strains suggests that it can form the basis for a novel and useful sublethal bioassay for xenobiotics. Recently the assay has been adapted for soil testing, and environmental soil and water samples contaminated with various levels of heavy metals and other chemicals have been examined. Both saltwater and freshwater samples can be assayed, and the results suggest that induction of the stress response may be a good indicator of the biological availability of metal ions. The results of this stress assay are being correlated with other existing bioassays. Supported by the Science Council of British Columbia and StressGen Biotechnologies Corp.

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B6-027 HEAT SHOCK PROTEINS AND THERMOTOLERANCE IN A NATURAL VERTEBRATE, Philip J. diIorio[1], Carol E. Norris[2], Eileen Fielding[1], R. Jack Schultz[1], and Lawrence E. Hightower[2], [1]Department of Ecology and Evolutionary Biology and [2]Department of Molecular and Cell Biology, University of Connecticut, Storrs, CT 06269.

Tropical and desert species of topminnows in the genus *Poeciliopsis* are useful vertebrate models for studies of hsps and thermotolerance. The maximum temperature allowing 100% survival varied from 39-41°C among several species. When placed in a temperature gradient tank, well-fed fish most frequently select 30-32°C water. The threshold induction temperature for hsp70 varied from 32-34°C among species, temperatures which fish frequently selected. The threshold induction temperature for hsp30 was 38°C for all species tested. Interestingly, temperatures ≥38°C were rarely selected by fed fish, but starving fish frequently ventured into this range. A large amount of biochemical diversity, represented by different protein isoforms, was detected in hsp30 among species and also within the same species. For one species, *P. monacha*, a different hsp30 isoform pattern was found in fish from three geographically separate locations in Northwestern Mexico, suggesting that rapid hsp30 evolution may create useful isoform markers for identifying subspecies. The hsp70 family is among the evolutionarily most highly conserved, and yet, considerable isoform diversity was found for this family both between and within species. Five qualitatively distinct inducible hsp70 patterns were found in one tropical species, *P. gracilis*. In contrast, only one hsc70 isoform was found, indicating that inducible and constitutive members are under different evolutionary constraints. As an initial test of the possible functional significance of the different *P. gracilis* hsp70 patterns, fish were raised to 41°C, and there were no differences in the time at 41°C tolerated by fish with different hsp70 patterns. There are other processes which remain to be tested including inflammation, wound responses, and responses to chemical proteotoxicity. It is quite possible that other vertebrate species including humans contain hsp70 variation and some alleles might be linked to genetic susceptibility to various diseases. We have exploited an unusual reproductive strategy of *Poeciliopsis* called hybridogenesis. For example, *P. monacha* females mate with P. *lucida* males both in nature and the laboratory to produce an all-female species *monacha-lucida*. In this hybrid species, the maternal genome is passed to progeny as an intact linkage group which does not recombine or reassort with the paternal genome. Thus, naturally occurring combinations of alleles are preserved. Different *monacha-lucida* genotypes were subjected to four heating regimes that differed in the times required to reach maximum temperature. All genotypes survived the slowest heating protocol equally, but differences in survival were found using faster heating rates. The amount of hsp70 induced depended upon the heating regime, but all four genotypes made the same amount, which did not correlate with thermotolerance differences. Interestingly, the amounts of constitutive hsc70 differed among the hybrids, and those with higher amounts of hsc70 were more thermotolerant. Three of the hybrids contained half the diploid amount of hsc70 found in inbred *lucida* strains, suggesting that expression of maternal hsc70 is repressed or silenced in these hybrids.

B6-028 STRESS PROTEINS AS MOLECULAR BIOMARKERS IN TOXICOLOGY, Cindy S. Orser, Pauline Gee, and Spencer B. Farr, Xenometrix, Inc., 2860 Wilderness Place, Boulder, CO 80301.

Individual cells respond in measurable ways to changes in their environment, just as whole organisms respond to stimuli such as heat, cold, and light. Such environmental changes include toxic stimuli which activate or induce specific genes. Many of these gene products play a role in countering the effects of a given toxicant, e.g. by detoxifying it, by transporting it out of the cell, by repairing the damage it causes to cell components, or by intercepting toxic intermediates. A large number of such bacterial and mammalian stress genes have now been isolated and characterized. By using simple techniques to measure the induced responses, information can be quickly obtained regarding the levels of classes of toxins or types of toxic damage caused by cellular exposure to particular compounds. Many macromolecules and cellular processes that are induced in response to toxic stress are highly conserved across broad phylogenetic spectra. For example, genes encoding the heat shock proteins are among the most broadly conserved structural genes known. Such conservation permits the application of bacterial-based assays as predictors of certain types of molecular damage in mammalian cells. These assays are designed to test for the toxic potential of a chemical compound and to indicate the specific cellular macromolecule, process or pathway that has been affected. We have developed rapid and inexpensive bacterial assays, the Pro-Tox assays, which use regulatory sequences of several stress genes (stress promoters) which are placed in front of a structural reporter gene, *lacZ*, whose product β-D-galactosidase can be readily measured. The activity of β-D-galactosidase degrades *o*-nitrophenyl-β-D-galactopyranoside (ONPG) to the products galactose and the intensely yellow *o*-nitrophenol, which is measured as a simple colorimetric change at 420 nm. The assays are run in standard 96-well microtiter plates, testing a given compound at seven doses across 16 fusion strains. This multi-gene, microtiter format requires only small amounts of test compound and permits the rapid acquisition of two key pieces of information regarding toxicity. First, the specific promoters that are induced indicate the target and the class of damage a compound inflicts on these cells. Second, dose-response relationships permit the rapid determination of those doses below which specific targeted stress responses are not elicited. The induction profile for a given test compound can be graphically represented as a histogram that shows the fold induction over baseline of each gene as a function of the dose. The data collected from these and other stress response assays developed at Xenometrix are being compiled in a XenoMatrix™ database, which promises to be a novel means of assessing the relative toxicity of chemical compounds.

Late Abstract

REGULATION AND FUNCTION OF COLLAGEN-SPECIFIC MOLECULAR CHAPERONE-LIKE PROTEIN, HSP47.

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HSP47, a collagen-binding stress protein, is located in the endoplasmic reticulum (ER) because of the presence of an RDEL (Arg-Asp-Glu-Leu) sequence at the C-terminus, which acts as an ER-retention signal. HSP47 transiently binds to procollagen in the ER. Recently we have revealed that HSP47 is dissociated from procollagen in the *cis*-Golgi network area under normal condition using *in vivo* cross-linking, pulse label and chase, and immunoprecipitation analysis. When cells are heat shocked or treated with an inhibitor for triple helix formation of collagen, HSP47 is retained to be bound to the denatured or abnormal procollagen, resulting in inhibiting these collagen to be secreted out of the cell. Thus, HSP47 is assumed to be a molecular chaperone-like protein which has substrate specificity.

To analyze the interaction of HSP47 with collagen in more detail, we measured the kinetic parameters of recombinant HSP47 with purified collagen using a biosensor, *BIA*core. *K*d values of rHSP47 were 10^{-6} to 10^{-7} M for types I to V collagens consisting of high association rate constant in the order of 10^{4} $M^{-1}s^{-1}$ and very fast dissociation rate constant in the order of 10^{-2} s^{-1}. As the concentration of HSP47 is very low, actually not observed, in the Golgi compartment, HSP47 is thought to be rapidly dissociated from procollagen in the concentration-dependent manner due to this rapid dissociation rate constant immediately after it enters the *cis*-Golgi.

In addition to the functional relevance of HSP47 with collagen, the expression of HSP47 is regulated in parallel with that of collagen. The synthesis of both HSP47 and collagen decreases after malignant transformation in the fibroblasts and markedly increases during the differentiation of mouse teratocarcinoma cell line F9. In addition, the cells that do not synthesize detectable level of collagen do not synthesize HSP47, either. These correlation in the expression of HSP47 with collagen was also confirmed in the pathophysiological conditions such as liver cirrhosis caused by the administration with carbon tetrachloride. During progression of hepatic fibrosis, both HSP47 and types I and III collagens were dramatically induced. *In situ* hybridization using a probe for HSP47 revealed that only the cells, mainly Itoh cells, alongside the collagen fibril showed the positive signals, which again suggests the functional involvement of HSP47 as a molecular chaperone in the processing and/or secretion of collagen molecules into the extracellular matrix.

We also examined the expression of HSP47 during the embryogenesis or the development of each organ in mice using anti-HSP47 antibody. Staining of HSP47 was first detected in the developing heart, somites and cardiovascular system at the 8.5 day embryo. The expression of HSP47 was observed throughout the embryonic development mainly in the tissues originated from the mesoderm including notochord, somite, mesenchyme, cartilage and bone. The expression of collagen was evident in these tissues, which again paralleled well with that of HSP47 *in vivo*.

Structure/Function

B6-100 INTERACTION OF ERYTHROPOIETIN RNA BINDING PROTEIN WITH mRNA REQUIRES AN ASSOCIATION WITH HEAT SHOCK PROTEIN 70, Barbara S. Beckman,[1,4] Aline B. Scandurro,[1] Eric C. McGary,[1] Russell B. Wilson,[2,4] Scott A. Tennenbaum,[3] Robert F. Garry[3,4] and Isaac J. Rondon,[1] Departments of Pharmacology,[1] Pathology and Laboratory Medicine,[2] Microbiology,[3] and Program in Molecular and Cellular Biology,[4] Tulane University School of Medicine, New Orleans, LA 70112

Synthesis of erythropoietin (Epo), the glycoprotein hormone that regulates red blood cell formation, is induced in response to low oxygen stress (hypoxia) and is regulated at both transcriptional and post-transcriptional levels. The regulation of Epo mRNA stability is an important control mechanism and may be affected by the binding of a regulatory protein, termed Epo RNA binding protein (ERBP), to its 3' untranslated region. Because heat shock proteins (hsps) are induced in response to a variety of stresses, including hypoxia, we tested the possibility that hsps are involved in control of Epo synthesis. Using an electrophoretic mobility shift assay (EMSA) in which human anti-hsp70 antibody was added to ERBP-containing human hepatoma cell (Hep3B) lysates, we found the elimination of the ERBP-Epo mRNA complex. Further, evidence for direct binding of hsp70 to Epo mRNA is suggested from EMSAs where purified human hsp70 was added. These studies suggest a novel function of an hsp, regulation of Epo mRNA stability, and suggest that hsp70 might be involved in the complex process that controls red blood cell production during oxygen deprivation.

B6-101 HORMONE DEPENDENT TRANSACTIVATION BY THE HUMAN ANDROGEN RECEPTOR IS REGULATED BY A dnaJ PROTEIN,by Avrom J. Caplan‡*, Elizabeth Langley§, Elizabeth M. Wilson§ and Johanna Vidal‡, from ‡the Department of Cell Biology and Anatomy, Mount Sinai Medical Center, New York NY 10029 and the § Department of Biochemistry and Biophysics and Department of Pediatrics, University of North Carolina, Chapel Hill, NC 27599

The molecular chaperone Hsp90 functions in signal transduction by steroid hormone receptors by maintaining them in an inactive state prior to hormone binding. The molecular chaperone Hsp70 is also thought to play a role in this process. Since dnaJ proteins are functional partners of Hsp70, genetic studies were performed to examine the role of a eukaryotic dnaJ protein, Ydj1p, in signal transduction by a steroid hormone receptor, in this case the human androgen receptor. To test the role of Ydj1p in this process, the hAR gene was heterologously expressed in wild type and *ydj1-151* mutant strains of *Saccharomyces cerevisiae*. Hormone dependent trans-activation by hAR was measured in both strains as a function of lacZ reporter gene expression. Similar levels of hAR mRNA were detected in wild type and *ydj1-151* cells, but the mutant displayed 2-3 fold greater level of hAR protein as assessed by western blot. Addition of a synthetic androgen, R1881, to growing cultures of wild type cells resulted in rapid expression of the lacZ gene with an average maximal induction of 15 fold above the background levels. By contrast, induction in the *ydj1-151* mutant was only 3 fold above the background. Control experiments demonstrated that the defective induction phenotype co-segregated with the *ydj1-151* allele and further studies showed that *ydj1* null strains were similarly defective. In similarity with Hsp90, the target for Ydj1p action was determined to be the hAR hormone binding domain since an N terminal fragment lacking this region was constitutively active in both wild type and *ydj1-151* mutant strains. These data correlate hormone dependence of hAR activation with a requirement for Ydj1p function. This is consistent with a role for dnaJ proteins in signal transduction by steroid hormone receptors.

B6-102 A FACTOR PRESENT IN MURINE ASCITES FLUID CAN ALTER REGULATION OF HSP72 IN A CELL LINE DEFECTIVE IN THE HEAT SHOCK RESPONSE. S.M.Davidson, P.B.Høj, M.Smith and R.L.Anderson. Peter MacCallum Cancer Institute, St. Andrew's Place, E. Melbourne, Australia, 3002.

We have previously described a murine B lymphoma cell line, CH1, which is unable to transcribe *hsp72* in response to heat stress *in vitro* although it can still develop thermololerance. We have been unable to detect any defect in the known pathway of heat shock gene regulation. Specifically, the heat shock transcription factor (HSF) is phosphorylated, migrates to the nucleus, and is capable of binding *in vitro* to an oligonucleotide representing the heat shock element.

We have discovered that when the CH1 cells are grown as a tumour by injecting them into the peritoneal cavity of Balb/c nude mice, the cells are capable of responding to whole body hyperthermia by synthesizing HSP72 which can be detected by 2-dimensional gels and westerns. Interestingly, the CH1 grown *in vivo* have a higher intrinsic heat resistance than those grown in tissue culture. A diffusible factor from the ascites fluid is responsible for this potentiation, since the cells are still able to respond to heat even if they are injected into a 0.22 micron diffusion chamber implanted within the peritoneal cavity of the mice.

We have begun purification of the factor by incubating CH1 *in vitro* with the ascites fluid, and have shown that in this situation, hsp72 can still be detected in response to heat shock. The results of our latest experiments towards characterizing the ascitic factor will be presented. We will also present some preliminary data suggesting that methylation of the endogenous *hsp72* gene may be responsible for its inactivation in this cell line.

B6-103 OLIGOMERIZATION OF HEAT SHOCK TRANSCRIPTION FACTOR ON HEAT SHOCK ELEMENTS, Becky L. Drees, Ralph Peteranderl, Hays S. Rye, and Hillary C.M. Nelson , Dept. of Molecular and Cell Biology, Univ. of California Berkeley, Berkeley, CA 94703

The heat shock transcription factor, HSF, is the only known trimeric DNA binding protein. HSF exists as a trimer in solution and binds to heat shock elements as a trimer. At higher concentrations, HSF forms larger complexes on DNA which are multimers of trimers. To investigate the relationship between oligomerization and HSF's DNAbinding characteristics, we replaced the trimerization domain of HSF from *S. cerevisiae* with the "leucine zipper" of GCN4. This chimeric protein, ScHSF-GCN4, is a dimer in solution. We compared the complexes formed by the chimeric protein bound to HSEs of differing lengths with those formed by trimeric HSF and the relative DNA binding affinity and specificity of the two proteins. ScHSF-GCN4 binds as a dimer to palindromic heat shock elements of two inverted repeats, with similiar affinity as trimeric HSF. Surprisingly, the chimeric protein binds most stably as a trimer on an HSE containing three inverted repeats and forms complexes which are multimers of trimers, although there is no evidence that it forms trimers in solution. These results suggest that the interaction of the HSF DNA binding domain with a three repeat site is able to force the usually dimeric leucine zipper into a trimeric conformation. As a trimeric DNA binding protein, HSF binds to three-repeat HSEs with higher affinity and specificity than it does to sites with only two inverted repeats.

B6-104 FORMATION OF PROTEIN DISULFIDES, GENERATED BY OXIDATIVE STRESS, REPRESENT A SIGNAL FOR INDUCTION OF THE STRESS RESPONSE Michael L. Freeman, Elaine Sierra-Rivera, Michael J. Meredith, Michael J. Borrelli, and James R. Lepock, Radiation Oncology, Vanderbilt Univ. School of Medicine, Nashville, TN 37232

To identify the chemical nature of signals generated during oxidative stress that are capable inducing heat shock protein synthesis and thermotolerance, Chinese hamster ovary cells were exposed to menadione sodium bisulfite. Menadione redox cycling generates superoxide with subsequent thiol oxidation. Accumulation of Hsc/Hsp 70 mRNA and protein as measured by Northern and Western blotting, was found to be directly proportional to the extent of protein thiol oxidation. To directly test the concept that thiol oxidation can induce HSPs, cells were exposed to diamide, a compound that specifically generates disulfides. Differential scanning calorimetry demonstrated diamide generated disulfides destabilized protein structure such that proteins unfolded to a more disordered arrangement, causing them to be denatured. This was followed by induction of HSF binding activity and subsequent accumulation of Hsc/Hsp 70 mRNA, as determined by mobility gel shift assay and Northern blotting, respectively. Finally, diamide induce thiol oxidation produced thermotolerance. Conversely, cells induced to synthesize heat shock proteins exhibited resistance to cytotoxicity produced by protein thiol oxidation. Thus, oxidative stress can produce protein thiol oxidation, specifically, non-native disulfides. Formation of such species can result in protein denaturation, induction of HSP synthesis and the development of thermotolerance. Supported in part by PHS/NIH CA 38079, ES 03272, CA 40251, & CA 60331.

B6-105 ANALYSIS OF STRUCTURE AND FUNCTION OF THE SMALL HSP KINASE MAPKAP K2, Matthias Gaestel, Katrin Engel, Kathrin Plath and Heidi Krause, AG Stressproteine, MDC for Molecular Medicine, Berlin-Buch, D-13122, Germany

Several small mammalian Hsps show a rapid stress- and mitogen-induced phosphorylation at two or three serine residues. It has been shown that the MAP kinase-activated protein kinase 2 (MAPKAP K2) is the major enzyme responsible for small HSP serine-phosphorylation within the motif RXXS *in vitro*. Further evidence is presented that small Hsps are also phosphorylated as a result of activated MAPKAP K2 in cells subjected to heat shock and after treatment with TNFα. Under both conditions an increased MAP kinase activity could also be detected, suggesting that sHsp phosphorylation is a result of the activated MAP kinase cascade.

As an approach toward a better understanding of the function of sHsp phosphorylation, the structure and function of the sHsp kinase MAPKAP K2 is analyzed. Several sequence motifs are present in the primary structure of the kinase: A SH3 binding domain, a MAP kinase phosphorylation site, an A-helix and an nuclear translocation signal. The functional significance of these motifs has been analyzed. It could be shown that the SH3 binding domain could bind the SH3 domain of C-abl, and that this binding probably influences the subcellular localization but not the catalytic activity of the kinase. The A-helix, which is complementary to the catalytic region of the kinase, is demonstrated as an intramolecular inhibitor, which is regulated by MAP kinase phosphorylation. A constitutively active A-helix-deletion mutant of MAPKAP kinase 2 has been constructed and is overexpressed in eukaryontic cells. The effect of hyperphosphorylation of sHsps in these cells will be discussed.

B6-106 ATTENUATION OF THE HEAT SHOCK RESPONSE IN AGING ORGANISMS, Gershon D.,Efrati E.,Elaad S.,Haddad D.,Shpund S.,Blake* M., Department of Biology, Technion,Haifa 32000,Israel and*Department of Pharmacology, University of North Dakota, Grand Forks, ND.58202-9037, U.S.A.

Analysis of the response to heat shock was done by determination of the levels of heat shock proteins (hsp) m-RNA in brain, lung, skin and liver of rodents exposed to ambient temperatures of 35-40°C. The response was strong in young, but very much reduced in old animals. *In situ* hybridization studies in mouse liver revealed a uniform response throughout the liver in young mice but was patchy in about 50% of the old mice. Over half of the cells of patchy old livers did not produce HSP70mRNA.Correlation with possible pathology is investigated.

Human peripheral blood monocytes from 33 old individuals showed considerably altered synthesis of hsp70,90 and 110 in response to temperatures of 42-44°C. The nature of the alterations was varied and included no response, early but unsustained response, low levels or considerably delayed synthesis of hsps. In monocytes of old individuals the hsps were much more stable ($t_{1/2}$ of >99 hours) than in young cells ($t_{1/2}$ = ~48 hours).

An assay was developed to study the specific activity of hsp70. The assay is based on the capacity to protect heated enzymes (e.g. creatine kinase) in an ATP-dependent manner to retain catalytic activity. It was found that the specific "chaperone" activity of hsp70 purified from rat liver declined by 50% with age.

In Drosophila the heat shock (37°C) response as a function of age was studied at the protein, m-RNA and transcription levels of the hsp70 and hsp83 genes. The capacity to synthesize hsp70 and hsp83 proteins declined with age by over 30%. At the mRNA levels, we found a 92% and 77% decline for hsp83 and 70, respectively. Gel shift analysis showed that with age, the capacity of the transcription factor, HSF, to bind to HSE declined by 71%.The decline was due to an inhibitory element, that normally prevented the activation of HSF, which became more heat resistant with age and did not allow HSF activation.

B6-107 **Heat Shock Factor Can Bind to Nucleosomes and Remodel Chromatin in S. cerevisiae,** David S. Gross, Tuba Diken, and Alexander M. Erkine, Department of Biochemistry and Molecular Biology, Louisiana State University Medical Center, Shreveport, LA 71130-3932.

To investigate the role of yeast heat shock factor (yHSF) in regulating transcription and generating the DNase I hypersensitive, nucleosome-free region over the promoter of the *HSP82* heat shock gene, we have mutated all 4 upstream heat shock elements, both individually and in combination. While deletion of the 3 most distal elements (HSEs 2 - 4) has a minimal effect on either basal or induced function, deletion or substitution of the TATA-proximal site, termed HSE1, reduces both levels of transcription at least two orders of magnitude. Moreover, the ΔHSE1 mutation leads to a dramatic transition in chromatin structure: the DNase I hypersensitive region is replaced by two stable, sequence-positioned nucleosomes. One of these nucleosomes is centered over the mutated UAS, whereas the other is centered over the TATA-initiation site. Overexpression of yHSF strongly suppresses the null phenotype of the induced *hsp82*-ΔHSE1 gene and re-establishes DNase I hypersensitivity over its promoter. Such suppression is mediated through the HSE2 and HSE3 heat shock elements, which map very close to the dyad of the UAS nucleosome. HSF-mediated suppression is unaffected by prior arrest in G1, indicating that yHSF can directly bind to the UAS nucleosome and disrupt it and the adjacent TATA nucleosome in the absence of DNA replication. To investigate whether the *SWI/SNF* protein complex collaborates with HSF in mediating nucleosome binding and displacement over the *hsp82*-ΔHSE1 promoter, we have constructed *hsp82*-ΔHSE1, *swi1*Δ double mutant strains. Strikingly, an extragenic mutation in *SWI1* completely abolishes yHSF-mediated suppression. Using *in vivo* footprinting techniques, we are currently investigating at which step--e.g., yHSF loading onto the UAS nucleosome, heat shock-induced disruption of the TATA nucleosome, etc.--the *swi1*Δ-mediated block occurs.

B6-108 CHARACTERIZATION OF HSP70 ISOFORMS AND RELATIVE ABUNDANCE OF HSP70 mRNA AND PROTEIN, Vince Guerriero, Jr. and Jesus A. Gutierrez, Dept. of Animal Sciences, University of Arizona, Tucson, AZ 85721

A cDNA clone for the inducible Hsp70 has been isolated from a bovine skeletal muscle cDNA library. This mRNA encodes a protein with a calculated molecular mass of 70,250 Da. The cDNA has one continuous open reading frame capable of encoding a 641 amino acid protein. Expression of this cDNA in a bacterial expression system produced a protein of identical mobility to the inducible Hsp70 protein from bovine skeletal muscle as determined by polyacrylamide gels containing SDS. Two-dimensional gel electrophoresis demonstrated this protein to have identical focusing properties as a minor isoform from bovine skeletal muscle. Upon carbamylation of this bacterially expressed protein, a train of charged proteins with charge differences of minus one were produced. These carbamylated proteins were shown to have similar focusing mobilities as the Hsp70 isoforms isolated from bovine skeletal muscle. These results demonstrate the identification of a skeletal muscle inducible Hsp70 gene and suggest that the presence of multiple Hsp70 isoforms may be the product of posttranslational modifications to the Hsp70 proteins. A solution hybridization assay to measure bovine Hsp70 mRNA levels was used to demonstrate that skeletal muscle contains the highest amount of this mRNA while brain has the lowest amount and the level of Hsp70 mRNA is heat inducible in bovine skeletal muscle. Furthermore, this assay allowed for the comparison of relative Hsp70 protein and mRNA levels. Relative transcript levels compared to relative Hsp70 protein levels in tissues demonstrated 50 to 200 fold differences. These results, then, approximate the magnitude of the previously reported preferential translation of Hsp70 mRNA.

B6-109 ROLE OF MORTALIN AND MITOSIN DURING CELLULAR IMMORTALIZATION, Sunil C. Kaul, Yasuhiko Komatsu, Youji Mitsui and Renu Wadhwa, National Institute of Bioscience and Human Technology, AIST, 1-1 Higashi, Tsukuba 305, Japan

Spontaneously immortalized embryonic fibroblasts from three different strains of mouse were seen to represent two distinct stages of immortalization that could be characterized by growth properties, serum requirements, thermal characteristics of cells and the marker proteins such as mortalin, mitosin and p53. Mortalin, an actin binding hsp70 family member was seen to reside in the perinuclear locale in stage I cells indicating that change from the cytosolic to the perinuclear locale is an early event. On the other hand increased levels of p81 and p53 were only detected in stage II cells. p81 was identified and characterized as a proliferation stimulating protein belonging to actin binding protein family. An elevated expression of this protein along with the unique surface distribution was detected in stage II cells. Furthermore, these cells retract to the over expression of mortalin cDNA which induced cellular senescence in NIH 3T3 (stage I) cells. The study reveals that the expression of mitosin interferes with the overexpression of hsp70 family member, mortalin, in determining the divisional phenotype of cells.

B6-110 HIGHER PLANT HOMOLOGUES OF THE BACTERIAL DNAJ PROTEINS. Barbara Kroczynska, Rengang Zhou, G. Thomas Hayman and Jan A. Miernyk, Phytoproducts Research Unit, U.S.D.A., Agricultural Research Service, National Center for Agricultural Utilization Research, Peoria, IL 61604

The Stress70 molecular chaperone proteins have a low level ATPase activity. ATPase activity of the bacterial Stress70 protein DnaK is stimulated many-fold by the co-chaperone proteins DnaJ and GrpE. In addition, DnaJ might function independently as a chaperone. We have sequenced two cDNAs that encode Arabidopsis thaliana homologues of the DnaJ protein, AtJ1 and AtJ2. The protein encoded by AtJ1 contains the J- and C-rich domains, and is most homologous with E. coli DnaJ. The protein encoded by AtJ2 contains the J-, G/F- and C-rich domains, a carboxy terminal -CAQQ sequence, and is most homologous with the Saccharomyces YdJ1 protein. AtJ1 was expressed in E. coli as a chimera with the maltose binding protein, cleaved, and purified. The recombinant protein stimulates ATPase activity of both E. coli DnaK and maize cytoplasmic Stress70.

B6-111 THE C-TERMINAL PEPTIDE BINDING DOMAIN IS ESSENTIAL FOR SELF-ASSOCIATION OF THE MOLECULAR CHAPERONE HSC70

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The molecular chaperone HSC70, a member of the 70KD Heat Shock Protein family, self-associates in solution to form dimers, trimers and probably higher order oligomers. In order to identify the protein regions involved in this process, several truncated versions of the protein have been prepared by site directed mutagenesis and subsequently analyzed, in terms of their oligomerization properties, by FPLC and analytical ultracentrifugation. Whereas the N-terminal ATPase domain (residues 1 to 385) is monomeric in solution and behaves as a single ideal species of 41 KD in sedimentation equilibrium, the C-terminal peptide binding domain, comprising residues 385 to the C-terminus, shows association properties identical to those of the intact protein. Deletion of residues 541 to the C-terminus, either from the entire protein or from the C-terminal domain, gives, in each case, a stable protein that elutes approximately as a tetramer, thus indicating that the C-terminal fragment, made of residues 385 to 540, is sufficient for the self assembly of the protein. Since this fragment seems also to be sufficient for peptide binding, a model structure could be presented, in which HSP70 molecular chaperones would be organized in three domains, an N-terminal, Actin-like domain (residues 1 to 384) having ATPase activity, an intermediary, "HLA-like" domain (residues 385-540), responsible not only for peptide binding but also for self association of the protein, likely through an alpha-helical coiled coil structure, and a C-terminal domain (residues 541-C-terminus) of unknown function.

B6-112 INTERACTION OF THE PSEUDOPODIAL PROTEIN IEFSSP 3521 WITH HSC 70 AND HSP 90

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The ability of a cell to move is an important feature of such diverse biological events as embryogenesis, wound healing, and tumor metastasis. Cell motility is accompanied by dynamic surface extensions which are caused by changes in the cytoskeleton, especially in the actin-filament network. Filamentous actin (F-actin) is formed by the polymerization of monomeric actin (G-actin). The local amount of F- versus G-actin determines the motility state of the cell. It is regulated by a large number of different actin-associated proteins that interact with either G- or F- actin. By screening a cDNA expression-library of M27 mouse cells with an antibody against an actin-associated protein of the cellular slime-mold *Dictyostelium*, we isolated a cDNA encoding a protein that showed 97% identity to a human protein of unknown function named IEFSSP 3521 [(a)]. The human IEFSSP 3521 was shown to be two-fold up-regulated following SV40 transformation of MRC-5 fibroblasts and shows 42% identity to the stress-inducible protein STI1 of *S. cerevisiae*. The chicken homologue of IEFSSP 3521 was identified in a cytosolic complex with heat-shock-proteins 70 and 90 and as a receptor-associated protein of the *in vitro* reconstituted chicken progesterone-receptor [(b)]. The function of IEFSSP 3521 in these complexes is unclear [(c)]. Our biochemical fractionation and immunofluorescence studies show that the murine IEFSSP 3521 is a primarily cytosolic protein which is locally enriched in extending pseudopodia. Synthesis of the murine IEFSSP 3521 is not inducible under our heat-shock conditions. In protein binding studies using mouse cell extracts and purified recombinant murine IEFSSP 3521 we show that murine IEFSSP 3521 interacts with HSC 70 and HSP 90. Experiments with deleted recombinant IEFSSP 3521 proteins show that HSC 70 and HSP 90 interact with different domains of IEFSSP 3521. The interaction of HSC 70 and recombinant IEFSSP 3521 can be reconstituted *in vitro* with purified components in the absence of exogenous proteins. We speculate that the interactions of IEFSSP 3521 with HSC 70 and HSP 90 play a role in the cytoskeletal changes that take place in extending pseudopodia.

(a) Honore et al. 1992, J. Biol. Chem. 267, 8485.
(b) Smith et al. 1993, Mol. Cell. Biol. 13, 869.
(c) Schumacher et al. 1994, J. Biol. Chem. 269, 9493.

B6-113 MECHANISMS OF HEAT RESISTANCE IN OXIDATIVE STRESS RESISTANT CELLS

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A series of H_2O_2 resistant variants selected from HA-1 Chinese hamster fibroblasts (Radiat. Res. 114:114, 1988) which overexpress catalase, have been shown to be resistant to elevated temperatures, at 43°C but not 45°C (J. Cell. Physiol. 142: 255, 1990). We have been investigating both the physiological and biochemical aspects of this heat resistant state. Under conditions that cells display heat resistance, at 43°C, there is more rapid recovery from heat induced inhibition of protein and RNA synthesis, while no alterations are observed after exposures to 45°C. The dissociation of the excess levels of hsc70 associated with the nucleus induced by exposure to elevated temperatures also appears to be more rapid in the H_2O_2 resistant cells after exposures to 43°C. Comparison of the two dimensional gel electrophoresis patterns of wild type and H_2O_2 resistant variants indicated alterations in the expression of several polypeptides in the resistant cells, both under normal growth conditions and after exposure to H_2O_2. However, no obvious major alterations in the constitutive levels of the major heat shock proteins hsp 110, hsp 89, hsp 70, hsc 70 or hsp 60 were observed. Similar observations were made when Western blots using the respective antibodies were performed. These results indicate that mechanisms other than the elevated constitutive expression of the classical major heat shock proteins can be associated with heat resistance and that cellular mechanisms conferring resistance to oxidative stress may play an important role in protection from cell lethality associated with exposure to elevated temperatures. Supported by CA 51116.

B6-114 THE GENE ENCODING AN HSP70 ANALOG, HSC66, IN *ESCHERICHIA COLI* IS INDUCED UPON COLD SHOCK,

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Hsc66, the second identified Hsp70 protein in *Escherichia coli,* was found by screening second-site compensatory mutations in an *hns* mutant strain. Mutations in *hscA*, the gene encoding Hsc66, compensate for some *hns* mutations with respect to a subset of H-NS sensitive phenotypes. H-NS, a bacterial nucleoid-associated protein, is a stress response protein since it is induced upon a 37°C to 10°C cold shift. Because the amino acid sequence of Hsc66 is 62% similar to DnaK, we examined the induction pattern of *hscA* for similarities to other genes encoding Hsp70 proteins.

We have monitored the expression of *hscA* using a *lacZ* reporter construct and ribonuclease protection assays under various environmental conditions. Although the expression of genes encoding other Hsp70 proteins is induced upon a temperature upshift, i.e. heat shock, the expression of *hscA* was not affected by such an upshift. However, *hscA* mRNA levels increased following a cold temperature shift from 37°C to 10°C. Optimal *hscA* induction occurred 3 hours post-shock with mRNA levels approximately 25 fold higher than pre-shock levels as measured by ribonuclease protection assays. Whereas, a chromosomal *hscA-lacZ* transcriptional fusion produced a 3 fold increase in beta-galacatosidase activity first detectable at 9 hours post-shift. Based on the predicted homology to Hsp70 proteins, the ability of *hscA* mutations to compensate for mutations in another cold inducible gene, and the induction pattern of *hscA*, it is possible that Hsc66 is a cold-shock chaperone protein in *Escherichia coli* .

B6-115 REGULATION OF HSP70 EXPRESSION IN MAMMALIAN CELLS,

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We have examined the expression of hsp70 upon heat shock and during post heat-shock recovery in various rodent cell lines. Specifically, we determined the cells' thermal sensitivity, the cells' ability to develop thermotolerance, the ability of HSF1 to bind to the heat shock element, and to transactivate heat shock genes, especially hsp70. Our results show that a high level of HSF1 in a phosphorylated state, and in a form capable of binding to the heat shock element, is insufficient to induce the synthesis of hsp70 upon heat shock. The thermal induction and regulation of hsp70 expression appears to involve additional control mechanisms besides the transactivation by HSF1.

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B6-116 **CHARACTERIZATION OF THE HSF2 - HSE BINDING ACTIVITY PRESENT IN MOUSE EMBRYONAL CARCINOMA CELL LINES UNDER NON-STRESSED CONDITIONS,** *Marie-Thérèse Loones, Murielle Rallu, Valérie Mezger and Michel Morange. Unité de Génétique Moléculaire, Ecole Normale Supérieure, 46 rue d'Ulm 75230 PARIS- FRANCE*

Mouse embryonal carcinoma (EC) cells represent a paradigm for studying HSF2-HSE binding activity under non stressed-conditions. At normal temperature, in contrast to the mouse differentiated cells, all EC cells tested expressed a **constitutive** HSE binding activity (BA), as determined by gel shift assay. A similar constitutive HSE binding activity is detected transiently during early embryogenesis (see Rallu *et al.*). Such an activity disappears in the EC cells upon *in vitro* differentiation. In some EC cell lines like F9 there is also a heat inducible HSE-BA, similar to that found in heat shocked fibroblasts.

Specific polyclonal **antibodies** to factors HSF1 and HSF2 have been raised by Morimoto's laboratory (Northwestern University, Chicago). We used these antibodies to disturb the formation of the HSF/HSE complexes in extracts from F9 cells in gel shift assay. AbHSF1 cross-reacts only with stressed cell extracts and AbHSF2 only with the unstressed cell extracts, bringing evidence for distinct activities.

From F9 cell tumor extracts we have **purified** to homogeneity the factor carrying the constitutive activity, as visualized on silver-stained gel. After an heparin-sepharose column and two cycles of affinity purification, the protein is eluted between 1M and 2M NaCl.

In order to characterize the constitutive and heat induced activities, we analyzed, in parallel, the **stability at** various **temperatures** of the unbound factors, HSF1 and HSF2 respectively, and compared with the stability of the complexes formed with HSE. We showed that the factor contained in non-stressed F9 cell extracts is markedly more resistant at all temperatures than the factor contained in heat-shocked cells. In contrast, the complexes (bound factors) formed with extracts from unstressed cells are very labile (<10 min at 33°C; 2 min at 45°C), while the complexes detected with extracts from stressed cells are very stable (more than 20 min at 45°C). We investigated also the **cooperativity** of the binding of HSF1 and HSF2 to HSE. Arrays of 5bp units (nGAAn) have been shown to act synergically on the *in vivo* expression of heat shock genes (Xiao *et al.*, Cell 64,1991), so we have examined the binding of HSF1 and HSF2 to various arrays (2 to 9) of the 5bp units. Only HSF1 binds to HSE cooperatively suggesting different ways in the regulation of the genes activated respectively by HSF1 and HSF2 .

B6-117 ANTISENSE INHIBITION OF HSP-27 PRODUCTION AFFECTS GROWTH RATE AND CYTOSKELETON ORGANIZATION IN MCF-7 CELLS.

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The small heat shock protein HSP-27 is predominantly expressed in different types of tumor tissues or cells including the human breast cancer-derived MCF-7 line, our experimental model. This protein is an early target of phosphorylation, following cells exposure to mitogenic or differentiating agents, suggesting a role in signal transduction. The aim of the present work was to test this hypothesis by investigating the effects of the antisense HSP-27 gene in MCF-7 transfectants. The cells were cotransfected with pSVL plasmids expressing G418 resistance and HSP27 in the antisense orientation. Out of the 10 clones that were obtained, 3 proliferated normally and showed a normal HSP-27 content (Western blot). The 7 other clones were characterized by a very slow growth rate and their cells progressively hypertrophied, exhibited lamellar protrusions and tendend to loose contact with each other. Finally numerous multinucleated cells appeared in the population; this was followed by cell lysis within a short delay. Among those clones, 2 could be immunocytochemically analyzed for HSP-27 and one of them also for actin organization. Both clones were indeed negative for HSP-27 and in addition, actin immunostaining revealed changes in cytoskeleton i.e. accumulation of actin patches at the cell cortex, contrasting with the regular cytoplasmic network seen in controls. In highly hypertrophied and multinucleated cells, actin was detected as a multitude of punctated dots, which, when examined at the electronic microscope corresponded to abundant densely packed bundles, practically absent in controls. Our data thus support the current hypothesis of a role for HSP-27 in the control of actin polymerisation-depolymerisation. We propose that deficiency of this function might be causally related to the growth and division abnormalities seen in the antisense-transfected cells.

B6-118 HEAT SHOCK PROTEINS AND CYTOSKELETON OF MOTILE MYCOPLASMAS Alexandr Yu. Malinin, Maxim S. Vonski, Anna V. Kukekova, Dmitriy G. Usoskin, Alexey G. Sotnikov & Sergei N. Borchsenius, Inst. of Cytology, Russ. Acad. Sci. St.-Petersburg, 194064, Russia.

Mycoplasmas lack a cell wall and are amongst the smallest prokaryotes. Albeit the apparent simplicity of their organization and extremely small genome sizes, expression of the HSPs was shown in a number of mycoplasmas. Western blot analysis using pAbs to human HSP70 and pAbs to *P. aeruginosa* CBA was done with several mycoplasma species (*A. laidlawii*, *M. pneumoniae*, *M. genitalium*, *M. gallisepticum*, *M. fermentans*). Chloramphenicol addition repressed all these syntheses. Most of mycoplasmas possessed HSPs of molecular weights typical to HSP65 and HSP70 families.

Recently a system of tubular structures in gliding *M. gallisepticum*, which might perform the functions of cytoskeleton was revealed in our laboratory. A protein with a molecular mass of about 40kDa was detected by immunoblotting with the anti-pig brain tubulin antibodies. The intracellular spatial distribution of the tubular structures was reconstructed. We suggest that mycoplasmal HSP70 mediating protein folding and translocations are involved into process of assambly of the tubular structures as molecular chaperone.

We are going to clone and analize hsp70 and tubulin-like protein genes of *M. gallisepticum*. Since no hybridization was found on mycoplasmal DNA with *E. coli* dnaK gene probe, we were forced to use PCR approach for gene cloning. Primer set was designed for both HSP70 and eucariotic α-tubulin on the basis of analysis of known amino acid and nucleotide sequences. Fragments of expected size were generated by PCR on the mycoplasma DNA templates.

This study was supported by Int. Sci. Found. grant RIY000 and by Russian FBS grant 94-04-13293.

B6-119 **ANALYSIS OF ELEMENTS IMPLIED IN THE MOUSE ZYGOTIC GENOME ACTIVATION. ROLE OF HSE SEQUENCES**

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Zygotic genome activation (ZGA) occurs during the two-cell stage in mouse embryos. The HSP70 gene is one of the first genes expressed in the embryo. Our goal is to determine the different regulatory elements implied in the early activation of the HSP70 gene.

Transgenic mice have been generated that express the luciferase gene under the control of the promoter N3 from the mouse heat shock gene HSP70. We have observed a transient luciferase expression at the two-cell stage similar to the expression of the HSP70 gene. The microinjection of high concentrations of the wild promoter at the one-cell stage inhibits the two-cell stage luciferase expression.

We analyzed the regulatory elements present in the promoter implied in this specific stage expression. At first we created punctual mutants by mutagenesis in the heat shock element (HSE) sequences. We injected these mutant promoters in one-cell embryos and observed a less important decrease in luciferase expression at the two-cell stage.

Therefore mutations in the HSE inhibit the competition of the promoter with the transgene. We currently advance in the construction of other mutants, on the one hand with all HSE sites mutated and on the other hand in other elements which are not implied in the heat shock response.

We hope to obtain a better understanding of the ZGA, and analyze the effects and the importance of this spontaneous early expression of HSPs in the following steps of the embryonic development .

B6-120 THE EFFECTS OF C-TERMINAL TRUNCATION UPON THE ATPASE ACTIVITY OF MAIZE ENDOSPERM ENDOPLASMIC RETICULUM STRESS70. Jan A. Miernyk, G. Thomas Hayman and Rengang Zhou, Phytoproducts Research Unit, U.S.D.A., Agricultural Research Service, National Center for Agricultural Utilization Research, Peoria, IL 61604

Members of the Stress70 family of molecular chaperone proteins have an intrinsic low level ATPase activity. This activity can be stimulated by unfolded proteins or short synthetic peptides. It has been reported that the peptide-binding domain of the Stress70 proteins is located within the carboxy-terminal portion of the protein, and that truncation leads to an ATPase that is higher in activity and is no longer responsive to short peptides. We have constructed a plasmid encoding a carboxy-terminal truncated derivative of endoplasmic reticulum-resident Stress70. The truncated protein was expressed in E. coli as a chimera with the maltose binding protein. The recombinant protein was cleaved with Factor Xa, then purified by affinity chromatography plus anion exchange FPLC. ATPase activity of the truncated maize Stress70 protein was not stimulated by short synthetic peptides, but was not higher than that of the native or full-length recombinant proteins. Analysis of the difference between mammalian and maize Stress70 proteins in response to carboxy terminal truncation is in progress.

B6-121 NOVEL REGULATION OF MITOCHONDRIAL HSP GENE EXPRESSION IN *SACCHAROMYCES CEREVISIAE*, Yoshikazu Nakamura and Koichi Kawakami, Institute of Medical Science, University of Tokyo, P.O. Takanawa, Tokyo 108, Japan

The *SSC1* gene of *S. cerevisiae* encodes a mitochondrial Hsp70 and is essential for cell growth. The basal level of *SSC1* transcript is high and further increased upon heat shock. However, unlike other yeast hsp genes, *SSC1* does not contain canonical heat-shock transcription elements (HSEs).

The *HSX1* gene of *S. cerevisiae* encodes a minor arginine tRNA(CCU) that recognizes the minor arginine codon, AGG. Regardless of a single copy of this tRNA gene in yeast haploid genome, disruption of *HSX1* was not lethal and caused an unexpected defect in heat shock induction of, at least, two mitochondrial hsp genes, *SSC1* and *HSP60*. Since most other hsp genes are not affected by the *hsx1* mutation, it seems likely that *SSC1* and *HSP60* are controlled by some other mechanism than that of normal heat-shock regulation.

Transcription start site of the *SSC1* gene was determined by primer extension and a series of *SSC1* expression analyses were conducted. The data indicated that *SSC1* transcription is enhanced by a specific sequence (referred to as MHS) upstream of TATA, and that there exists a protein factor (referred to as MHF) that binds to the MHS element in mobility shift assay. Interestingly, the MHF activity was not found in the extract prepared from the *HSX1* disruption strain, suggesting that the activity or expression of MHF is regulated by the rare arginine tRNA. The MHS element also exists in the 5' upstream region of *HSP60* and MHF was capable of binding to this region. Isolation and characterization of MHF protein, a putative regulator of mitochondrial hsp genes, are currently in progress.

B6-122 BENZOQUINONE ANSAMYCINS DISRUPT THE RAF-HSP90 MOLECULAR COMPLEX, RESULTING IN RAPID LOSS OF CELLULAR RAF PROTEIN, Len Neckers, Theodor W. Shulte, Mikhail Blagosklonny and Edward Mimnaugh Clinical Pharmacology Branch, NCI, NIH, Bethesda, MD 20892

Cellular raf is a mitogen activated protein (MAP) kinase kinase kinase (MAPKK kinase). This serine-threonine kinase is a central effector in the ras-regulated MAP kinase cascade in which extracellular growth factor signalling is translated into altered nuclear transcription factor function. Activation of ras by extracellular signals results in recruitment of inactive, cytoplasmic raf to the cell membrane, where it subsequently becomes activated as a kinase. Raf has recently been shown to exist as a multimolecular complex in which 1 raf molecule associates with 2 molecules of hsp90 and 1 molecule of p50[1,2]. We recently showed that benzoquinone ansamycins interact specifically with hsp90 and interfere with its association with the v-src protein kinase[3]. We now report that treatment of cells with the benzoquinone ansamycin geldanamycin (GA) results in rapid, apparent loss of raf protein. Although steady-state protein level is markedly reduced following drug treatment, raf synthesis, as measured by a short pulse of radiolabelled methionine, is actually somewhat elevated compared to untreated cells, suggesting a reduced raf half-life following drug addition. Additionally, although hsp90 can be co-precipitated in a raf immunoprecipitation, this association is lost shortly after GA addition, before raf protein levels have fallen significantly. Finally, while ras can also be co-precipitated in a raf immunoprecipitation, this association is also lost a short time after GA treatment. These data suggest that raf association with hsp90 is critical for protein stability and/or proper localization in the cell.

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B6-123 MODULATION OF PARAMYXOVIRUS TRANSCRIPTION BY HSP70, Michael J. Oglesbee and Zheng Liu, Department of Veterinary Biosciences, The Ohio State University, Columbus, OH 43210.

The RNA genome of paramyxoviruses (e.g., measles and canine distemper virus) is transcribed in the cytoplasm of infected cells. Although viral transcription is regulated by the cell, specific cellular factors involved in this process are poorly defined. For canine distemper virus (CDV), we have shown that induction of the cellular stress response promotes viral genomic transcription. The enhancement of transcription was correlated to the formation of complexes between HSP70 and the viral core particle or nucleocapsid (NC)(the NC represents the self-contained means of virus gene expression). In this work we document a direct role for HSP70 in promoting NC transcription. Addition of antibody specific to 72k HSP suppressed cell-free NC transcription in a dosage-dependent manner. Furthermore, addition of purified HSP70 enhanced cell-free NC transcriptional activity at low supplementation levels (e.g., $2x10^{-3}\mu g/\mu l$). These results were correlated to enhanced NC transcriptional activity following the *in situ* induction of HSP70-NC complexes by thermal or sodium arsenite stress. The physical interaction between HSP70 and CDV NC was shown to be an ATP-dependent event. Cytoplasmic extracts of infected cells (containing NC) were either depleted of ATP or were adjusted to 2.5 or 0.5mM ATP. The NC was then purified by isopycnic density centrifugation. ATP depletion allowed the detection of 72k/73k HSP-NC complexes for the two ultrastructural variants of the CDV NC. One variant is characterized by a tight helical ribonucleoprotein structure encapsidating a nuclease resistant genome. The second variant (L-NC) is characterized by an open helical structure and an exposed RNA genome, analogous to transcriptionally active chromatin. Here, the L-NC variant was detected only in states of ATP depletion, suggesting that L-NC is dependent upon HSP70 complex formation for its expression. These results are the basis for a model in which HSP70 regulates the transcription of a eukaryotic virus through effects upon NC conformation.

B6-124 DEGRADATION OF E. COLI σ^{32} IS MEDIATED BY THE MEMBRANE-BOUND, ATP-DEPENDENT ZINC-METALLOPROTEASE, FtsH, T. Ogura[1], T. Tomoyasu[1], J. Gamer[2], B. Bukau[2], Masaaki Kanemori[3], H. Mori[4], A. J. Rutman[5], A. B. Oppenheim[5], T. Yura[3], K. Yamanaka[1], H. Niki[1] and S. Hiraga[1], [1]Department of Molecular Cell Biology, Institute of Molecular Embryology and Genetics, Kumamoto University School of Medicine, Kumamoto 862, Japan, [2]Zentrum für Molekulare Biologie, Universität Heidelberg, INF 282, D69120 Heidelberg, Germany, [3]HSP Research Institute, Kyoto Research Park, Kyoto 600, Japan, [4]Nara Institute of Science and Technology, Ikoma 630-01, Japan, and [5]Department of Molecular Genetics, The Hebrew University-Hadassah Medical School, Jerusalem, Israel

*When cells are exposed to various forms of stress, synthesis of a set of heat-shock proteins is rapidly induced. In *Escherichia coli* cells, stress induction is regulated by the specific transcription factor σ^{32}. σ^{32} is an extremely unstable protein, and stress-induced σ^{32} stabilization is a key regulatory event of the heat shock response. The specific protease responsible for σ^{32} degradation has not yet been identified. Recently, we reported that the *ftsH* gene of *E. coli* encodes an essential integral membrane protein of 70.7 kDa with an ATPase domain homologous with some regulatory ATPase subunits of the eukaryotic 26S protease, and that it is involved in proteolysis of the λ CII protein. We now report that in an *in vitro* reconstituted proteolytic system FtsH degrades σ^{32} in the presence of ATP and Zn^{2+}, and that σ^{32} is stabilized in an *ftsH* mutant. We conclude that FtsH is a novel membrane-bound, ATP-dependent zinc-metalloprotease with activity for σ^{32}. This involvement of a membrane-bound protease in degradation of a transcription factor provides evidence for a novel mechanism of gene regulation in *E. coli*.*

B6-125 STRUCTURAL AND FUNCTIONAL ANALYSIS OF A CHLOROPLAST SMALL HEAT SHOCK PROTEIN, Katherine W. Osteryoung and Elizabeth Vierling, Department of Biochemistry, University of Arizona, Tucson, AZ 85721

In higher plants, the heat shock response is dominated by the induction of the small heat shock proteins (sHSPs), which accumulate in the cytosol, ER and chloroplasts. The abundance of and evolutionary conservation among the plant sHSPs suggest they are critical for plant survival at high temperatures. Recent studies have shown that sHSPs exhibit activities *in vitro* consistent with a molecular chaperone-like function. However, little is known regarding the native targets of sHSP activity nor of their contribution to the development of thermotolerance *in vivo*. We are taking a multifaceted approach, involving biochemical analysis, transgenic plant experiments, and mutant screening, to probe the function of the chloroplast-localized sHSP, HSP21 (21 kD), in the chloroplasts of higher plants. HSP21 is not detectable in the absence of stress, but accumulates in heat-stressed plants in proportion to temperature. Analysis of soluble extracts from heat-stressed pea plants indicates that HSP21 is present in high molecular mass complexes of 200 and 230 kD. In both isolated chloroplasts and intact plants, these soluble complexes become associated with a detergent-insoluble fraction in a time- and temperature-dependent manner. The soluble and insoluble forms of the protein are not differentially modified based on 2D gel analysis. A second protein, recently identified by N-terminal sequence analysis as rubisco activase, also exhibits temperature-dependent insolubilization in isolated chloroplasts and whole plants. We have begun investigating the possibility that HSP21 interacts directly with rubisco activase in heat-stressed plants. In addition, we are developing assays for thermotolerance in *Arabidopsis* that will be useful both in analyzing transgenic plants with altered expression of heat shock genes and in designing mutant screens to identify genes involved in the acquisition of thermotolerance in plants. Analysis of transgenic plants that constitutively overexpress HSP21 is underway and should help to elucidate the *in vivo* function of sHSPs in all organisms.

B6-126 CYCLOSPORIN A HAS "STRESS LIKE" EFFECTS ON CELLULAR SYSTEMS

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The cellular stress factors as well as the immunosuppressive drugs have pleiotropic effects on cells. In a previous paper, we presented some effects of the immunosupressant Cyclosporin A : the inhibition of general protein synthesis, the stimulation of BiP (GRP78) synthesis and also the strengthening of HSP 70 and HSP 90 synthesis after a heat shock. Experiments with Actinomycin D demonstrate that Cyclosporin A acts at the transcriptional level.

All these effects suggest that Cyclosporin A might be included in the stress factor category. In order to compare the "stress like" effects of Cyclosporin A to the "classical" stress effects we turned to phosphorylation processes : there are evidences that many protein kinase and phosphatase activities are modified after a stress treatment. In this respect heat-shock and related stresses (arsenite shock) enhance a kinase activity, able to phosphorylate a peptide called "hepta-4" derived from the C-terminal part of RNA polymerase II largest subunit (HS-CTD kinase).

We studied the effect of Cyclosporin A on this protein kinase activity. Addition of different concentrations of CsA (in micromolar range) to HeLa cells induces an increase of phosphorylation of the "hepta-4" peptide substrate comparable to a stress effect. Under stress conditions (heat-shock or arsenite shock) this CsA effect is strengthened.

B6-127 **A MATHEMATICAL MODEL OF THE HSP70 REGULATION IN THE CELL,** **Peper A.*, Grimbergen C.A.*, Spaan J.A.E.*, Souren J.E.† and Van Wijk R.†; * Laboratory of Medical Physics, Academic Medical Centre, Amsterdam, The Netherlands and † Department of Molecular Cell Biology, Utrecht University, Utrecht, The Netherlands.**

A mathematical model of the hsp70 organization in the cell has been developed. The model is based on our assumption that the level of free hsp70 in the cell is determined by active regulation. In the model the hsp70 regulation is divided in five main blocks. The first block simulates the damaging effect of elevated temperature on proteins, the interaction of free hsp70 with these injured proteins and its chaperone role in nascent protein translation. The second block simulates the relation between the amount of free hsp70 and the binding of hsp70 with heat shock factor proteins (HSF). The third block describes the binding of free HSF with the heat shock elements on the DNA. In the fourth block the synthesis of mRNA of hsp70 is described and the interaction between mRNA and injured proteins not bound to hsp70. This interaction results in a decreased translation and consequently a decreased synthesis of hsp70. The synthesis of hsp70 is simulated in the fifth block. The behaviour of the model on a temporal rise of temperature shows an initial decline and a subsequent sharp rise to an increased level of the amount of free hsp70 in the cell. This is consistent with the heat induced temporal changes in sensitivity to a second heat shock, i.e. an early sensitization and a subsequent development of tolerance. The general agreement found between the behaviour of the model and in vivo findings supports our hypothesis that the level of free hsp70 in the cell is determined by active regulation.

B6-128 STUDIES ON THE PHYSICAL STRUCTURE OF THE TRIMERIZATION DOMAINS OF THE YEASTS *S.CEREVISIAE* AND *K.LACTIS* R.Peteranderl and H.C.M.Nelson, Dept. of Molecular and Cell Biology, University of California, Berkeley

Heat shock factors contain two conserved regions of heptad repeats of hydrophobic residues C-terminal to the DNA binding domain. This region had been shown to be necessary for trimerization *in vivo* in *S. cerevisiae*. We have demonstrated that it is also sufficient for trimerization *in vitro* if expressed as a peptide covering 92 amino acids of this region from *S. cerevisiae* or *K. lactis* HSF. The isolated domain is highly alpha-helical, suggesting that trimerization is based on a triple stranded coiled-coil.

We have now narrowed down the actual extent of the trimerization domain of the heat shock factor from *K.lactis* through limited proteolysis and genetic truncations to the amino acids 303 - 373. The region N-terminal to the start of the heptad repeat (aa 303 - 313) was shown to be necessary for the function of the isolated domain and to be protected from site specific proteases. This suggests that there are important structural elements in the trimerization domain besides the heptad repeats.

To study the orientation of the subunits in the trimer we used ^{19}F NMR and specific labeling of the unique tyrosine (Y326) and tryptophan (W346) residues in the trimerization domain of *K.lactis* HSF. We were able to show that in the cases of both Y326 and W346 all three residues in a trimer appear to be in identical environments, strongly suggesting that all subunits are parallel as proposed in the initial model.

We were also able to define subdomains of the trimerization domain. A 59-mer corresponding to the aa 303 - 361 of the *K. lactis* HSF (helix 1, predicted to form the central coiled-coil) was shown to form a triple stranded coiled-coil, although the T_m dropped by 50°C. On the other hand, a 25-mer derived from aa 354 - 378 (helix 2, predicted to form the buttressing helix) was shown to be very stable as a helix, but did not appear to form any specific complexes.

We have now continued to study the structure and function of the trimerization domain using site-directed mutagenesis.

B6-129 THE STABILITY OF HSC70 DEPENDS ON THE TEMPERATURE AT WHICH IT IS SYNTHESIZED, N. S. Petersen, J. Williams, and P. Young, Dept. of Molecular Biology, Laramie, WY. 8207

We have examined the question of why most hsc70 in *Drosophila melanogaster* is stable while hsp70 is unstable. Hsc70 made at 25°C in wing, brain and salivary glands is stable with a half-life of more than 24 hours. Hsp70 made during a 40 minute heat shock at 35°C decays rapidly with a half life of 2-3 hours during a chase at 25°C (Mitchell et al., 1985; Palter etal., 1986, Petersen, et al., 1992). In order to directly compare the stability of hsp70 and hsc70, we labeled 72 hour pupal wings, which make both hsp70 and hsc70, for 40 minutes at 35°C with 35S-methionine. The label was chased with excess cold methionine at 25°C. Both hsc70 and hsp70 made under these conditions are unstable with a half life of about 2 1/2 hours. This raises the question of whether conditions at 35°C such as the induction of a proteolytic system might affect the stability of both hsp70 and hsc70. To test for this possibility, Hsc70 was labeled at 25°C and chased at 35°C for the first 40 minutes followed by a longer chase at 25°C. This hsc70 made at 25°C was stable at 35°C and during the remainder of the chase at 25°C. The instability of hsc70 synthesized at 35°C, therefore, is not simply due to the cells ability to regulate levels of hsps so that excess is degraded. Rather, it indicates that hsc70 synthesized at 25°C is somehow different from hsc70 synthesized at 35°C. Possible differences include differences in post-translational modifications, differences in cellular location and differences in associated proteins. Experiments are underway to elucidate this difference. This research is supported by AFSOR grant #F49620-92-0234. Mitchell, H.K. et al. (1985) PNAS 82; 4969-4973.
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Petersen N. S. et al. (1992) Mol. Biol. of the Cell 3; 178a.

B6-130 DEVELOPMENTAL CONTROL OF SMALL HEAT SHOCK PROTEIN EXPRESSION IN PLANTS Ralf Prändl and Fritz Schöffl, Lehrstuhl für Allgemeine Genetik, Biologisches Institut, Universität Tübingen, 72076 Tübingen, Germany

Small heat shock proteins (sHSP) in plants are usually induced at elevated temperature whereas constitutive expression is restricted to certain developmental stages. We investigated the histological distribution of sHSP mRNA by *in situ* hybridization to tissue sections of *Arabidopsis* and tobacco plants. Under heat shock conditions sHSP mRNA was detectable in all tissues of the leaf with the highest signal in the vascular system. In contrast no signal was visible with tissue sections obtained from control leaves, indicating that sHSP expression is a consequence of environmental stress. Without heat shock sHSP mRNA could be detected in seeds of *Arabidopsis* and tobacco and exhibited a high tissue specifity. The meristematic tissues and the provascular system showed a high level of hybridization whereas the signal was much lower in other parts of the embryo.

A similar pattern of expression was detected in transgenic tobacco for a GUS reporter gene driven by a soybean HS promoter. The GUS activity was strictly heat inducible in the leaf but in seeds GUS activity could be detected without prior heat shock. Hence the soybean 17.3B promoter appears to be developmentally regulated in tobacco and therefore is a suitable model for the identification of *cis*-acting elements. Transgenic tobacco plants were generated containing chimeric genes with promoter deletions fused to the GUS reporter gene. The heat inducible expression of these genes was verified in leaf tissue and the developmental regulation of these genes will be investigated using mature seeds.

B6-131 **An HSF2-like DNA-binding activity is regulated during mouse development in absence of stress** Murielle Rallu, Marie-Thérèse Loones, Valérie Mezger Elisabeth Christians, Jean-Paul Renard and Michel Morange Unité de Génétique Moléculaire, Ecole Normale Supérieure, 46 rue d'Ulm 75005 PARIS - FRANCE.

Our work is mainly focused on the detailed analysis of the HSE-DNA binding activity (HSE-BA) displayed both by mouse embryo and by embryonal carcinoma cells (used as a model for early embryonic cells). We have examined the profile of HSE-BA in matured ovulated oocytes and during the preimplantation development by gel shift assay and quantification of the data by PhosphorImager. The heat-inducibility of HSE-BA appears to be tightly regulated: oocytes, one-cell and two-cell embryos respond correctly to heat shock whereas no HSE-BA can be induced at the four-cell stage. A progressive reappearance of the ability to induce HSE-BA by stress is observed between the eight-cell stage and the blastocyst stage, which parrallels the appearance of heat shock gene inducibility. We showed that this activity cross-reacts in gel-shift assays with polyclonal antibodies raised against HSF1.

In contrast to fibroblasts, there is a **constitutive HSE-BA** present at normal temperature in mouse embryo at the morula stage which increases at the blastocyst stage. This activity is distinct from HSF1 but is specifically recognized by anti-HSF2 antibodies.We were able to detect in gel-shift assays a DNA-binding activity in whole-cell extracts with as few as one hundred blastocysts. Until now, the sole case of HSF2 DNA-binding and transcriptional activation was evidenced upon *in vitro* differentiation with hemin of K562 erythroleukemia cells. No HSF2 DNA-binding activity is detected in mouse adult tissues. We have thus shown that mouse preimplantation embryo constitutes the first evidence of an *in vivo* DNA-binding activity due to HSF2.

Such a constitutive activity is also detected after implantation of the embryos and remains present at a high level between day 8.5 and day 10.5. The level of this HSF2-like activity strongly decreases in later stages in the entire embryo, except in the brain. At day 15.5, no more DNA-binding activity can be detected in the limb buds or in the tail and it begins to decrease in the brain. At any stages, absolutely no activity can be detected in yolk sac. Our preliminary *in situ* hybridization analysis with HSF1 and HSF2 antisense probes confirm the gel-shift assay experiments: a higher HSF2 RNA level is detected in regions of the embryo containing a high constitutive HSE-binding activity. On the contrary, HSF1 RNA level is much more homogenous along the whole embryo. Immunolocalizations and more detailled *in situ* hybridization experiments are currently in progress to exactly determine in which regions of the embryo HSF1 and HSF2 are expressed.

Many experiments suggest that HSF2 could have other targets than heat-shock genes. So it could be now interesting to determine the *in vivo* targets of this factor to try to understand its potential role in the development, since HSF2 is expressed, active and regulated very early in the mouse development and down-regulated later.

B6-132 **THE DECREASE IN HSP70 TRANSCRIPTION WITH AGE OCCURS BECAUSE OF A DEFECT IN HSF,**

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A characteristic feature of senescence is the progressive decline in the ability of an organism to respond to stress. Because heat shock proteins protect cells from a variety of stresses, we studied the expression of hsp70 by hepatocytes and spleen lymphocytes isolated from young (4 to 6 months of age) and old (24 to 28 months of age) male Fischer F344 rats. The induction of hsp70 synthesis and hsp70 mRNA levels by heat shock (42.5°C for 60 to 30 min) was 40-50% lower for cells isolated from old rats compared to cells isolated from young rats. Using *in situ* hybridization, it was found that the decline in hsp70 mRNA levels was not due to a age-related decrease in the number of cells that responded to heat shock; essentially all cells isolated from young or old rats responded to the heat shock and expressed hsp70. The age related decline in the induction of hsp70 synthesis and mRNA levels was paralleled by a decline in the nuclear transcription of hsp70. Therefore, the age-related decline in hsp70 expression occurs at the level of transcription. The effect of aging on the binding activity of the heat shock transcription factor (HSF) to the heat shock element (HSE) was studied using a gel shift assay. Cell extracts from hepatocytes or lymphocytes isolated from old rats showed significantly reduced (approximately 50%) HSF binding activity. Using a polyclonal antibody to HSF1, we have measured the levels of HSF protein in hepatocytes isolated from young and old rats by Western blots. The reduced HSE binding activity of HSF observed in old cells was not due to a decrease in the level of HSF1 protein; in fact, the HSF1 protein level was significantly higher in hepatocytes from old rats. Rather, it appears that the decrease in the induction of HSF binding activity by heat shock is due to a defect in the ability of the HSF to be converted from its inactive, monomeric form to its active (HSE binding) oligomeric form (supported by NIA grant AG 01548).

B6-133 Site Directed Mutagenesis Of A Highly Conserved Putative Nuclear Localization Sequence In Human HSP72, Marwan R. Salfity and Anne A. Knowlton, Cardiology Research, Baylor College of Medicine and the VAMC, Houston, Tx.

HSP72 rapidly translocates to the nucleus after heat shock and associates with the nucleoli. To elucidate the region responsible for nuclear localization, we reviewed the primary structure of the HSP72 molecule and selected the amino acids 243-263 as the probable nuclear localizing region based on the fact that this region is highly conserved and is very rich in basic amino acids. The following five mutations were done and confirmed by sequencing: A-Deletion of amino acids 243-263; B-Mutation of amino acid glutamine at position 254 to alanine; C-Deletion of amino acids 254-250 KRKHKK; D-Deletion of amino acids 256-261 KRAVRR; E-Mutation of amino acids 245-250 from KRKHKK to ARAHKA. Mutant and wild type HSP72 were tagged with an eight amino acid sequence at the carboxyl terminal end of the protein and subcloned into pRc/CMV. Lipofectamine was used to transfect Cos-1 cells grown on coverslips. Transfected Cos-1 cells were heat shocked at 43°C for two hours by immersion in a water bath, fixed after two hours at 37°C with formaldehyde and ethanol, and then incubated with tag -specific antibody followed by antimouse IgG-FITC. Nuclear and cytoplasmic staining were studied under ultraviolet light and patterns were compared for wild type protein and mutant proteins. All constructs localized to the cytoplasm in control, non-heat-shocked cells, and all constructs moved to the nucleus with heat-shock. We conclude that the highly conserved basic amino acid enriched region between amino acids 243-263 does not represent the nuclear localizing domain of HSP72.

B6-134 CHARACTERIZATION OF *dnaK* OF AN INTRACELLULAR SYMBIOTIC BACTERIUM IN APHID, Shigeharu Sato, and Hajime Ishikawa, Zoological Institute, Faculty of Science, University of Tokyo, Tokyo, Japan 113

The bacterium, *Buchnella aphidicola*, is an obligatory intracellular symbiont which can grow only in the mycetocyte of an aphid. It is reasoned that the intracellular environment should be stressful to these symbionts, and that the expression of the stress proteins by the symbionts should be elevated. In fact, it was observed that a large amount of a GroEL homolog, symbionin, is accumulated in *B. aphidicola*. The gene for symbionin (*symL*) and *symS* , which is a *groES* homolog, have been cloned and their sequences have been reported. However, there is no information on other stress proteins in this bacterium. To know structures of other stress proteins and the mechanism by which the bacterium regulates the expression of the stress genes, we cloned the homolog of *dnaK*, and characterized its structure and expression.

As a result, it was found that the *dnaK* gene of *B. aphidicola* encodes a protein highly identical to the *Escherichia coli* DnaK protein. In the meanwhile, the base substitutions of G/C to A/T were conspicuous, especially in the third position of the codons, as are the cases with other genes so far reported. In contrast to high accumulation of symbionin, the DnaK-homologous protein was scarcely detected in the bacterium isolated from its host, and its mRNA was much smaller in amount than *symSL* mRNA. These results suggest that the expression of *dnaK* in this bacterium is regulated differentially from that of *symL*, a *groEL* homolog, in response to the intracellular stress.

B6-135 ECTOPIC EXPRESSION OF CHIMAERIC HSF CAUSES CONSTITUTIVE ACTIVATION OF HSP SYNTHESIS IN TRANSGENIC *ARABIDOPSIS* PLANTS, Fritz Schöffl, Jeong H. Lee, Anja Hübel, Lehrstuhl für Allgemeine Genetik, Universität Tübingen, Auf der Morgenstelle 28, D-72074 Tuebingen, Germany

Both, C- and N-terminal fusions of the *Arabidopsis* HSF (Athsf1) with a reporter gene were constitutively expressed in transformed *Arabidopsis thaliana* plants and stably inherited into F1 generation. The recombinant protein allows detection by a new method of activity staining in gels after electrophoresis. The CaMV 35S promoter driven expression of the chimaeric HSF exceeds the expression of the authentic ATHSF1. The chimaeric HSF trimerizes and binds constitutively to HSE at normal temperature. The authentic wt HSF requires activation by heat stress. Under hs conditions chimaeric HSF forms also multimeric complexes with another protein in the cells, probably with ATHSF1. Transgenic plants show significant constitutive levels of both, HSP18 mRNA and protein at normal temperature (25 °C).

B6-136 ROLE OF HEAT SHOCK TRANSCRIPTION FACTOR 1 (HSF1) IN THE HEAT INDUCTION OF ALBUMIN IN EARLY EMBRYONIC RAT LIVER, Usha K Srinivas, S.K.Swamynathan and C.J.Revathi, Centre for Cellular and Molecular Biology, Hyderabad 500 007, India.

Expression of heat shock proteins is mediated by the interaction of heat shock transcription factor with well conserved heat shock elements present in the promoters of heat shock genes. Earlier, we reported the premature enhanced expression of albumin in early embryonic rat liver upon heat shock. Here show that the mechanism of heat induction of albumin is similar to the induction of hsps upon heat shock. There are functional HSE like sequences and GAGA factor binding sites within the -450 bp region of rat albumin promoter. Gel retardation assays done with synthetic oligonucleotides representing putative HSEs in the rat albumin promoter and the rat liver nuclear extracts show that both HSF1 and HSF2 bind to this region in a sequence specific and reversible manner. Similar assays done with oligonucleotides representing the GAGA repeats in albumin promoter and H411 cell extracts show that the GAGA factor binds rat albumin promoter, again, in a sequence specific manner.

We have constructed vectors carrying CAT reporter gene under the control of different fragments of rat albumin promoter. The functional significance of HSEs and GAGA elements was established using chloramphenicol acetyl transferase assays done with the extracts from normal and heat shocked H411 cells transfected with the above plasmids. Our results show that the CAT activity in the transfectants varies with the size and orientation of the albumin promoter region that is directing the CAT gene. Results also show that the albumin promoter activity is modulated by temperature through HSFs and GAGA factor. We have cloned and expressed the rat HSF1 in pGex vector. The possible role of rat HSF1 in the induction of HSPs during embryonic development will be discussed.

B6-137 THE CLONING AND ANALYSIS OF THE MAMMALIAN 110 kDa HEAT SHOCK PROTEIN.

J. Subjeck, M. Murawski, X. Chen, R. Burd, D. Easton and D. Yoon. Roswell Park Cancer Institute, Buffalo, NY 14263.

We have screened a cDNA expression library using an antibody prepared against the mammalian 110 kDa heat shock protein (hsp110). Two clones were identified and a Northern blot analysis indicated that both were strongly heat inducible and hybridized to a message of approximately 3.5 kb in hamster and mouse. These clones and the Rapid Amplification of cDNA ends technique were used to obtain a full length sequence. By comparison with GenBank protein sequences hsp110 was found to share about 30%-33% identity with the hsp70s, most of which occurs in the amino terminal-halves of these molecules. The carboxyl-terminal half of hsp110 differs considerably from the hsp70s. A very highly conserved segment found at approximately 400 a.a. in every hsp70 sequence examined and containing the proximal portion of the putative peptide binding site (Rippmann et al, EMBO J., 10: 1053, 1991) is deleted from hsp110. Hsp110 contains an insertion of 100 a.a. in length not observed in the hsp70s. Five GenBank sequences were found to be highly similar to hsp110 including the *sea urchin egg receptor for sperm* (Foltz et. al. Science, 259, 1421-1425, 1993) and additional sequences from human, C. elegans and yeast (2) of unknown function. All bear the same insertion and deletion described above and also contain a pattern of highly conserved regions of sequence unique to this group and not present in the hsp70s. Moreover, a Southern blot analysis using a probe derived from this conserved region strongly cross reacts with sequences from human to yeast. Therefore, hsp110 would appear to belong to a new category of large and structurally unique stress proteins which are the most distantly related known members of the hsp70 family. Murine tissue analysis indicates that hsp110 is present in all tissues but is highly expressed in brain and liver. Initial localization studies using designed peptide antibodies suggest that hsp110 is constitutively associated with nucleoli and various cytoplasmic structures which may include the endoplasmic reticulum. The functional significance of this family of stress proteins and the manner in which they differ from previously studied hsp70s is unknown. Lastly, progress in the sequencing of GRP170, another mammalian hsp110-like protein will be presented.

B6-138 DIFFERENT PROTEIN KINASE ACTIVITIES ARE INDUCED BY HEAT SHOCK AND ARSENITE : SEPARATION BY MONO Q CHROMATOGRAPHY AND COMPARISON WITH THE MAP KINASES

Sylviane Trigon[1] and Michel Morange[1], John Rouse[2] and Philip Cohen[2].

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We are interested in the protein modifications occurring after a stress, before the beginning of heat shock protein synthesis. We already described the induction, immediately after a heat or a sodium arsenite shock, of a protein kinase activity. It has been called HS-CTD kinase for its ability to phosphorylate, *in vitro*, a synthetic peptide composed of four repeats of the motif Ser-Pro-Thr-Ser-Pro-Ser-Tyr. This motif is highly repeated in the Carboxy-Terminal Domain (CTD) of the largest subunit of eucaryotic RNA polymerase II.

By using peptides differing by one amino-acid from the consensus SPTSPSY, we showed that the sequence phosphorylated by the HS-CTD kinase activity is (-P-X-S/T*-P-). This sequence is very similar to the consensus sequence phosphorylated by the major members of the Extracellular Regulated Kinase family (or MAP kinases). Several protein kinases are responsible for this CTD kinase activity induced by stress in HeLa cells. Mono Q chromatography allows the separation of three CTD kinase activities, one of which is precipitated by anti ERK1-ERK2 antibodies. The two other CTD kinase activities are immunologically distinct from ERK1 and ERK2 and they use differently serine or threonine as the phosphate acceptor.

Pr. P. Cohen demonstrated that another protein kinase, also induced by stress, different from the HS-CTD kinases, activates the MAPKAP-kinase-2. MAPKAP kinase-2 might be responsible for the phosphorylation of HSP 27 *in vivo*. The new protein kinase has been called (RK) for Reactivating Kinase. It is induced by heat or sodium arsenite but does not respond to growth factors on the opposite to MAP kinases. RK is recognized by antibodies raised against a Xenopus MAP kinase (mpk2). Moreover, a RK kinase has also been identified, showing that RK might be a component of a new protein kinase cascade, leading to the phosphorylation of the low molecular weight heat shock proteins.

B6-139 RELATIONSHIP BETWEEN THE THERMAL DENATURATION OF CELL PROTEIN AND THE HEAT SHOCK INDUCED HSP70 SYNTHESIS,

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Biochemical analysis of essential elements of the hsp70 regulatory cycle was performed in order to be used in the mathematical model of Peper et al (abstract in parallel). Reuber H35 rat hepatoma cells were cultured and heat shocked under highly standardized conditions. We determined cell volume, cellular protein content, rate of protein synthesis and decay, polyribosome size and concentration of nascent protein chains under culturing conditions. The levels of hsp70 were determined by long term labelling as well as by immunoblotting.

H35 cells were exposed to temperatures up to 43 °C for maximally 1 hour. The heat shock response of these cells was determined by measuring the inhibition and recovery of the protein synthesis, the induction and decay of HSF activation, the increase and decline of hsp70 and mRNA levels, hsp70 synthesis and hsp70 levels after the different treatments. Furthermore, differential scanning was used for measuring the protein denaturation during heat shock. The level of free and complexed hsp70 was determined after lysis of the cells at low detergent concentrations followed by electrophoresis of the lysate under non-denaturating conditions in the first dimension and SDS-PAGE in the second. The biochemical data obtained were used for feeding and fine tuning the mathematical model. The interaction between biochemical measurements and mathematical modelling largely facilitated the research and resulted in a better insight into the dynamics of the relationship between thermal denaturation of cell protein and heat shock induced hsp70 synthesis.

B6-140 **THE FUNCTION OF HEAT-SHOCK PROTEINS IN STRESS TOLERANCE OF HEPATOMA CELLS,** Anikó Venetianer, Melinda Pirity and Anna Hevér-Szabó, Institute of Genetics, Biological Research Center, Hungarian Academy of Sciences, Szeged, Hungary

We earlier demonstrated that hsp68 is **deficiently** induced upon stress in the glucocorticoid-resistant rat hepatoma clone 2 cells, but is strongly activated in the glucocorticoid-sensitive Faza 967 cells from which it was derived. We used the clone 2 cells to address the questions of whether hsp68 is specifically involved in the development of thermotolerance, thermoresistance or drug resistance. Our experiments show that clone 2 cells were not protected from the killing effect of heat by pre-treatment with sodium arsenite, whereas Faza 967 cells were. These results strongly suggest a role of hsp68 in the development of thermotolerance in hepatoma cells. Stable **heat-resistant** variants of clone 2 cells were also isolated by repeated cycles of heat exposure, where an increased basal expression of several hsps was observed together with the (at least partial) restoration of the heat-inducibility of hsp68 indicating that several hsps are needed to protect the cells at high temperature. Using increasing concentrations of colchicine we isolated cell lines with higher resistance to several anticancer drugs (doxorubicin, vinblastine, actinomycin D, colchicine, puromycin) from clone 2. The induction deficiency of hsp68 was maintained in these **multidrug resistant** clone 2 cells suggesting that hsp68 inducibility is not a prerequisite for drug resistance in hepatoma cells.
The heat-resistant hepatomas became also resistant to several anticancer drugs. The multidrug resistance of the hepatoma variants correlates with the overexpression of the P-glycoprotein and the corresponding mRNA and decreased retention of doxorubicin. Our results showing that severely stressed heat-resistant hepatoma cells overexpressed the mdr gene(s) raise the possibility that P-glycoprotein may participate in the protection against stress.

B6-141 HSP27 IN SERTOLI CELLS: GERM CELL-STIMULATED HSP27 PHOSPHORYLATION, MRNA LEVELS DURING SPERMATOGENESIS, AND HSP27 CO-LOCALIZATION WITH MICROFILAMENTS. M J Welsh, C DeGuzman, R Gilmont, Y Jia, M Parvinen and W Wu, Department of Anatomy & Cell Biology, University of Michigan Medical School, Ann Arbor, MI 48109.

In many phyla an exquisite coordination of spermatogenesis occurs that is believed to be controlled by interactions between germ cells and Sertoli cells. Modest gains in knowledge have been made concerning how Sertoli cells provide for and control germ cell differentiation, but little is known about how or why germ cells communicate with Sertoli cells. We have found that cultured Sertoli cells rapidly respond to germ cells or germ cell-conditioned medium by phosphorylation of two proteins, one of which we have identified as hsp27. In rats, segments of seminiferous tubule containing the same stages of developing germ cells can be seen and isolated for biochemical analysis, using a dissecting microscope and transmitted light. We have determined that the amount of hsp27 mRNA in isolated seminiferous tubule segments varies in relation to the stage of germ cells present in the tubules, and that the mRNA is predominantly present in Sertoli cells of the tubules and not the germ cells. Hsp27 has been localized, by immunofluorescence, in frozen sections of rat testis, in whole rat seminiferous tubules, and in cultured rat Sertoli cells. Microfilaments were also localized using rhodamine-phalloidin or BODIPY-phallicidin. Hsp27 colocalized with microfilaments only in Sertoli cells. In all other cells types, hsp27 was diffusely distributed in the cell cytoplasm even though distinct microfilaments were present in the cells. Colocalization of hsp27 with microfilaments in Sertoli cells was seen only in differentiated Sertoli cells (i.e. in cells from rats >3 weeks of age) and not in undifferentiated Sertoli cells. In rats treated with a single low dose of cadmium (a testicular toxicant known to induce hsp27 synthesis) after 24h Sertoli cell microfilament bundles were disrupted and failure of sperm release occurred in seminiferous tubule segments that normally would be expressing decreasing and/or relatively low levels of hsp27 mRNA. We conclude that Sertoli cells respond to germ cell signals by a pathway involving hsp27 phosphorylation and that hsp27 expression is regulated during spermatogenesis. The results also indicate that at least two forms of microfilaments exist in non-muscle cells, one form with significant amounts of associated hsp27 and one without, the latter being more widely expressed than the former. We also propose that induction of hsp27 synthesis is a marker for and may play a role in cell-specific toxicant damage, particularly in Sertoli cells of the testis. (Supported by NIH grant ES06265 and by March of Dimes Birth Defects Foundation grant #15-FY94-0705 to MJW).

B6-142 STABLE AND SPECIFIC BINDING OF HSP90 BY THE BENZOQUINONE ANSAMYCINS INHIBITS GLUCOCORTICOID RECEPTOR FUNCTION, Luke Whitesell, Paul H. Cook and Rochelle Bagatell, Department of Pediatrics, University of Arizona School of Medicine, Tucson, AZ 85724.
In the absence of ligand binding, the glucocorticoid receptor (GR) exists as a multiprotein, cytosolic complex which contains, among other components, two molecules of the stress protein HSP90. Hormone binding to the receptor induces HSP90 dissociation and translocation of the receptor to the nucleus where it binds DNA and activates target gene transcription. We have previously reported that the benzoquinone ansamycins bind HSP90 in a stable and specific fashion. This binding disrupts HSP90 association with v-src kinase and abrogates its transforming activity. We now report that ansamycin interaction with HSP90 also inhibits glucocorticoid receptor function.

Cells were stably transfected with a reporter construct expressing CAT under control of the steroid-inducible MMTV-LTR promoter. Treatment of these cells with dexamethasone induced CAT activity while treatment with the ansamycin Macbecin II had little effect on basal CAT activity. Simultaneous treatment with the two agents, however, resulted in near complete inhibition of dexamethasone induction of reporter expression. Western blot analysis of lysate from Macbecin-treated cells demonstrated a marked depletion of cytosolic GR protein. In order to define the mechanism underlying these findings, we are examining ansamycin effects on GR-HSP90 complex formation in reticulocyte lysate as well as drug effects on GR half-life, intracellular localization and [3]-dexamethasone 21-mesylate binding. The benzoquinone ansamycins are proving useful probes to study the role of HSP90 in steroid receptor function. They may provide a novel therapeutic approach to the interruption of hormone signalling downstream of current agents which target ligand-receptor interaction.

B6-143 *YERSINIA ENTEROCOLITICA* STRESS PROTEINS INDUCED IN THE INTRACELLULAR ENVIRONMENT OF THE PHAGOCYTIC CELL, Tomoko Yamamoto, Tomoko Hanawa, Sachio Ogata, and Shigeru Kamiya, Department of Microbiology, Kyorin University School of Medicine, 6-20-2 Shinkawa, Mitaka, Tokyo 181, Japan

Yersinia enterocolitica is a facultative intracellular pathogen which survives in phagocytic cell. We previously showed that phagocytosed *Y. enterocolitca* exhibits a global stress response to the hostile environment within macrophage (Yamamoto et al., 1994. Microbiol. Immunol. 38:295-300). At least 16 bacterial proteins were selectively induced in response to phagocytosis and several out of these proteins were induced by various *in vitro* stress stimuli. To know the role of bacterial stress proteins within the phagocytic cell, we characterized the stress proteins of *Y. enterocolitica* induced by intracellular growth. The immunological analysis revealed that two of these proteins were homologue of DnaK and CRPA (Cross-Reacting Protein Antigen, GroEL homologue). In addition, GroES-like protein and GrpE-like protein were confirmed by analysis of their N-terminal amino acid sequences. The proteins with a molecular mass of 18.5 kDa, 36.0 kDa, and 38.0 kDa, respectively which were induced in all of the stress conditions that we examined were also induced in the intracellular environment. The results suggest that multiple regulons are involved in the response which possibly contributes to the survival of *Y.enterocolitica* within phagocytic cell.

B6-144 GLUTAMINE INCREASES HSP70 EXPRESSION IN RAT SKELETAL MUSCLE L6 CELLS, Xiwu Zhou and James.R. Thompson, Department of Animal Science, University of British Columbia, Vancouver, Canada V6T 1Z4

Heat shock proteins (hsp) protect normal intracellular proteins against various stressors. Increasing the availability of glutamine to cells has been suggested to increase hsp70 expression. Since skeletal muscle produces glutamine quantitatively and often experiences physiological stressors (e.g. elevated temperature during exercise), we determined if glutamine benefits hsp expression in stressed skeletal muscle cells.

L6 skeletal muscle cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum. When the cells fused into myotubes, they were cultured in either DMEM or Krebs Ringer bicarbonate (KRB) buffer supplemented with 0, 2, or 5 mM glutamine and treated with heat shock (43°C for 45 min). The cells were labelled with ^{35}S-methionine for 2 h following heat shock to measure protein synthesis. Total RNA was isolated for measurement of hsp70 mRNA by Northern blotting analysis. Protein samples were prepared for SDS-PAGE and Western blotting analysis.

Our results show that hsp70 protein expression in the heat-shocked L6 cells increases as the concentration of glutamine increases. When glutamine was increased from 0 to 2 and 5 mM, in cells cultured in DMEM hsp70 synthesis increased by 49% and 93% ($P<0.05$), respectively. In cells cultured in KRB hsp70 synthesis increased by 31% ($P<0.05$) and 55% ($P<0.01$), respectively. The results obtained from SDS-PAGE autoradiography were verified by Western blotting. Northern blotting analysis showed that the expression of hsp70 mRNA did not change as glutamine concentration in the culture media increased. Addition of 2 or 5 mM glutamine to L6 cells cultured at normal temperature (37°C) but starved of glutamine did not increased hsp70 expression. Our results suggest that glutamine may regulate hsp70 expression at the translational or post-translational levels in stressed L6 muscle cells.

B6-145 COMPARISON OF THE ATPASE ACTIVITIES OF CYTOPLASMIC, MITOCHONDRIAL, AND ENDOPLASMIC RETICULUM STRESS70 MOLECULAR CHAPERONE PROTEINS FROM MAIZE ENDOSPERM. Rengang Zhou and Jan A. Miernyk, Phytoproducts Research Unit, U.S.D.A, Agricultural Research Service, National Center for Agricultural Utilization Research, Peoria, IL 61604

The Stress70 proteins have a low basal level of ATPase activity, and it is thought that this activity is important in molecular chaperone function. Subcellular fractions were isolated from cultured maize endosperm cells, and the resident Stress70 proteins purified by a combination of immobilized ATP affinity chromatography and anion-exchange FPLC. Purity was evaluated by SDS-PAGE and Western blotting using organelle-specific anti-peptide antibodies. The ATPase activity of each of the three classes of native Stress70 proteins was characterized with respect to temperature, pH, nucleotide specificity and cations. The ATPase activity of each of the Stress70 proteins was stimulated more than 5-fold by mM concentrations of potassium. Short synthetic peptides stimulated ATPase activity 2- to 10-fold, depending upon the sequence. ATPase activity was also stimulated up to 2-fold by free amino acids. The E. coli DnaJ protein stimulated the ATPase activities of the plant Stress70 proteins approximately 5-fold. DnaJ plus GrpE resulted in slightly more stimulation than DnaJ alone. In no case was stimulation of ATPase activity by the various factors additive.

B6-146 TOWARDS THE THREE-DIMENSIONAL STRUCTURE OF THE PEPTIDE BINDING DOMAIN OF HSC, Erik R.P. Zuiderweg, Robert C. Morshauser, Hong Wang, and Gregory Flynn† , Biophysics Research Division / Department of Biological Chemistry, The University of Michigan, Ann Arbor MI 48109 and †Institute of Molecular Biology, University of Oregon, Eugene OR 97403

We concentrate on the family of 70 kDa heat shock proteins (Hsp70) which bind to nascent peptide chains and peptide chains destined for transport to cellular organelles. It is our interest to gain insight in the function of these chaperones by studying the structure and dynamics of their peptide binding domains (and their complexes with peptides) using multi-nuclear, multi-dimensional NMR methods.

We have obtained good expression and high quality NMR spectra on the fragment 400 - 562 from the heatshock cognate protein (hsc) from rat cytosol. This fragment, that maps directly C-terminal of the ATP-binding domain, was shown to be necessary and sufficient for the peptide binding of the full Hsp70 molecule.

To date, we have obtained the heteronuclear NMR resonance assignments for the backbone nuclei of the Hsc peptide binding domain (HscPDB, 18 kDa). Assessment of the secondary structure from the NMR data shows that HscPBD contains (only) one long alpha helix and at least eight strands of extended structure. Initial alignment of extended elements in mostly anti-parallel β-sheet revealed a surprising topology, very different from that predicted from modeling studies.

At the meeting, we will present the validation (or not) of this topology and report progress towards the determination of the three-dimensional structure of the peptide binding domain . It is also anticipated that we will be able to present some NMR-monitored data on the binding of peptides to this fragment.

Molecular Chaperones

B6-200 GROES HOMOLOGUE SPECIFICALLY REQUIRED TO FOLD THE MAJOR CAPSID PROTEIN OF PHAGE T4 BECAUSE OF LOCALIZED SEQUENCE DETERMINANTS, Joanne D. Andreadis, Zhaojun Ren, and Lindsay W. Black, Department of biological Chemistry, University of Maryland AT Baltimore, Baltimore, MD 21201.

GroEL and groES are members of a ubiquitous class of folding factors called chaperonins, which are required to fold proteins *in vivo*. Bacteriophage T4 requires the *E. coli* protein groEL and a phage groES homologue, gp31, for the correct folding and oligomerization of the T4 major capsid protein, gp23, during head morphogenesis. Using expression vectors we are able to show that coexpression of gene *31* with gene *23* in the presence of groEL protein is necessary and sufficient to form open-ended tube structures called polyheads *in vivo* and that groES is unable to function effectively in this role. Thus, although the T4 co-chaperonin gp31 may have some general chaperonin capability, it is specifically required during the groEL-dependent folding of the major capsid protein. We have also studied two T4 *bypass31* mutants originally detected by their ability to suppress gene *31* amber mutations. These mutations, which allow the major capsid protein to fold less efficiently in a groEL chaperonin independent mode, were mapped within gene *23* (1). We have now sequenced these mutations to sites of gp23 which appear critical for folding. This suggests that the requirement for specific chaperonin assisted folding lies in highly localized sequence determinants within the potential target protein. Our results suggest that certain co-chaperonins can display specificity for folding critical target sequences.

1. Simon, L.D. and Randolph, B. (1984) *J. of Virol.* **51**, 321-328.

B6-201 αB-CRYSTALLIN IS CHAPERONE FOR MICRO-TUBULE ASSEMBLY (Y. Atomi, H.Arai and Y. Hashimoto) Department of Life Sciences, College of Arts and Sciences, University of Tokyo, Tokyo 153

In our previous studies, we identified 22-kDa protein which specifically decreased in muscle atrophy and localized at Z-bands, as αB-crystallin (αB). The mRNA and protein expression of αB increased with mechanical stimulus of muscle. Although recent *in vitro* studies showed that αB not only played a role as molecular chaperone for proteins denatured by heat stress, but also had a autokinase activity, the role of it in non-lenticular tissues and cultured cells were unknown yet. Associated proteins with αB (L6E9etc.) were examined by biochemical and immunocytochemical analysis. Anti αB C-terminal peptide polyclonal antibodies were produced and purified by peptide affinity chromatography. Analyzing by confocal microscopy, it was surprisingly found that αB colocalized microtubule network as well as intermediate filament (vimentin) in immunofluorescent stained L6E9 cells. Immuno-precipitates for cell and muscle lysates were a large heteropolymer composed of tubulin, vimentin and some other proteins in addition to αB analyzed by immunoblotting. *In vitro* binding analysis of purified tubulin and αB showed that it increased microtubule assembly, especially under the existence of calcium ions. Further immunostaining against anti-αB antibody was seen both at the nucleus and cell attachment regions in cultured cells except around mitotic phase. Taken together, αB plays a role of shaperones for cytoskeletal networks as well as seems deeply associated with mechanical signal transduction from cell membrane to nucleus, relating to calcium ions and/or possible phosphorylation. In conclusion, αB-crystallin unexpectedly functionates associating with microtuble networks as well as intermediate filament as cytoskelton stabilizing protein like as previously suggested.

B6-202 INTERACTIONS BETWEEN β-GALACTOSIDASE AND THE GroELS CHAPERONE, Amanda Ayling and François Baneyx, Department of Chemical Engineering, BF-10, University of Washington, Seattle, WA 98195.

We have investigated the formation of complexes between the GroELS molecular chaperone machine and *E. coli* β-galactosidase, a tetrameric protein whose 116 kDa protomer should not completely fit into the central cavity of the $(GroEL)_{14}$ toroid. Light scattering experiments indicate that GroEL suppresses the aggregation of chemically denatured β-galactosidase upon dilution in appropriate refolding buffers. However, this interaction is a weak one as we were unable to isolate a stable GroEL-β-galactosidase complex by gel filtration chromatography. The presence of GroEL alone, without the addition of GroES and ATP, is sufficient to double the initial rates of β-galactosidase refolding and to increase the overall recovery of active enzyme two-fold compared to refolding buffer alone, as judged by activity experiments. Non-denaturing PAGE and Western blotting further indicate that GroEL alone increases the yields of the monomeric, dimeric, and tetrameric forms of the protein. Addition of GroES and ATP to pre-formed GroEL-β-galactosidase complexes reduces the positive effect of GroEL on the β-galactosidase activity regain, and does not markedly affect the ability of GroEL to suppress aggregation in light scattering experiments. The unusual behavior of this system is discussed in terms of the nature of the interaction, availability of substrate binding sites on GroEL, and competition between aggregation and proper refolding pathways in the presence and absence of chaperones.

B6-203 MODULATION OF hsc70 SUBSTRATE BINDING AND ATPase ACTIVITY BY HSJ1 PROTEINS, Michael E. Cheetham and Brian H. Anderton, Department of Neuroscience, Institute of Psychiatry, De Crespigny Park, London, SE5 8AF, U.K.

Human HSJ1 proteins are expressed as two distinct isoforms, HSJ1a and HSJ1b, with different C-termini. These isoforms are generated by alternative splicing and appear to be neuron-specific in expression. HSJ1 proteins are members of the DnaJ family of molecular chaperones. They have an N-terminal 70 amino acid 'J' domain and a glycine/phenylalanine rich spacer in common with *E. coli* DnaJ. However, the homology between DnaJ and HSJ1 proteins is limited to these regions and does not extend to the other two of the four canonical *E.coli* DnaJ domains. DnaJ interacts with the hsp70 family of stress proteins to modify hsp70 substrate binding and enhance hsp70 ATPase activity, this interaction is conserved for the yeast proteins YDJ1 and SSA1(hsp70). YDJ1 shows full domain conservation with DnaJ. However, expression of HSJ1 proteins in YDJ1 null yeast can partially recover the wild type phenotype, suggesting that the DnaJ-like protein/hsp70 interaction is mediated by elements common to both proteins that are conserved throughout evolution.

In this study, we have investigated, *in vitro,* if recombinant HSJ1 proteins, despite their limited homology to DnaJ, modulate the function of the constitutive porcine brain hsp70, hsc70. The weak intrinsic ATPase activity of hsc70 is enhanced more than 5-fold by stoichiometric amounts of both HSJ1a and HSJ1b. This enhancement is mediated by an increase in the rate of bound ATP hydrolysis, whereas the rate of ADP release is unaffected. HSJ1 proteins also appear to regulate the affinity of hsc70 for the permanently unfolded substrate CMLA. In the presence of ATP, HSJ1 proteins reduce hsc70:CMLA complex formation both in the presence and absence of K^+. This argues that HSJ1 proteins induce a conformational change in hsc70 that can mimic the effect mediated by K^+, and therefore modulate hsp70 substrate binding by another mechanism than merely stimulating the hsp70 ATPase activity. As HSJ1 proteins have limited homology to DnaJ, we suggest that this action is mediated by the 'J' domain alone, and that this modulation of hsp70 substrate binding will be common to all proteins that contain a 'J' domain and, therefore, that DnaJ-like proteins are potent modulators of hsp70 function in eukaryotes.

B6-204 ATPase AND LUCIFERASE REFOLDING ACTIVITIES OF BACTERIAL AND YEAST HSP70 CHAPERONES AND MODULATORS, William J. Chirico[1], John McCarty[2], Bernd Bukau[2], and Ellen J. Levy[1], [1]Department of Anatomy and Cell Biology, State University of New York Health Science Center at Brooklyn, Brooklyn, NY 11203 and [2]Zentrum für Molekulare Biologie, Universität Heidelberg, D-69120 Heidelberg, Germany

Hsp70 molecular chaperones possess a low intrinsic ATPase activity that is greatly stimulated by protein modulators. The ATPase activity of DnaK, an Hsp70 of *E. coli*, is increased by DnaJ and GrpE. The ATPase activity of Ssa1p, an Hsp70 of *S. cerevisiae* cytosol, is increased by the DnaJ homolog Ydj1p. Modulation of chaperone ATPase activity may control release of bound polypeptide substrate and subsequent folding. To learn more about the evolutionary conservation of Hsp70 and modulator function, we examined whether the ability of modulators to stimulate ATPase and luciferase refolding activities is conserved between the DnaK and Ssa1p chaperone systems. Both DnaJ and Ydj1p stimulated the ATPase activity of DnaK and Ssa1p. In contrast, GrpE greatly stimulated the ATPase activity of DnaK, but not of Ssa1p, in the presence of either DnaJ homolog. Although GrpE formed stable complexes with DnaK, complexes with Ssa1p were not detected using native polyacrylamide gel electrophoresis. Ydj1p poorly substituted for DnaJ during refolding of denaturant-unfolded firefly luciferase in the presence of DnaK and GrpE. Ssa1p with either Ydj1p or DnaJ refolded luciferase. Together, these results indicate that interactions between Hsp70s and DnaJ homologs are more evolutionarily conserved than those with GrpE. They also suggest potential differences in chaperone requirements for protein folding between prokaryotes and the cytosol of eukaryotes.

B6-205 THE UNFOLDED PROTEIN RESPONSE SIGNAL TRANSDUCER Ire1p PROMOTES SECRETION OF HETEROLOGOUS PROTEINS IN *Saccharomyces cerevisiae*. Howard Clarke, Jeffrey McGrew, and Virginia Price, Immunex Research and Development Corporation, 51 University Street, Seattle, WA 98101.

Efficient secretion of heterologous proteins in the yeast *Saccharomyces cerevisiae* requires proper transport into the endoplasmic reticulum (ER) and subsequent post-translational modification and folding. ER resident proteins such as protein disulfide isomerase (Pdi1p) and chaperones like Kar2p facilitate folding of secreted proteins into the correct conformation. Regulated expression of the *PDI1* and *KAR2* genes requires Ire1p (Ern1p), a putative transmembrane kinase that activates transcription in response to unfolded proteins in the ER. We are investigating the role of *IRE1* in the secretion of human cytokines in *S. cerevisiae*. Mutants have been generated with targeted disruptions of *IRE1* that show reduced levels of expression of these human proteins. Our results indicate that *IRE1* is involved in foreign protein secretion and suggest that overexpression or increased activation of *IRE1* may result in increased expression of foreign proteins. A reporter gene construct has been generated that responds to unfolded proteins through an *IRE1* mediated signal transduction pathway. This reporter gene construct enables us to examine the role of an increased unfolded protein response on heterologous protein secretion.

B6-206 CRYSTALLOGRAPHIC STUDIES OF GROEL WITH BOUND NUCLEOTIDE ANALOGUES, Paul M.G. Curmi, Sarah J. Tilley and Nick E. Dixon, IBiS, School of Physics, University of New South Wales, Sydney, NSW 2052 and CMSF, Research School of Chemistry, Australian National University, Canberra, ACT 2601, Australia

A full understanding of the workings of the chaperonins in protein folding will require several atomic structures in various functional states. Crystallographic studies on other NTPases such as EF Tu and the F1-ATPase have shown that quite dramatic structural changes can be involved in coupling NTP hydrolysis with protein function. The elucidation of these large-scale structural changes can only be achieved by the co-crystallisation of the protein with substrate analogues.

The bacterial chaperonin GroEL has been overexpressed in *E. coli*. Several crystal forms have been grown in the presence and absence of nucleotide analogues under similar crystallisation conditions. The presence of nucleotide analogues alters the physical form and space group of the crystals. We have collected native data from these crystal forms and are currently analysing the symmetry and working towards an atomic resolution structure determination.

B6-207 THE CHAPERONIN GroEL IS INVOLVED IN RNA BINDING AND STABILIZATION, Dimitris Georgellis, Bjorn Sohlberg, Franz U. Hartl and Alexander v. Gabain, Institute of Microbiology and Genetics, Vienna Biocenter, Dr Bohr gasse 9, A-1030 Vienna, Austria.

GroEL is an absolute requirement for bacterial cell growth . Mutations in the groE gene have shown various phenotypes, including temperature-sensitive growth, altered permeability and fragility, altered RNA synthesis and inability to assemble phage particles. The role of GroEL as a chaperonin in mediating protein folding and assembly is currently raising considerable interest. In fact, GroEL was suggested to affect mRNA decay[1]. In the present study we provide evidence for a novel function of GroEL. In a modified form it is required to form an RNA binding complex that provides protection against nuclease degradation. Our conclusions are supported by the following observations: using specific GroEL antibodies, GroEL was identified as a constituent of a protein-RNA complex; this RNA binding complex was able to confer RNase-resistance to the RNA; the bound RNA was released from the binding complex by addition of Mg^{++} in combination with certain nucleotides; and GroEL-specific monoclonal and polyclonal antibodies were able to neutralize the RNA protection activity. 2-D gel electrophoresis showed the RNA-binding form to differ from the one with protein folding properties. In addition, the physiological relevance is supported by the observation of an increased binding activity in anaerobically growing cells where mRNA decay is down-regulated. Our results suggest that GroEL, in addition to its role in protein folding, is involved in RNA binding and regulation of mRNA stability.

1. Sohlberg et al. (1993) PNAS 90, 277-281.

B6-208 HSP104-MEDIATED REACTIVATION OF HEAT-DAMAGED REPORTER ENZYMES *IN VIVO* AND *IN VITRO*. J.R. Glover, D.A. Parsell, A.S. Kowal, M.A. Singer, and S. Lindquist. Department of Molecular Genetics and Cell Biology and The Howard Hughes Medical Institute. The University of Chicago, Chicago, IL 60637

Hsp104 is required for induced thermotolerance in *S. cerevisiae* cells. The function of Hsp104 in thermotolerance was probed by comparing reporter enzyme activity in isogenic strains with and without a functional *HSP104* gene. A constitutively expressed bacterial luciferase fusion protein was progressively inactivated and rendered insoluble at 44°C in both strains. During recovery at 25°C in the presence of cycloheximide, luciferase activity was regained and the protein resolubilized only in the strain expressing Hsp104. Dispersal of cytosolic and nuclear aggregates observed by electron microscopy was also Hsp104-dependent. To study in more detail the mechanism of Hsp104 function, an *in vitro* assay for refolding of heat-inactivated firefly luciferase was established. Purified Hsp104 did not reactivate luciferase. Lysates from yeast which do not express Hsp104 could stimulate the activity of purified Hsp104. Refolding of luciferase under these conditions is dependent on ATP hydrolysis. Attempts to identify the factor(s) required for Hsp104 activty *in vitro* are currently under way.

B6-209 STRESS-REGULATED PROTEIN FOLDING BY INTER-CONVERSION OF TWO CHAPERONIN HETERO OLIGOMERS, Abdussalam Azem, Sophie Diamant, Celeste Weiss and Pierre Goloubinoff, Department of Botany. Alexander Silberman Institute of Life Sciences. The Hebrew University of Jerusalem, 91904 Jerusalem. Israel.

Chaperonins GroEL and GroES form two types of hetero oligomers in vitro, which serve as intermediates of a stress-regulated protein-folding cycle. Chemical crosslinking and kinetic analysis show that in the asymmetric GroEL$_{14}$GroES$_7$ ("bullet") hetero oligomer, ATP hydrolysis and the protein folding mechanism are uncoupled. A stress-destabilized protein can bind and be subsequently released in a folding-competent state, only from the symmetric GroEL$_{14}$(GroES$_7$)$_2$ ("football") chaperonin hetero oligomer. During a heat-shock, the football dissociates into a bullet chaperonin, which than serve as a trap for stress-destabilized proteins. When to stress is over, footballs reconstitute and chaperonin-assisted protein folding resumes.

B6-210 IDENTIFYING PATHWAYS IN THE SPONTANEOUS AND GROEL-ASSISTED REFOLDING OF GLUCOSE-6-PHOSPHATE DEHYDROGENASE BY USING GROEL TO TRAP FOLDING INTERMEDIATES, John E. Hansen and Ari Gafni, Department of Biological Chemistry and Institute of Gerontology, University of Michigan, Ann Arbor, MI 48109-2007

We have previously reported that the efficiency of the GroEL-assisted reactivation of bacterial glucose-6-phosphate dehydrogenase (Glu-6-PDH) is strongly dependent on temperature. A switch from enhanced to fully arrested reactivation occurs over a narrow temperature range - from 25° to 30° C. This switch was employed in the current study to trap folding intermediates from both the spontaneous and GroEL-assisted renaturation of Glu-6-PDH. These renaturations were initiated at 2° C to slow down the refolding process, which allowed us to more easily follow individual folding intermediates. At various times following the inception of renaturation the renaturation temperature was quickly increased to 35° C, having already added GroEL either at the inception of renaturation (for the case of GroEL-assisted refolding) or immediately before increasing the temperature (for the case of spontaneous refolding), and thereby use the GroEL to trap folding intermediates. However, not all folding intermediates of Glu-6-PDH are trapped by GroEL. After a delay time the temperature was reduced to 20° C to determine what fraction of the folding intermediates entrapped by GroEL are able to resume renaturation. By measuring the limiting yields of reactivation following these temperature changes and comparing those measurements with the time courses for spontaneous and assisted reactivation, those folding intermediates that interact with GroEL at a particular temperature are determined. From these studies we have identified three folding pathways in the spontaneous renaturation. In the assisted renaturation GroEL directs the folding along only two of those pathways. We propose that GroEL directs folding long pathways that generate folding intermediates which are less vulnerable to the side reactions leading to aggregation.

B6-211 THE *IN VIVO* EXPRESSION OF BIP ATPASE MUTANTS INHIBITS THE SECRETION OF CO-EXPRESSED IMMUNOGLOBULIN LIGHT CHAINS, Linda M. Hendershot*, James R. Gaut*, Jue-Yang Wei*, Jeff Melnick†, and Yair Argon†, *Department of Tumor Cell Biology, St. Jude Childrens Research Hospital, Memphis, TN 38105 and †Department of Immunology, Duke University, Durham, NC 27710.

We have recently made a series of point mutations in the ATP binding domain of hamster BiP that inhibit its ATPase activity. These mutations do not alter the ability of BiP to bind to nonsecreted Ig heavy chains, but do block the *in vitro* ATP-mediated release of BiP from the heavy chains. We wished to determine the *in vivo* effects of our ATPase mutants on the secretion of proteins. COS monkey fibroblast cells were transfected with cDNAs encoding wild-type λ light chains along with either wild-type or ATPase-defective hamster BiP. Immunoprecipitation of metabolically labeled cell lysates revealed that only trace amounts of the λ light chains were co-precipitated with wild-type BiP and that the light chains were secreted from the COS cells. Co-expression of the λ light chains with BiP ATPase mutants resulted in a readily detectable association of the two proteins and a diminished secretion of the λ light chains. Two λ light chain mutants that are normally retained in the ER were examined in the same way. We found an increased binding of ATPase-defective BiP to the mutant light chains over that seen with either endogenous BiP or transfected wild-type hamster BiP. Experiments are in progress to determine whether this increased binding is due to the stabilization of additional BiP binding sites on the mutant light chains or is a reflection of BiP binding to a larger proportion of the mutant λ light chains. These results demonstrate that BiP's ATP binding/hydrolysis functions are important for the *in vivo* release of bound proteins, and that enzymatically inactive BiP can interfere with the secretion of a normally transported protein. Because BiP binds to proteins as a monomer, the dominant negative effect on secretion must be due to mutant BiP acting as a "chaperone trap" in the processing and maturation of secretory pathway proteins.

B6-212 CONTROL OF FOLDING OF HOMOLOGOUS PROTEINS BY groEL/groES. Ana Iriarte, Joseph R. Mattingly and Marino Martinez-Carrion, School of Biological Sciences, University of Missouri, Kansas City, MO 64110

The homologous cytosolic and mitochondrial isozymes of aspartate aminotransferase (c and mAspAT, respectively) follow different folding pathways after synthesis in rabbit reticulocyte lysate, suggesting different interactions with molecular chaperones. To discern the basis for this phenomenon, we study the refolding of the guanidine hydrochloride denatured, purified proteins as they interact with the groEL/groES molecular chaperone system. In the absence of chaperones, temperature affects the refolding of the two isozymes, particularly mAspAT. groEL and groES can extend the temperature range over which the AspAT isozymes refold; however, cAspAT can still refold at higher temperatures than mAspAT. In the absence of groES and MgATP, groEL arrests the refolding of mAspAT throughout the temperature range of 0-45°C and adding only MgATP releases very little mAspAT; both groES and MgATP are required for significant refolding of mAspAT in the presence of groEL. By contrast, inhibition of refolding of cAspAT by groEL depends upon the temperature of refolding, only slowing the reaction at 0°C but arresting it completely at 30°C. Furthermore, MgATP alone effects the release of cAspAT from groEL at any temperature examined; inclusion of groES along with MgATP has no effect on the refolding yield but does increase the rate at higher temperatures. These results show that groEL has different affinities for proteins with highly homologous final tertiary and quaternary structures and suggest that dissimilarities in the sequence of the protein substrates may control the structure of the folding intermediates captured by the chaperone and/or the composition of the surfaces through which the folding proteins interact with groEL.

B6-213 A COMMON GROEL-INDUCED CONFORMATION FOR E. COLI AND BACTERIOPHAGE CO-CHAPERONIN MOBILE LOOPS, Samuel J. Landry*, Karol Maskos*, Costa Georgopoulos# and Saskia van der Vies# (*Department of Biochemistry, Tulane University School of Medicine, 1430 Tulane Avenue, New Orleans, LA 70112, and #Départment de Biochimie Médicale, Centre Médical Universitaire, 1211 Genève 4 Switzerland)

The GroEL and GroES proteins of *Escherichia coli* function together to facilitate folding of a diverse range of polypeptides, including components of the bacteriophage capsid. In the presence of ATP, the GroES 7-mer binds to one end of the GroEL 14-mer which aligns the 7-fold axes of the molecules and could allow each GroES subunit to engage a GroEL subunit. We have shown previously by ^{1}H nuclear magnetic resonance that upon binding of GroES to GroEL, a mobile polypeptide segment of GroES (a.a. 17-32) becomes immobilized in the GroEL/ES/ADP complex suggesting that this mobile segment interacts directly with GroEL. By analysis of transferred nuclear Overhauser effects, we find that a synthetic peptide corresponding to the mobile loop adopts a hairpin turn conformation in association with GroEL. The bacteriophage T4 gene product Gp31 has recently been shown to functionally substitute for GroES *in vivo* and *in vitro*, despite the absence of apparent amino acid identity between the two proteins. Gp31 contains a mobile segment of similar length and position to that of the mobile loop in GroES. This result highlights the importance of mobility in this region of co-chaperonin polypeptides. A synthetic peptide corresponding to the Gp31 mobile loop also forms a hairpin turn conformation in association with GroEL. The similarity of GroEL-bound conformations of the GroES and Gp31 mobile loop peptides suggests that geometrically specific interactions with the polypeptide backbone of the loops constrain the GroEL-bound conformations.

B6-214 KAR2 & SEC63: FUNCTION IN TRANSLOCATION INTO THE ENDOPLASMIC RETICULUM OF S. CEREVISIAE. S. K. Lyman and R. Schekman. Department of Molecular and Cell Biology, University of California, Berkeley. Berkeley, CA 94720.

Protein translocation across the membrane of the endoplasmic reticulum (ER) in Saccharomyces cerevisiae is dependent on the orchestrated action of several proteins. These proteins likely comprise an assembly that is responsible for mediating the functions required for the movement of a secretory protein across the ER membrane : 'docking' of the secretory protein at the membrane, traversing the lipid bilayer, and exiting on the lumenal side. Perhaps the best defined component is Sec61p, an integral membrane protein. Genetic and biochemical analysis has indicated that Sec61p may comprise the membrane pore through which secretory precursors pass. Work by Musch et al. and Sanders et al. has demonstrated that a secretory protein (ppαF) artificially blocked in the process of crossing the membrane is in close contact with Sec61p and with the lumenal hsp70 homolog Kar2p, implying that perhaps Kar2p receives the translocating polypeptide as it enters the lumen.

Kar2p likely works in conjunction with Sec63p, an integral membrane protein which contains in its lumenal domain a region 43% identical to the conserved 'J-box' found in DnaJ and its homologs. Brodsky et al. have shown that a mutant allele of SEC63 (sec63-1), which maps to an invariant alanine residue in the J box, is defective in its interaction with BiP; while Scidmore et al. have isolated suppressors of the sec63-1 mutation which map to the KAR2 gene.

In order to clarify the roles of KAR2 and SEC63 in translocation, we have utilized mutant alleles of these two essential genes: sec63-1 and kar2-203. Sanders et al. showed that the kar2-203 mutant, although defective in translocation, can still support the initial interaction of precursor with Sec61p; yet is apparently unable to complete the process of precursor transfer across the lipid bilayer. This is consistent with a model in which Kar2p is responsible for receiving the polypeptide in transit and helping it to fold on the lumenal side. Intriguingly, we have found the the sec63-1 mutation results in a phenotype similar to that of kar2-203: the precursor is apparently 'stalled' in the pore, unable to complete its transit across the membrane. Neither of two cytoplasmic sec63 alleles yielded a similar phenotype--thus, the defect may result specifically from the inability of the lumenal sec63-1 mutant to interact productively with BiP, leaving the precursor stranded in the pore.

B6-215 *IN VIVO* STUDIES ON THE EFFECTS OF TRUNCATION AND DEPLETION OF THE GROEL PROTEIN IN *ESCHERICHIA COLI*, Neil F. McLennan, Sean McAteer and Millicent Masters, Institute of Cell and Molecular Biology, University of Edinburgh, King's Buildings, Mayfield Road, Edinburgh, Scotland, EH9 3JR

The active form of the HSP60 molecular chaperone of *Escherichia coli*, GroEL, is a pair of 7-membered rings. We have used site directed mutagenesis to construct forms of the 547 amino acid monomer truncated at the carboxyl terminus. We show here that forms 520 amino acids long or longer are close to fully functional *in vivo*. Removing one further amino acid, however, results in a protein, GroEL519, which retains little function. This truncated form is metabolically stable, but is not recovered from the cell in particle form and seems to interfere with the assembly of wild-type GroEL when co-expressed. We conclude that amino acid 520 is crucial for correct particle assembly. We have also used the arabinose-inducible P_{BAD} promoter to regulate the levels of GroE proteins *in vivo* and report here on the effects of GroE depletion on several vital cellular processes.

B6-216 MECHANISM OF CHAPERONIN-ASSISTED β-TUBULIN FOLDING, Ronald MELKI*, Paul D. WALDEN#, Stephanie SOULIE* and Robin LEGUY*, * Laboratoire d'Enzymologie, CNRS, 91198 Gif-sur-Yvette Cedex, FRANCE, and # Department of Urology, New York University Medical Center, 550 First Avenue, New York, NY 10016, USA

α and β tubulin polypeptide chains acquire their native conformation in the presence of the heteromeric TCP-1-containing chaperonin and several other cofactors. We recently identified one of the protein cofactors, termed cofactor A, that are required for the proper folding of tubulin chains (Y. Gao, R. Melki, P.D. Walden, S.A. Lewis, C. Ampe, H. Rommelaere, J. Vandekerckhove and N.J. Cowan, J. Cell. Biol. 125: 989-996, 1994). Here we demonstrate that cofactor A does not interact with the chaperonin nor does it affect either it's intrinsic ATPase activity or it's ATPase activity in the presence of a range of target proteins. Thus, cofactor A does not act on the chaperonin as a catalyst in tubulin folding. We also show that cofactor A participate in the tubulin folding process by interacting with a β tubulin folding intermediate. Our data imply that cofactor A is a chaperone involved in tubulin folding.

B6-217 SEQUENTIAL INTERACTION OF THE CHAPERONES BiP AND GRP94 WITH IMMUNOGLOBULIN CHAINS IN THE ER, Jeffrey Melnick and Yair Argon, Department of Immunology, Duke University Medical Center, Durham, NC 27710

During their transit through the endoplasmic reticulum, newly synthesized light (L) and heavy (H) chains of immunoglobulins (Ig) associate with two resident ER stress proteins, BiP/GRP78 and GRP94. We have shown by sequential immunoprecipitation that GRP94-L binary complexes exist in addition to BiP-L and GRP94-BiP-L complexes, and that binding of BiP and GRP94 to Ig chains can be independently modulated biochemically. These findings suggest that GRP94 is a polypeptide-binding protein. Phosphorylation regulates GRP94 activity: GRP94 is phosphorylated on serine and threonine residues, but only non-phosphorylated GRP94 associates with L chains. Like BiP, GRP94 association with H and L chains is C_H1- and V_L-dependent but not observed with assembled, tetrameric Ig. GRP94 binds preferentially to mutant L chains whose transport is arrested in the ER; we show that this is not due to the mutant's extended residence in the ER, but rather to a subtle structural alteration in the V_L domain. Both BiP and GRP94 bind secretable L chains transiently, but GRP94 associates subsequently to BiP and dissociates more slowly than BiP. Indeed, BiP displays a strong preference for a disulfide folding intermediate, whereas GRP94 association with newly synthesized L chains is dependent on their complete oxidation. These results indicate that GRP94 is a molecular chaperone which acts subsequently to BiP, and suggest the presence of a chaperone relay mechanism in the ER.

B6-218 **HEAT-INDUCED PROTEIN DENATURATION IN DIFFERENT INTRACELLULAR COMPARTMENTS USING FIREFLY LUCIFERASE AS A REPORTER PROTEIN.** A.A.Michels[1], V-T. Nguyen[2], O.Bensaude[2], H.H.Kampinga[1]. [1] Dept.Radiobiol., Univ.Groningen, The Netherlands. [2] Génétique Moléc. Ecole Normale Supérieure, Paris, France

In order to obtain specific information on the role of individual hsp's in protection against inactivation, aggregation and reactivation of proteins in various cell compartments, the effect of heat on well characterized reporter enzymes can be examined. Heat-induced protein denaturation *in situ* was studied using cells transfected with the foreign firefly luciferase gene. When transfected into mammalian cells, firefly luciferase is usually found in the peroxisomes. A cytosolic luciferase has been constructed previously. To provide a tool for the *in situ* investigations on chaperone functions in the cell nucleus, we were successful to target luciferase into the nucleus: after transfection into mammalian cells nuclear luciferase turns out to be enzymatically active. Using O23 hamster cells as well as Ltk- mouse cells, it was found that both nuclear and cytosolic luciferase were quite stable at 37°C: under conditions of inhibited protein synthesis (by cycloheximide), about 50% activity was retained after 4 hours at 37°C for both luciferases. Luciferase targeted into the nucleus was more sensitive to 42°C and 43°C heating than cytosolic luciferase. *In situ* reactivation (within the first 4 hours after heating the cells) was found; yet, this recovery was only partial. In cells made thermotolerant by prior heating, heat-inactivation of both the cytosolic and nuclear targeted enzyme is attenuated. Thus, the current system seems an excellent tool to study the role of individual heat shock proteins in protection against protein denaturation and in reactivation of heat-denatured proteins in different intracellular compartments.

This work was partially supported by grant CHRX-CT93-0260 of the European Community.

B6-219 NOVEL FUNCTION OF STRESS PROTEIN: HSP60 BEHAVES AS A SENSOR MOLECULE OF TWO-COMPONENTS PATHWAY, M.Morioka, K.Yamamoto and H.Ishikawa, Zoological Institute, Graduate School of Science, University of Tokyo, Tokyo, JAPAN

The environmental stress induces the prokaryotic and eukaryotic cells to synthesize an array of stress proteins that function as molecular chaperones. Likewise, bacterial endosymbiont of pea aphid harbored in the host eukaryotic cells, selectively synthesizes *symL* protein (symbionin), a GroEL homologous molecular chaperone. We recently found that symbionin is autocatalytically phosphorylated to produce a protein with a high-energy phosphate bond, which, in tern, is transferred to other component such as ADP.

In the present study, we demonstrated that symbionin retains the autophoshorylating and phosphoryl-group transferring activity even after disassembly into the monomeric state. Furthermore, we identified the autophosphorylation site of symbionin as His-133 by amino-acid sequencing and TLC analysis, suggesting that symbionin is a kind of histidine protein kinase, while the amino acid sequence of symbionin around the autophosphorylation site has no similarity to the conserved domain of any known HPK. It was also shown that there are eight proteins in the endosymbiont, with apparent molecular mass of 66, 63, 60, 55, 42, 31, 25, and 42-kDa, that are capable of accepting the high-energy phosphate bond from the phosphorylated symbionin. This suggested that these proteins correspond to response regulators known in the two-components pathway. The partial amino acid-sequence of the major response regulator with 42-kDa demonstrated that this protein is homologous to OmpF that is a constituent protein of *E. coli* outer membrane. It was demonstrated by immunoblotting analysis that polyclonal antibody against OmpF cross-reacts with the 42-kDa protein.

All these evidence suggest that symbionin functions not only as a molecular chaperone to assist the folding and/or assembly of polypeptides but also a sensor molecule of the two-components pathway to regulate the signal transduction through its phosphoryl-group transfer.

B6-220 BIP ATPase ACTIVITY IS REQUIRED TO PROTECT CELLS FROM ER STRESS AND FOR THE SECRETION OF SELECTIVE PROTEINS, Jill A. Morris[1], Andrew J. Dorner[2], James R. Gaut[3], L. M. Hendershot[3], and Randal J. Kaufman[1], [1]Howard Hughes Medical Institute, Ann Arbor, MI, [2]Genetics Institute, Cambridge, MA; [3]Dept. Tumor Cell Biol. St. Jude Children's Research Hospital, Memphis, TN

BIP/GRP78 is a lumenal protein of the endoplasmic reticulum (ER) that is induced at the transcriptional level by inducers of the stress response in the ER. In addition, BIP interacts with some proteins that transit the secretory compartment. We have studied the secretion and the stress response in Chinese hamster ovary cells that over-express wild-type BIP and a BIP deletion molecule (residues 176-201) that can bind peptides and ATP but is defective in ATP hydrolysis and concomitant peptide release. Over-expression of wild-type BIP or ATPase-defective BIP prevented secretion of Factor VIII, a coagulation factor that extensively binds BIP in the lumen of the ER. In contrast, the secretion of M-CSF, a protein that cannot be detected in association with BIP, was not affected by over-expression of wild-type or ATPase-defective BIP. These results show that BIP ATPase activity is not required for secretion of some proteins and suggest that some proteins do not interact with BIP upon transport through the ER.

Over-expression of wild-type BIP prevented the stress-mediated transcriptional induction of the glucose regulated proteins (GRPs) in response to calcium ionophore A23187 treatment or tunicamycin treatment. In contrast, over-expression of ATPase defective BIP did not prevent the stress induction of GRPs. To determine whether over-expression of wild-type BIP interferes with the signal transduction pathway for GRP induction or whether it relieves stress in the ER, we studied the survival of cells in response to A23187 treatment. Over-expression of wild-type BIP, but not ATPase defective BIP, dramatically increased survival of cells treated with A23187. These results indicate that over-expressed BIP alleviates the stress in the ER that the ATPase activity of BIP is required.

B6-221 A GENETIC ANALYSIS OF HSP90 FUNCTION: INTERACTIONS WITH GLUCOCORTICOID RECEPTOR AND $p60^{v\text{-}src}$, Debra F. Nathan and Susan Lindquist, Department of Molecular Genetics and Cell Biology, The Howard Hughes Medical Institute, The University of Chicago, Chicago, IL 60637

Hsp90 is a ubiquitous and highly conserved molecular chaperone that regulates the activity of specific proteins. To better understand the relationship between Hsp90 and its target proteins, we isolated Hsp90 mutations that confer a temperature-sensitive (ts) growth phenotype on *Saccharomyces cerevisiae* cells lacking wild-type Hsp90. The effects of the mutations on two well-characterized target proteins, the transcription factor glucocorticoid receptor (GR) and the tyrosine kinase $p60^{v\text{-}src}$, served to classify the mutations and to further define the functions of Hsp90. Seven mutations reduce the activity of Hsp90 at all temperatures. They are ts because much higher levels of Hsp90 function are required for growth at high temperatures than at low temperatures. These mutations are broadly distributed through the protein, yet all compromise Hsp90's ability to mature both $p60^{v\text{-}src}$ and GR. Thus, the integrity of the entire protein is required for functional interactions with these two very different targets. Another mutation has a classic ts phenotype, with near wild-type activity at 25°C and virtually no activity above 34°C. Temperature-shift experiments with this mutant demonstrate that once Hsp90 has productively interacted with GR, it is continuously required to maintain GR in an activatable state.

B6-222 AUTOPHOSPHORYLATION OF GROEL IN VITRO, Kaori Sadakane, Mizue Morioka and Hajime Ishikawa, Zoological Institute, Graduate School of Science, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113, JAPAN

It was suggested that GroEL, the E. coli chaperonin, undergoes phosphorylation upon temperature shift-up[1]. While the phosphorylation was suggested to proceed in an autocatalytic manner, no evidence has been published. Here we report that purified GroEL preparation does exhibit GroEL kinase activity as suggested, and compare its activity with that of symbionin, a GroEL homologue produced by an aphid endosymbiont, which is more than 85% identical with groEL at the amino acid sequence level[2].

It was found that GroEL and symbionin share the following properties in common: 1) temperature-dependent autophosphorylation in vitro; 2) requirement for Zn^{2+} for the efficient autophosphorylation; 3) retention of the phosphorylation activity after dissociation into monomeric state.

Unlike symbionin, however, GroEL did not exhibit phosphotranspherase activity when incubated with ATP and GDP, suggesting that the sites of phosphorylation are different between the two chaperonins[3].

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2. Ohtaka, C. et al. (1992) J. Bacteriol. **174**, 1869.
3. Morioka, M. et al. (1994) J. Biochem., in press.

B6-223 RENATURATION OF THERMALLY DENATURED LUCIFERASE: AN ASSAY FOR HSP CHAPERONING ACTIVITY, Robert J. Schumacher and David O. Toft, Department of Biochemistry and Molecular Biology, Mayo Graduate School, Rochester, MN 55905

We have developed an assay for chaperone-mediated protein renaturation in rabbit reticulocyte lysate. Firefly luciferase is thermally denatured by incubation at 40°C, then diluted 10-fold into reticulocyte lysate at 25°C to allow renaturation. Dilution of denatured luciferase into reticulocyte lysate after addition of an ATP-regenerating system results in recovery of >70% activity. We have identified two heat shock proteins, hsp90 and hsp70, that are involved in this renaturation process. Purified preparations of these proteins support the renaturation of ~30% of denatured luciferase. Both hsp90 and hsp70 are required for this process and additional factors may be needed for more efficient renaturation. This renaturation is also ATP-dependent suggesting that hsp90 and hsp70 are part of a cooperative and ATP-dependent process for the renaturation of denatured or thermally damaged proteins. The luciferase renaturation assay is a simple and reliable assay for measuring the functional activity of hsp70. We have used this assay to analyze the chaperoning activities of mutated human hsp70s (supplied by Brian Freeman and Rick Morimoto, Northwestern University) and mutated hsp70 homologs such as BiP (supplied by J. R. Gaut and Linda Hendershot, St. Jude Children's Research Hospital).

B6-224 HSP90 AND HSP70 ARE PRESENT IN A HETEROMERIC COMPLEX IN THE STEROID HORMONE RESPONSIVE FUNGUS *ACHLYA*, Julie C. Silver[1], Gary H. Perdew[2], and Shelley A. Brunt[1], [1]Department of Microbiology and Division of Life Sciences, University of Toronto, Scarborough, Ontario, Canada M1C 1A4. [2] Department of Food and Nutrition, Purdue University, West Lafayette, Indiana.

In *Achlya,* HSP90 and HSP70 family proteins are constitutively expressed, upregulated by heat shock, and by steroid hormone treatment of the mycelium. HSP90 is reported to exist in heteromeric complexes with a number of cellular proteins, including kinases, transcription factors and steroid hormone receptors, as well as other heat shock proteins (HSP70, HSP56). In the fungus *Achlya* steroid hormones regulate sexual development. The response to hormone is thought to be mediated by a steroid hormone receptor. Immunoprecipitation of either cellular or *in vitro* translated proteins with monoclonal antibodies to HSP90 from *Achlya* (AC88), mouse (8D3) or rat (2D12), showed that HSP90 (85kD) in *Achlya* is found in a heterocomplex with proteins of 110kD, 74kD, 64kD, 61kD, 56kD, 47kD and 23kD. Proteins of similar molecular weight are observed in vertebrate steroid receptor heterocomplexes. Western analyses, using AC88 and BRM-22 an antibody to HSP70, showed that the 85kD and 74kD proteins which immunoprecipitate with AC88 are HSP90 and HSP70 respectively. Our results suggest that the association of steroid receptors in a heteromeric complex with HSP90, HSP70, and other proteins, extends to this microbial steroid responsive system.

(Supported by NSERC Canada)

B6-225 ALTERED MOLECULAR INTERACTIONS IN AN *E. Coli dnaK756* MUTANT LEAD TO INCREASED PRODUCTION OF ENZYMATICALLY ACTIVE HETEROLOGOUS PROTEINS, Jeffrey G. Thomas and François Baneyx, Department of Chemical Engineering, BF-10, University of Washington, Seattle, WA 98195

We have previously reported a two- to fourfold increase in the production of active, soluble heterologous enzymes in *E. coli* strains bearing the *dnaK756*(Ts) allele at permissive temperatures. This effect, presumably due to the increased production of chromosomally-encoded molecular chaperones in the *dnaK756* background, was not observed in isogenic *dnaJ259*(Ts), *grpE280*(Ts), and *dnaK103*(Ts) mutants which display phenotypes similar to that of *dnaK756* mutants. The molecular mechanisms involved have been investigated in a *dnaK756* strain using preS2-β-galactosidase - a fusion protein consisting of the preS2 sequence of the hepatitis B virus surface antigen fused to the amino-terminus of the *lacZ* gene product - as a model system. Plasmid copy number and efficiency of transcription/translation were not affected by the *dnaK756* mutation, suggesting that the effect occurs post-translationally. Altered rates of protein degradation were not responsible for the difference in yield as the half-life of the fusion protein was more than 120 minutes in both the *dnaK756* and isogenic wild-type strains. However, the initial kinetics of aggregation of preS2-β-galactosidase were doubled in the wild-type versus mutant strain. The reduced aggregation of the fusion protein appears to be linked to an increased affinity of the DnaK756 protein for preS2-β-galactosidase relative to DnaK, as evidenced by co-immunoprecipitation and Western blotting. Additionally, pulse-chase experiments show an increased residence time of the model protein on DnaK756 relative to DnaK. In contrast to the results obtained with preS2-β-galactosidase, the *dnaK756* mutation was detrimental to the activity of an isogenic preS2-β-galactosidase protein containing a hydrophobic domain inserted between the preS2 and *lacZ* coding sequences.

B6-226 THE EFFECT OF LACK OF HSP70 ON THE EXPRESSION OF EXOGENOUS PROTEIN The Study of Yeast Ssa1ssa2 Cell Expressing Luciferase

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To determine the role of hsp70 and hsp104 in the process of heat denaturation and recovery of proteins, the yeast cell constitutively expressing luciferase was used. A bacterial luciferase fusion protein (Escher et al, 1989) is a very useful probe, because of its high temperature-sensitivity and the easy assay available for its activity. The yeast cell, SL314, which is a double mutant lacking the constitutive hsp70 proteins (Ssa1 and Ssa2), and carrying a plasmid constitutively expressing luciferase fusion protein (pLux(URA)), was used for the experiment.

The activities of luciferase were tested in several different transformants. In wild type cells (WT), the average activity of 10 colonies was 0.19±0.04 unit/cell. In ssa1ssa2 cells, the activities varied from 0.31×10^{-2} to 0.84×10^{-1} unit/cell, and the average activity excluding the lowest was 0.048±0.02 unit/cell. This value was about a quarter of that of WT. The doubling time was always about 3 hours in WT cells, however, the doubling times were obviously extended in ssa1ssa2 cells. Notably, cells expressing higher activity exhibited longer doubling times and cells with the lowest activity doubled at a rate similar to that of wild type cells. This result suggests that, in ssa1ssa2 strains, the expression of the luciferase protein might suppress the cell growth. Furthermore, the amount of luciferase in ssa1ssa2 cells was lower than that in WT cells, the level of activity was almost proportional to the amount of the enzyme. We are currently trying to determine if it is the synthesis, folding, or degradation that is altered in ssa1ssa2.

Although preheating cells at 37°C did not affect luciferase activity, heating at 44°C drastically decreased its activity in WT and ssa1ssa2 cell. The rate of heat-denaturation was a little higher in WT than that in ssa1ssa2. By the heating at 44°C without preheating, the rate of heat-denaturation was more higher in WT than that in ssa1ssa2. On the other hand, the decreased activities were similarly recovered in WT and in ssa1ssa2 during the incubation at 25°C for 120 min. The amounts of luciferase were not changed through the heating in ssa1ssa2 and WT cells. Hsp104 was overexpressed inssa1ssa2, suggesting that the lower denaturation of luciferase might be contibuted to the large amount of hsp104 in ssa1ssa2.

B6-227 SIMILARITY BETWEEN BiP AND G PROTEIN NUCLEOTIDE INTERACTIONS, Vincent Vidal, Nathan Brot, Betty Redfield, Na-Hong Qiu, Guo-Jun Chen, Anthony Carlino, and Herbert Weissbach, Roche Research Center, Roche Institute of Molecular Biology, Nutley NJ 07110 USA

Immunoglobulin heavy chain binding protein (BiP) is localized in the endoplasmic reticulum (ER) and is a member of the highly conserved family of heat shock-related proteins known as Hsp70. In mammalian cells, BiP is not induced by heat, however treatments increasing the amount of malfolded proteins in the ER (e.g. inhibitors of N-linked glycosylation) stimulate a dramatic increase in its synthesis.

Previously we showed that both bovine liver and a recombinant BiP exist as a mixture of monomers and dimers, and that ATP effects the conversion of dimers to monomers, a reaction that is reversed when ATP is removed. Moreover, we demonstrated that BiP monomers form a stable complex with ATP. In our present studies we examine the *in vitro* interaction of BiP with ATP and a model substrate, Substance P (SP), an 11 amino acid peptide.

We show that SP stimulates BiP ATPase activity approximately 4-fold and that this stimulation requires KCl. By gel filtration and a newly developed spin column assay we show that SP•BiP complex formation is stimulated about 2-4 fold at low concentrations of ATP (5-10 μM), but that the BiP•SP complex is dissociated in the presence of ATP levels greater than 50 μM. Neither ADP nor a number of nonhydrolyzable ATP analogues such as AMP-PCP, AMP-PNP and γ-S-ATP can substitute for ATP. The data suggest that a BiP-ATP complex is initially formed which reacts with SP to form a ternary SP•BiP•ATP complex. Hydrolysis of the bound ATP yields a SP•BiP•ADP complex. Using a rapid filter binding assay, an exchange of ATP with ADP bound to BiP has also been demonstrated. These results suggest that the interaction of BiP with ATP resembles that of G proteins with GTP.

B6-228 THE FUNCTIONS OF mt-HSP70 AND THE GrpE-HOMOLOGUE MGE IN PROTEIN TRANSLOCATION ACROSS THE INNER MITOCHONDRIAL MEMBRANE. W. Voos, B.D. Gambill*, E.A. Craig*, and N. Pfanner, Biochemisches Institut, Universität Freiburg, D-79104 Freiburg; *Dept. of Biomol. Chem., University of Wisconsin, Wisconsin 53706

The involvement of the matrix-localised mitochondrial hsp70 (Ssc1p) in translocation of cytosolically synthesised preproteins was analysed using two temperature sensitive mutant strains, *ssc1-2* and *ssc1-3*. *In vitro* import reactions with the mutant mitochondria showed a differential accumulation of preproteins destined for the matrix. Ssc1-2p still strongly binds to precursor proteins, but preproteins are accumulated as membrane spanning translocation intermediates. The translocation defect can be overcome by an artificial unfolding of the precursor protein. In contrast, even with unfolded preproteins, mitochondria of strain *ssc1-3* show a complete translocation arrest due to absent protein binding by Ssc1-3p. ATP-depletion of the mitochondrial matrix results in a similar translocation phenotype as in *ssc1-3* mitochondria. Using crossreacting antibodies a mitochondrial homo-logue (MGE) of the *E. coli* GrpE protein could be identified. MGE can be isolated in a salt-resistant complex with mt-hsp70, which is dissociated in the presence of ATP but not with nonhydrolyzable nucleotide analogues. The mutant Ssc1-3p is not able to interact with MGE. In a complex with mt-hsp70, MGE interacts with translocation intermediates but not with fully imported proteins. We conclude that Ssc1p together with MGE forms an enzymatic system which is an essential component of the inner membrane translocation machinery performing the dual function of unfolding of preproteins and membrane translocation in an ATP-dependent manner.

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W. Voos, B.D. Gambill, B. Guiard, N. Pfanner, E.A. Craig (1993). JCB 123, 119

W. Voos, B.D. Gambill, S. Laloraya, D. Ang, E.A. Craig, N.Pfanner (1994). MCB, in press

B6-229 GENOMIC AND mRNA STRUCTURE OF THE HUMAN 'CHAPERONIN CONTAINING TCP-1 GAMMA' (CCTγ). Neal A. Walkley and Afshan N. Malik. School of Molecular and Medical Biosciences, University of Wales College of Cardiff, PO Box 911, Cardiff CF1 3US, UK.

Chaperonins, a sub-class of molecular chaperones, function as multisubunit ring structures and are encoded by families of sequence-related genes. The eukaryotic cytosolic chaperonin, CCT, is a multisubunit hetero-oligomeric complex composed of at least seven subunits. CCT has been shown to be directly involved in the folding of tubulin and actin and is postulated to be involved in folding a large number of other cytosolic proteins. Chaperonins, therefore, play a fundamental role in the functioning of the cell.

We have cloned Cctg, the human cDNA encoding the largest subunit of CCT, denoted CCTγ, and have also isolated genomic clones, corresponding to Cctg, from a human P1 genomic library (insert size 70kb) and a Lawrist4 cosmid library (insert size 20-40kb). We are currently investigating the primary structures at the mRNA and the genomic level.

DNA sequence analysis of Cctg cDNA has revealed an open reading frame of 1635bp making a predicted peptide of 60KDa (544 residues). The predicted human CCTγ shares significant homology with human TCP-1 (31·0%), TF55 (32·7%), TCP-20 (25·8%) and also with murine CCTγ (97·1%). Sequence alignments show that human CCTγ shares conserved domains identified in TCP-1 related chaperonins. Using RT-PCR and northern analysis we have detected the expression of Cctg mRNA in a large number of human fetal and adult tissues. We have also found over-expression of Cctg mRNA in mouse and rat testes when compared to brain and kidney. Southern analysis has revealed that Cctg is highly conserved in mouse, rat, hamster, sheep, frog, stone-loach and pike. Southern analysis of human genomic DNA and the corresponding genomic clones is being carried out to generate a restriction map of the genomic region. In addition, we are characterising the intron/exon organisation of the gene using a combination of PCR and DNA sequencing. These studies are likely to yield information about the primary structure of the Cctg gene and will also result in identification of upstream regulatory sequences.

B6-230 **CHAPERONE AND FOLDASE EXPRESSION IN RECOMBINANT CHO CELL LINES : EVIDENCE FOR AN ADAPTIVE RESPONSE TO CHANGES IN RECOMBINANT PROTEIN EXPRESSION.** Steven G. Webster, Sabrina M. Tom, and Amy A. Murnane, Department of Cell Culture Research and Development, Genentech, Inc., South San Francisco, CA 94080.

The protein folding machinery of the endoplasmic reticulum (ER) is comprised of a functionally heterogeneous group of chaperone and foldase (C/F) proteins. We have used SDS-PAGE and Western immunoblotting to survey C/F expression during large scale cell culture of a variety of recombinant Chinese hamster ovary (CHO) cell lines. The effect of culture conditions and process manipulations on chaperone (GRP78, GRP94, HSP70, and HSC 70) and foldase (PDI) expression as well as the relationship of C/F expression to intracellular levels of recombinant protein (rPRO) and cell-specific productivity (CSP) have been evaluated. We have found that higher rPRO expression resulting from various process conditions is followed by increased expression of several ER C/F proteins. In addition, levels of C/F expression correlate well with cell line-specific quantitative differences in rPRO expression and CSP. Moreover, secretion remains efficient in cell lines where process-induced increases in intracellular rPRO are succeeded by elevated C/F expression. These observations are consistent with a mechanism for efficiently accomodating changes in rPRO expression and the polypeptide load of the ER through a coordinate adaptive regulation of C/F expression. Curiously, increases in intracellular rPRO levels are not associated with a higher CSP in one mAb-producing cell line with an unusually high level of rPRO expression, intimating a bottleneck may emerge which acts to reduce the efficiency of secretion at exceptionally high levels of rPRO expression. Intriguingly, we have evidence which suggests that the expression of GRP78, a chaperone known to play a key role in the folding and assembly of immunoglobulins, may be maximally up-regulated in this cell line and possibly limiting for Ab folding and assembly. An immunocytochemical analysis of intracellular Ab distribution has indicated that most Ab is co-localized with ER -specific markers, consistent with an ER limitation for secretion. These observations have led us to further investigate the relationship of GRP78 expression to ER transit rates of rPRO, CSP, and the efficiency of secretion in this and other cell lines.

Stress Proteins in Medicine/Response

B6-300 BCL-2 AND THERMOTOLERANCE COOPERATE IN CELL SURVIVAL. Robin L. Anderson, Tim Gabriele and A. Strasser*. Peter MacCallum Cancer Institute, St. Andrews Place, East Melbourne, 3002 and * Walter and Eliza Hall Institute, Royal Parade, Parkville, 3050, Victoria, Australia.

The protein product of the oncogene *bcl-2* is an effective inhibitor of apoptosis in mammalian cells, including that caused by exposure to heat. Hyperthermia in the temperature range of 41° to 45°C induces apoptotic death in many cell types. Transient resistance to heat, known as thermotolerance, is induced by mild heat pre-treatment and can inhibit apoptosis induced by subsequent more severe heat exposure. This protection is thought to be mediated by the expression of increased amounts of heat shock proteins (HSP) which act as chaperones to prevent heat-induced denaturation and to repair heat damaged proteins. We set out to test whether there was any relationship or cooperation between *bcl-2* expression, heat shock proteins and/or the thermotolerant state in inhibiting the process of apoptosis and in promoting cell proliferation after heat stress.

Three murine cells lines, a myeloid line, a T cell hybridoma and a B cell lymphoma, were stably infected with a retrovirus containing a human *bcl-2* construct. The presence of Bcl-2 conferred resistance to heat shock in all three lines with decreased numbers of cells entering apoptosis. The induction of thermotolerance also increased resistance to heat, but much more so in the lines containing *bcl-2*. The results suggest that *bcl-2* and the thermotolerant state act via independent mechanisms to inhibit apoptosis and provide evidence that two separate pathways can cooperate to promote cell survival.

To examine directly the role of HSP in apoptosis, we are testing whether over-expression of one major HSP, HSP70, can substitute for the thermotolerant state in blocking heat-induced apoptosis and in enhancing the effect of *bcl-2*. The cell lines mentioned above are being transfected with a construct containing human HSP70 under the control of a constitutive promoter. In one cell line, we have isolated several clones that express high levels of human HSP70 and have preliminary evidence of protection from apoptosis.

B6-301 HEAT- SHOCK PROTEINS (Hsp)70 FAMILY: DOMINANT SELF-ANTIGEN CANDIDATE IN THE SELECTION OF INTRAEPITHELIAL LYMPHOCYTES (IEL)

Clara G.H. Bell, Univ. of Illinois, Department of Microbiology and Immunology, College of Medicine, Chicago, IL. 60612

The function of the immune system to protect the host from exogenous invaders & endogenous aberrations is related to the capacity of the system to recognize foreign antigens (Ags) & discriminate them from self-Ags. Central to this function are the T-cells, the population of $CD3^+$ noncovalently associated $Tc R\alpha\beta$, $Tc R\alpha\beta$ characterized by the cluster differentiation markers CD4 &/or CD8, being the major set with the most diverse clonal receptors weapons against foreign invaders. These differentiate intrathymically from fetal liver & from bone-marrow stem cell migrants via a series of steps that lead to the expression of the $Tc R\alpha\beta$ -that convey the clonal Ag-specificity & to the expression of the CD4 & CD8 differentiation Ags skewed to recognition of the Ag in the context of the self-major histocompatibility complex (MHC) gene product. The role of the thymus in the selection & maturation of the $Tc R\alpha\beta^+CD4^+CD8^-$, $Tc R^+CD4^-CD8^+$ cell-surface cytofluorometric identifiable phenotype repertoires, is central. The potential repertoire of the $Tc R\alpha\beta$ - expressed after rearrangements in the thymus of the T lineage gene segments- is selected in the thymus positively to bind to self-Ag- or self-idiotype (Id)- associated to self-MHC & negatively to eliminate the cells that could cause toxic autoreactivity, to result in an available repertoire that is restricted to foreign Ag. The function of the $Tc R\gamma\delta$ which rearrange in the thymus prior to the $Tc R\alpha\beta$ & which are a minor population in the thymus that fails to exhibit the classical $Tc R\alpha\beta$ MHC-restricted recognition of Ag, is unclear, as is the ligand recognized by these. Accordingly, the role of the $Tc R\gamma\delta$, that do not exhibit MHC-restricted Ag-recognition, and are unresponsive to most Ags recognized by the $Tc R\alpha\beta$, major thymic derived T lineage population, is one of the central issues in immunology today.

Using a murine system I made a key observation that raised the possibility that Hsp68/Hsp70 family members (constituently tissue expressed self-Ags that bear sequence similarity to bacterial & yeast Hsps) might be target Ags for the $Tc R\gamma\delta$ T-cells resident within the gut intestinal (i) IEL. Herein, I present a cytofluorometric dissection of the lymphocytes (LC) in murine peripheral lymphoid tissues lymph nodes (LN) & spleen (SpL) & gut IEL, showing that the high representation of the $Tc R\gamma\delta$ in gut-associated iIEL & in the skin of both euthymic & nude mice, parallels an upregulated expression of the Hsp68-70 family (that are present as constituently & induced multiple copies within the mouse genome & which share sequence homology with the Drosophila & yeast Hsp). The distribution in these locales of the $CD4^-CD8^+$ ($\sim$80% of the IEL) (mostly $Pgp\text{-}1^+$, i.e., exhibiting a marker of memory T, suggesting an in vivo exposure to Ag), the majority of which (58-69%) exhibit the unique $CD4^-CD8\alpha\alpha(Lyt\text{-}2)^+\beta(Lyt\text{-}3)^-$ homodimers **associated with either the thymus independent (TI) $Tc R\alpha\beta$ or $Tc R\gamma\delta$** & of the $CD4^-CD8^-$ (as assessed by flow cytometry), relative to the frequency of the $CD4^+CD8^+(\alpha^+\beta^+)$ ($\sim$19-29% of the IEL $Tc R\alpha\beta$), **that are thymus dependent resembling the PP T**; & the frequency of the $CD4^+CD8^-$; $CD4^+CD8^-$ ($\sim$5-11% of the IEL $Tc R\alpha\beta$); & the distribution of the Hsp68-70 (as assessed by Western immunoblotting), **points to the Hsp68-70 as the dominant self-Ag for the murine TcR extrathymic maturation**. It leads to the speculation that $Tc R\gamma\delta$ $CD3^+Tc R\gamma\delta^+$ may be selected in the IEL on account of their reactivity with the highly conserved Hsp68-70 Ags to supplement some aspect of the immune defense not covered by the $Tc R\alpha\beta$ T major set. The $Tc R\gamma\delta$ predominant distribution in the iIEL & the high frequency of the Hsp-responsive $Tc R\gamma\delta$ T cells there, suggestive of a first line of defense at the site of first invasion, along with the distinct correlation between the distribution of the TI T-cells & the Hsp-activity, may argue that the Hsp expression may contribute multiple selective functions that may differentially affect the $Tc R\gamma\delta$ selection, however, whether Hsp expression is only causal in promoting $Tc R\gamma\delta$, or whether $Tc R\gamma\delta$ is an independent consequence of Hsp elevated expression is unclear.

B6-302 ROLE OF HSP70 IN THE PATHOGENESIS OF ACUTE GRAFT-VERSUS-HOST DISEASE (GvHD) John Clancy, Joanna Goral, Department of Cell Biology, Neurobiology and Anatomy, Loyola University Chicago, Stritch School of Medicine, Maywood, IL 60153.

When acute lethal GvHD is induced in (DAXL) rats with an i.v. injection of DA lymphoid cells, such GvHD rats exhibit an increases production of the HSP70 family of proteins within their lymphoid tissues within one week and the IgM, IgG2a and IgG2b antibodoes to HSP70 at 2 weeks with death within 3 weeks. If recipient rats are preimmunized to bovine HSP70 , a significant number are more susceptible to acute GvHD with the remaining exhibiting chronic GvHD symptoms. Thus, pre-exisiting immunity to HSP70 may indicate a more fulminant GvHD course for bone marrow transplant patients.

B6-303 STRESS PROTEINS DURING APOPTOSIS, Louise M. Desjardins and John P. MacManus, Apoptosis Research Group, Institute for Biological Sciences, National Research Council of Canada, Ottawa, Ontario, Canada K1A 0R6

Apoptosis is a mechanism by which unwanted cells are selectively removed from tissues. This type of cell death is controlled and regulated such that synthesis of certain proteins is required. We have developed a model to study the mechanisms of apoptosis in an adherent cell line, an alternative to the classical thymocyte model. We were particularly interested in studying the possible involvement of certain stress proteins in this process. Apoptosis was induced in HT29 cells by treating with 5 μM of VM26 (a topoisomerase II inhibitor) for 24 h. Changes in the amount of stress proteins (colligin (GP46), HSP60 and HSP90) over a 96-h period was determined by Western blotting. HSP60 and HSP90 decreased relative to total protein over time. In contrast, the amount of colligin increased progressively after the addition of VM26. A possible role of colligin is to protect the procollagen I chains in the ER from degradation. It can be speculated that not only is colligin involved in the mechanism of apoptosis but also extracellular matrix proteins such as collagen I may also be important.

B6-304 HEAT SHOCK PROTEIN IMMUNITY IN CARDIAC ALLOGRAFT REJECTION. R.J. Duquesnoy, R. Moliterno, J. Qian, M Donovan-Peluso, F. Pan and L. Valdivia, Departments of Pathology and Surgery, University of Pittsburgh , Pittsburgh, PA 15261. USA.
Although it is well known that cellular rejection is mediated by alloreactive lymphocytes, several investigators including our group, have shown that such cells comprise a rather small proportion of the T cell infiltrate of the allograft. We have therefore postulated that graft-infiltrating lymphocytes must recognize other antigens. Since heat shock protein (hsp)-specific lymphocytes participate in several autoimmune diseases and in tumor immunity, we hypothesized that hsp-reactive lymphocytes are involved with allograft rejection. Our initial studies on heart transplant patients have demonstrated that Mycobacterium tuberculosis extract (a source of hsp) and recombinant hsp65-induced lymphocyte propagation from endomyocardial biopsies correlates with rejection (Moliterno et al, J Heart&Lung Transpl, in press). We have also studied a rat model of heterotopic MHC-incompatible cardiac allografts (ACI into Lewis) whereby graft-infiltrating lymphocytes and spleen cells were tested *in vitro* with different recombinant Mycobacterial hsp preparations (Moliterno et al, Transplantation, in press). As expected, allograft lymphocytes showed proliferative responses to donor cells. This proliferation was markedly augmented by mycobacterial hsp65 and hsp70, whereas hsp10 and the protein control ovalbumin had no effect. Proliferation of allograft lymphocytes to hsp in context with syngeneic spleen cells was seen only if small quantities of IL-2 had been added to the cultures. In contrast, hsp-specific proliferation was never observed with syngraft lymphocytes, even after addition of IL-2. Culture conditions have been established to generate hsp65 and hsp 70-specific T lymphocyte lines and clones from allograft-infiltrating cells.
Hsp expression in transplanted tissues was analyzed by western blotting. A kinetic analysis during the first five days post-transplant showed higher hsp65 and hsp70 expression in allografts than in syngrafts especially when terminal rejection develops. Strong bands for grp78 and a 40 kDa molecule were seen in allografts. These data provide evidence for the involvement of hsp-reactive immunity in cardiac allograft rejection. A model will be presented whereby, during rejection, tissue stress induced by alloreactive effector lymphocytes promotes the upregulation of hsp antigens which will activate hsp-reactive lymphocytes especially in the presence of IL-2 released into the allogeneic environment of the transplant.

B6-305 HEME OXYGENASE: A STRESS PROTEIN INDUCED IN BRAIN INJURY, Barney E. Dwyer, Robert N. Nishimura, and Shi-Yi Lu, Molecular Neurobiology Laboratory, VA Medical Center, Sepulveda, CA 91343
Heme oxygenase (HO) catalyzes the rate-limiting step in the catabolism of heme producing bilirubin, a potentially important antioxidant, and carbon monoxide, a putative neuroregulatory molecule. In addition ferrous ion is produced which, if unsequestered, could promote oxidative injury. Previously we showed HO was a glial heat shock protein inducible by heat and oxidative stress, and possibly a PEST protein (Dwyer et al., GLIA 5:300-305,1992). To further study the role of HO in brain injury we raised an antibody in rabbit against recombinant rat HO-1. Our results suggest that the antibody (AB-1713) detects both inducible HO-1 and constitutive HO-2. HO-2 appears to be the predominant form of HO in normal rat brain and cultured cortical neurons. In contrast, HO-1 appears to be the predominant form of HO in cultured rat forebrain astrocytes. HO-1 is induced in cultured astrocytes by oxidative stress induced with submicromolar concentrations of hydrogen peroxide. In contrast, HO-1 induction in cultured cortical neurons exposed to the same oxidative stress was slight. Immunocytochemistry suggests that HO is not uniformly distributed among all neurons and that some respond to oxidative stress by induction of HO-1. In vivo, cerebral stab wound studies suggest that HO-1 is induced in injured brain early after injury in some neurons near the wound margin, that it later is increased in reactive glial cells and microglia/ macrophages near the wound margin, and that glial HO induction is transient, with augmented HO immunoreactivity in reactive glial cells largely gone 5 days after injury.

Supported by the Research Service of the Department of Veterans Affairs and by the United Cerebral Palsy Research and Educational Foundations.

B6-306 H_2O_2 AND UV STRESS INDUCTION OF AP1 BINDING IN VERTEBRATE LENSES, Peter H. Frederikse and Joram Piatigorsky, LMDB, NEI, NIH, Bethesda, MD 20892-2730
Oxidative stress by H_2O_2 and UV light is a major contributor to cataract in the vertebrate lens. Stress response can manifest itself in a proliferative-like response characterized by expression and DNA binding of AP-1, or a growth arrest-like response indicated by *gadd* gene expression. Extracts from Rhesus monkey lenses in organ culture and rabbit lens cells in cell culture exhibited increases in protein binding to AP-1 cognate sites, identified by competition with mutant oligonucleotides, following treatment with 50-1000μM H_2O_2. Co-migrating EMSA complexes were observed upon stimulation of monkey lenses with TPA. UV_{254nm} light also induced binding of proteins to AP-1 cognate sites. This effect was inhibited by tyrophostin (inhibitor of tyrosine phosphorylation) suggesting that the ras/src signalling pathway is involved as demonstrated by others in HeLa cells. H_2O_2 induction of putative AP-1 binding was also inhibited by tyrosine phosphorylation inhibition. As the lens contains many cells in the process of apoptotic-like terminal differentiation, where epithelial cells at the equatorial margin give rise to cells which elongate and enucleate as they become inner fiber mass cells, our data suggest a proliferative response to oxidative damage by lens cells can contribute to cataract formation.

B6-307 OXIDIZED LDL, HEAT SHOCK PROTEINS AND ATHEROSCLEROSIS. Johan Frostegård*, Birger Andersson, Sampath Jindal and Rolf Kiessling, *Dpt of Medicine, unit of Rheumatology, Karolinska Hospital, Karolinska Institute, 176 71 Stockholm, Sweden.

During recent years, it has become clear that both immunological mechanisms and lipoproteins, most notably oxidized low density lipoprotein (LDL) may play an important role in the development of atherosclerosis. Activated monocytes and T cells are abundant in the early atherosclerotic lesion, and the monocytes develop into foam cells filled with oxidized LDL derived lipids. We here report that oxidized LDL induced enhanced adhesiveness both in endothelial cells and monocytes, differentiation of monocytic cells to macrophages and activation of T cells. Oxidized LDL, in concentrations resulting in T cell activation, also stimulated the release of IL-1 from monocytes.
Heat shock proteins (hsp) have been detected in the atherosclerotic lesion. Expression of hsp60 in monocytic cells after treatment with oxidized LDL and Il-1 was investigated. The monocytic cell lines U937 and HL60 were treated with oxidized LDL or Il-1 and the expression of heat shock protein 60 was analyzed with FACScan, Western Blot and ELISA. In both cell types, a strong increase in the expression of heat shock protein was demonstrated.
The results show that oxidized LDL and Il-1 enhances the expression of heat shock protein on monocytic cells, which may lead to perpetuation of an inflammatory reaction and be of importance in the development of atherosclerosis.

B6-308 THE HEAT SHOCK PROTEIN HSP90 PLAYS A ROLE IN CELL CYCLE CONTROL AND DIFFERENTIATION OF MYELOMONOCYTIC CELLS.

Joanna Galea-Lauri, David S. Latchman and David R. Katz, Departments of Immunology and Molecular Pathology, UCL, 46,Cleveland Street, London W1P 6DB, UK.

Heat shock proteins (Hsps) are produced by both prokaryotic and eukaryotic cells in response to a variety of insults. Several of these proteins are also known to be expressed constitutively. One such protein, hsp90, is the most abundant constitutively expressed stress protein in the cytosol of cells and participates in the maturation of other proteins, modulation of protein activity (e.g. hormone-free steroid receptors) and intracellular transport of some newly synthesised kinases. Thus far, however a role for hsp90 has not been demonstrated in the cell cycle. In this study, we used the monoblastoid cell line U937. We used a molecular approach transfecting the parent cell line with anti-sense and sense constructs for hsp90 in order to develop novel stable cell lines with significantly reduced or elevated levels of the hsp90. Parental U937 cells express high levels of hsp90, and no further increase in this protein was seen after heat shock treatment despite a high induction in the expression of the inducible stress protein hsp72. Transfectants with low or higher levels of hsp90 were also incapable of increased expression of hsp90 after heat shock treatment despite induction of hsp72. This suggests that in these cells hsp90 has other critical functions. Decreased levels of hsp90 slows down the rate of cell division with less cells in the S phase of the cell cycle with less incorporation of ^{125}I-UdR and a shift in DNA profiles in cells stained with propidium iodide. Furthermore, cells with low expression of hsp90 differentiate to a mature macrophage like cell at a slower rate than control cells or cells expressing high levels of hsp90. Thus, at least in the monoblastoid cell line U937, hsp90 participates in cell cycle control as well as in the differentiation of monoblastoid cells to a macrophage-like population.

B6-309 IDENTIFICATION OF THE HUMAN HSPA2 GENE ON CHROMOSOME 14 AS THE HOMOLOGUE OF THE MOUSE HSP70-2 GENE AND THEIR PATTERN OF EXPRESSION IN TISSUES,

Clayton R. Hunt[1], L.L.C. Bonnycastle[2], C-E. Yu[2], B.J. Trask[3], K.P. Clancy[4], J.L. Weber[5], D. Patterson[4], and G.D. Schellenberg[2]. [1]Washington University School of Medicine, St. Louis, MO 63108; [2]School of Medicine, University of Washington, Seattle, WA 98195; [3]University of Washington, Seattle 98195; [4]Eleanor Roosevelt Institute, Denver, CO 80206; [5]Marshfield Medical Research Foundation, Marshfield, WI 54449.

The mouse Hsp70-2 gene was originally mapped to chromosome 2 in a region syntenic with human chromosome 14. In humans this region is near an unidentified human Hsp70 gene (HSPA2) and also in the vicinity of markers for early-onset familial Alzheimer's disease (FAD). Several lines of evidence have previously suggested the possible involvement of HSP70 proteins in Alzheimer's disease. To resolve the potential role of the HSPA2 gene in FAD we isolated the human homologue of the mouse Hsp70-2 gene and mapped it to YAC 741H4 which also contains the polymorphic marker D14S63. This YAC was mapped by fluorescence in situ hybridization to 14q24.1 Sequence analysis of the isolated gene identified it as the human homologue of the mouse Hsp70-2 gene which is expressed at high levels in germ cells. Examination of the expression pattern of HSPA2 also revealed high levels in RNA from germ cells but significant levels were detected in most other tissues especially brain. Mouse Hsp70-2 expression was also detected in brain tissue by Northern blot analysis. However more recent genetic analysis of FAD families has detected recombination events between the unidentified Alzheimer's locus and D14S63 which place the HSPA2 gene centromeric to the disease locus. The DNA sequence provided should, though, permit direct scanning of the HSPA2 gene for FAD mutations.

B6-310 HSP70-MEDIATED PROTECTION OF TUMOR CELLS FROM APOPTOSIS INDUCED BY TUMOR NECROSIS FACTOR.

Marja Jäättelä and Dorte Wissing. Dept. of Tumor Cell Biology, Danish Cancer Society Research Center, 2100 Copenhagen, Denmark.

We have previously shown, that transfection and following expression of human heat shock protein 70 (HSP70) in tumor cells protects them from the cytotoxicity mediated by tumor necrosis factor (TNF) and inhibits TNF-induced activation of phospholipase A2. To localize the domain responsible for HSP70-mediated tumor cell resistance to TNF, we generated two types of human HSP70 deletion mutants; HSP70-B (amino acids 120-426 deleted) and HSP70-S (amino acids 438-618 deleted) lacking the domains for ATP binding and nuclear localization, respectively. Our preliminary results show, that WEHI-S mouse fibrosarcoma cells expressing either one of the mutant HSP70s are as resistant to TNFs as cells expressing the full length HSP70. Thus, it is unlikely that either ATP binding or nuclear translocation is necessary for HSP70 associated resistance to TNF-mediated apoptosis. Interestingly, it has been shown that in HSP70-mediated thermotolerance, the ATP binding domain appears dispensable, while the domain responsible for translocation to the nucleus is necessary. Thus, our results suggest that mechanisms by which HSP70 protects cells from TNF and heat are different. In addition to the murine fibrosarcoma cells we have successfully expressed HSP70, HSP70-B and HSP70-S in human breast carcinoma cells. The results from these studies will be discussed. The work will be further extended using additional deletions in the gene encoding HSP70 and studying the effect of these mutants on TNF-induced apoptosis and activation of phospholipase A2 in human and murine tumor cell lines.

B6-311 CYTOKINE AND ACUTE PHASE PROTEIN RESPONSE IN EXPERIMENTAL SEPTIC SALMONELLOSIS OF PIGS BY *SALMONELLA CHOLERAESUIS*.

Ted T. Kramer, Dagmar E Frank and Franklin Ahrens. Veterinary Medical Research Institute, Iowa State University, Ames, IA 50011.

The acute phase response to a virulent strain of *Salmonella choleraesuis* was evaluated in 3 different ways: by nasopharyngeal gavage of a sublethal dose to young pigs; by intravenous injection; and by short-term extracorporeal perfusion of isolated pig livers. Fever, erythrocyte sedimentation rates, severity of clinical signs, and outcome of disease correlated with production of TNFα, IL-6, C-reactive protein, haptoglobin, and α-2 acid glycoprotein and other expressions of the acute phase response. Concentrates of liver perfusion fluid filtrates, obtained from pig livers perfused with *Salmonella choleraesuis*, and to a lesser extent with LPS, promoted growth of *Salmonella choleraesuis in vivo* and *in vitro*.

B6-312 QUERCETIN BLOCKS ISCHEMIC TOLERANCE AND SYNTHESIS OF HSP 70 IN RAT HEARTS, Rakesh C. Kukreja, Yong-Z Qian, Michael C. Kontos and Michael L. Hess.
Medical College of Virginia, Richmond Va 23236

Several investigators have suggested that heat stress induced cardiac protection results from synthesis of heat shock protein (HSP). We investigated the effect of quercetin, a known inhibitior of HSP transcription factor, in an *in vivo* rat model of heat-shock (HS) preconditioning. Rats (n=5 to 6) were treated with 1 dose of quercetin (5 mg/kg) 30 min prior to heat shock which was induced by wrapping the animals in a electric blanket to raise whole-body temperature to 42°C for 15 min; corresponding controls were treated with anesthetic alone. Twenty-four hours later, hearts were subjected to 30 min of coronary artery occlusion followed by 90 min of reperfusion (I/R). Area at risk (AR) was delineated by dye injection and area of necrosis (AN) was determined by tetrazolium staining. Results are presented as means±SEM.

Group	AR (% LV)	AN (% AR)
Control (I/R)	63.5±5.0	40.2±2.2
HS	77.3±0.8	12.4±4.2 *
Quercetin (5 mg/kg) + HS	58.6±2.0	37.1±6.0
Quercetin (5 mg/kg) (no HS)	74.4±1.2	36.4±1.0

*P<0.05

The results show a significant reduction in AN following whole-body hyperthermia (P<0.05). Treatment with quercetin prior to heat shock significantly blocked HS mediated protection. Quercetin pretreatment without heat shock did not significantly alter AN (P=NS). In addition, quercetin pretreatment prior to heat shock significantly inhibited the synthesis of HSP 70 in non-ischemic hearts. The results suggest that HSP 70 may be directly involved in ischemic tolerance following heat shock in rats.

B6-313 CLONING, SEQUENCING AND EXPRESSION OF A *CANDIDA ALBICANS* cDNA CODIFYING FOR AN IMMUNOGENIC HEAT SHOCK PROTEIN 70 kDa (CaHSP70). R. La Valle[1], C. Bromuro[1], S. Arancia[1], S. Sandini[1], H.M. Muller[2], A. Crisanti[2] and A. Cassone[1]. Bacteriology and Medical Micology Department[1], Istituto Superiore di Sanità, Rome, Italy; Parasitology Department[2], University of Rome, Italy.

To investigate the role of major immunogenic and immunomodulatory constituents of *Candida albicans* we have constructed a cDNA expression library of yeast form of the fungus. Using a rabbit anti-*Candida* polyclonal antiserum for library screening, we have cloned and sequenced a full-lenght cDNA (2325bp) encoding a stress protein of *C. albicans*. It shows high homology (80%) to the *Saccharomyces cerevisiae* SSA1 gene (one of the 70 kDa heat shock protein family genes of *S. cerevisiae*). In pulse-field electrophoresis, the *C. albicans* gene mapped on the largest electrophoretic chromosome (3.5 Mbp). We have called this gene Cahsp70 and the codified product CaHSP70. Northern-blot and western-blot analysis have shown induction of Cahsp70 gene transcription and expression after heat shock. To purify the CaHSP70 protein, fragments of the coding sequence were cloned into the EcoRI site of pDS56/RBSII-E$^-$-6his plasmid, bringing to fusion products with six histidine tag at the N-terminus, thus allowing purification through nickel column. Four different gene products, one of which representing the whole CaHSP70 protein, were purified and tested for their antigenicity in human lymphocyte cultures. At least the whole protein and the N-terminus fragment induced remarkable lymphocyte proliferation. Thus, the CaHSP70 may represent an immunodominant antigen of *C.albicans*, the role of which in host-*Candida* relationship is currently investigated.

B6-314 **ISCHEMIC PRECONDITIONING: EVIDENCE FOR LONG-TERM SYSTEMIC PROTECTION IN CANINE SKELETAL MUSCLE.** Shinta K. Liauw, Alex D. Romaschin, Barry B. Rubin, Thomas F. Lindsay, Jian X. Au, Paul M. Walker, Departments of Vascular Surgery and Clinical Biochemistry, The Toronto Hospital, Toronto, Ontario, Canada.

The cytoprotective effect of ischemic preconditioning (IP) has been known as a local phenomenon, but its remote beneficial effects are not well established. We hypothesize that skeletal muscle subjected to a period of ischemia and reperfusion (I/R) produces systemic mediators which trigger the production of protective factors in distant skeletal muscle. Using a paired canine gracilis muscle model, we subjected the first gracilis muscle (M1) to 5 h ischemia followed by 48 h of reperfusion. The muscle was removed and analyzed for necrosis. The contralateral muscle (M2) was then subjected to an identical insult. Biopsies at various time points were taken from both muscles for adenine nucleotide analysis.

Necrosis	M1 = 39.8 % ± 8.4 %		M2 = 16.5 % ± 5.4 % *	
	ATP (μmole/g dry weight)		$ECP_{(ATP+0.5ADP)/(ATP+ADP+AMP)}$	
Time	M1	M2	M1	M2
Pre-isch	21.1±2.2	22.9±2.7	.93±.01	.93±.01
End-isch	6.5±1.8	12.6±4.0*	.73±.04	.84±.03*
48H Rep	12.3±2.8	13.9±4.4	.85±.05	.80±.08

*Values are mean ± SEM, n = 6, * P < 0.05 for M2 vs M1.*

There was a linear correlation between end-ischemic ECP (ECP_{EI}) and necrosis in M1 (r = -0.920), but not in M2 (r = -0.748). The significant reduction of necrosis in M2 indicates that I/R in one muscle can induce a systemically mediated cytoprotection in a distant muscle. This protective effect is manifested as a preservation of ATP and ECP in M2 during the ischemic period. Previous studies have shown that ECP_{EI} can be used to predict the final extent of muscle necrosis. The lack of linear correlation between ECP_{EI} and necrosis in M2 supports the role of factors other than adenine nucleotide preservation in the muscle salvage. Candidate protective factors are stress proteins induced by I/R.

B6-315 STRESS PROTEIN INDUCTION IN KIDNEY PROXIMAL TUBULE CELLS IN RATS PRETREATED WITH ZINC IN VIVO AND EXPOSED TO CADMIUM CHLORIDE IN VITRO, [1]JENNY X. Liu, [2,3]Bruce A. Fowler, [2]Mirriam Akkerman, [2,3]Katherine S. Squibb and [1]Gunnar F. Nordberg, [1]Dept. of Environmental Medicine, Umeå University, S-901 87 Umeå, Sweden, [2]Toxicology Program, and [3]Department of Pathology, University of Maryland, Baltimore, MD 21201

Apart from the induction of metallothionein (MT), little attention has been given to the alterations in cellular gene expression in cadmium-induced cell injury. In vivo studies have shown that prior zinc treatment attenuates Cd toxicity but the underlying mechanisms are not completely known. In this study, the rat renal proximal tubule cells were used to study the relationship between the stress protein response and cytotoxicity of $CdCl_2$ in vitro. Rat kidney primary cell cultures were exposed to $CdCl_2$ for 2, 4 or 8 hours at a concentration of 129 μM (14.55 μg Cd/ml medium) $CdCl_2$ in the medium. The expression of stress protein gene products was demonstrated by ^{35}S-two-dimensional gel electrophoresis. In rats with no zinc pretreatment in vivo high cytotoxicity was elicited in cells exposed to $CdCl_2$, the prior administration of zinc in vivo significantly protected these cells in vitro with increased incorporation of ^{35}S-methionine and improved cell viability as measured by the alamar blue assay and electron microscopy. The ^{35}S labelling of gene products induced by cadmium exposure clearly demonstrated that specific gene products with approximate molecular masses of 12, 30, 46, 70, and 90 kDa were induced in cells from zinc pretreated rats relative to cells from rats not pretreated with zinc. These data provide further evidence that in addition to MT, the observed zinc-induced increases in expression of the major stress protein groups in kidney tubule cells exposed to $CdCl_2$ in vitro also correlated well with protection of cell viability suggesting that both protective mechanisms are operating.

B6-316 *MYCOBACTERIUM LEPRAE* HSP65 VACCINATES MICE AGAINST TUBERCULOSIS WHEN EXPRESSED FROM THE CLONED GENE IN TRANSPLANTED BONE MARROW CELLS. Douglas B. Lowrie*, Celio L. Silva, Rosemeire L.R. Pietro, Adriana Januario, Vania L.D. Bonato, Valeria M.F. Lima & Marcelo F. daSilva, *National Institute for Medical Research, Mill Hill, London NW7 1AA. School of Medicine of Ribeiro Preto, University of Sao Paulo, 14049-900 Brazil.

We previously showed that *M. leprae* hsp65 was remarkably effective in vaccinating mice against tuberculosis. The protein in adjuvant (IFA) had little effect but when J774 macrophage-like tumour cells expressing the protein from the transfected mycobacterial gene were injected iv or ip a high degree of specific protection was acquired (C.L. Silva & D.B. Lowrie, Immunology 1994 **82** 244). Protection against the tumour cells was also acquired (K.V. Lukacs, D.B. Lowrie, R.W. Stokes & M.J. Colston, J.Exp.Med. 1993 **178** 343). Because this implies that vaccines could be based on this single antigen, provided that the antigen arises endogenously within antigen presenting cells, we have tested the principle with bone marrow cells. Cells from femurs and tibias of 5-fluorouracil-treated BALB/c mice were transfected with retroviral vector pZIPNeo containing the mycobacterial gene, selected with neomycin, then injected iv into groups of 5 irradiated (9.5 Gy) mice. A high proportion of recipients were expressing the protein in their peripheral blood cells after 2 weeks and about half of those had specific DTH reactions to the protein. Challenge infection with *M. tuberculosis* H37Rv 5 weeks after transplantation showed that only mice with DTH were protected. Counts of viable (cfu) bacteria in livers 3 weeks after challenge were about 7-fold lower in DTH responders than in non-responders or in normal mice or mice that had been transplanted with bone marrow containing the vector only. Antigen in IFA was again ineffective. Hence, special properties of tumour cells are not needed for the effectiveness of vaccination, whereas endogenous origin of antigen is likely to be crucial.

B6-317 ANTIPROLIFERATIVE EFFECTS OF A HEAT SHOCK PROTEIN-LIKE FACTOR PRODUCED BY A NEW ESTABLISHED HUMAN LIPOSARCOMA CELL LINE, *Aldo Mancini, §Gian Luigi Russo, *Antonella Borrelli, *Maria Evangelista, *Monica Valentino, §Fulvio Della Ragione, *National Cancer Institute "G. Pascale", and §Institute of Biochemistry of Macromolecules, II University of Naples, Naples Italy.

Several data support the idea that mesenchimal tumors show a remarkable resistance to X-rays treatment and to chemotherapy. In order to develop alternative therapeutic approaches, we established new cell lines from human malignant sarcomas. One of these, named LSA, was obtained from a human malignant plejomorfic liposarcoma. LSA spontaneously accumulated lipid-containing vesicles thus mimicking adipocytes. LSA is able to proliferate for an exceptionally long period in serum-starvation conditions, suggesting the involvement of several possible mechanisms as: i. expression of modified forms of dominant oncogenes; ii. loss of activity of tumor suppressor genes; iii. production of autocrine factor(s) sufficient for sustaining cell growth requirement and for enhancing its stress resistance. We investigated the effect(s) of LSA-conditioned medium (LSA-CM) on the growth of different mammalian cells. Our data indicates that LSA-CM stimulates cell proliferation of different mammalian cells. Conversely, in other cell systems, the medium exerts a significant antiproliferative effect. One important finding is that all the cell line sensitive to LSA-CM cytostatic activity derived from mammary carcinomas and were positive to estrogen receptor.

The results discussed in the present communication indicate that one of the proteins endowed with cytostatic activity appears to belong to the low molecular weight heat shock protein family.

B6-318 TRANSCRIPTIONAL ADAPTATION TO STRESS IS MEDIATED BY HSFs IN MYOCARDIAL HYPERTROPHY.

Eduardo Mascareno, Manya Dhar, M.A.Q. Siddiqui. Department of Anatomy & Cell Biology, State University of New York at Brooklyn, NY 11203.

The response of animals and humans to stress is reflected at the cellular level by an increment in heat-shock gene expression. Studies in our laboratory have indicated that the promoter of the regulatory myosin light chain (MLC2) gene, among others, is the target for stress-related transcription activator proteins, the heat shock factors (HFSs). We have observed that the heat shock responsive sequence elements (HSEs) located in the proximal promoter of MLC2 gene, binds the activated HSFs in primary cardiac cells in culture following stress induced by heat shock treatment. Transient transfection of MLC2/CAT reporter into primary cardiac cells, respond positively upon heat shock treatment but not when HSEs were deleted from the promoter. When genetically predisposed hypertensive rats (SHR) were analyzed for the binding activity of the HSFs to the HSE on the promoter of the cardiac MLC2 gene, we observed a clear correlation between the appearance of activated HSFs and the onset of cardiac hypertrophy. Similar binding activity was absent in age matched normotensive rats (WKY). These results, defined for the first time a physiological target (MLC2 promoter),other than the genes for the heat shock proteins, and delineate a novel mechanism of stress-mediated adaptation of transcription during myocardial hypertrophy.

B6-319 LIPOPOLYSACCHARIDE INDUCES A CARDIAC CROSS-RESISTANT STATE IN RATS WHICH IS ASSOCIATED WITH HEAT SHOCK RESPONSE Xianzhong Meng, Lihua Ao, Robert T. Rowland, Anirban Banerjee, Alden H. Harken and James M. Brown, Department of Surgery, University of Colorado Health Sciences Center, Denver, CO 80262

We have observed that lipopolysaccharide (LPS) induces cardioprotection against ischemia/reperfusion (I/R) injury in rats and that the cardioprotective state is preceded by myocardial contractile depression and heat shock protein 70 (HSP 70) expression. This study tests the **hypothesis** that LPS induces a cardiac cross-resistant state, to both I/R injury and myocardial depression by subsequent LPS, which is associated with heat shock response. Adult rats were treated with a myocardial depressive dose of LPS (from *Salmonella typhimurium*, 500 μg/kg/IP) or subjected to whole body hyperthermia (42°C for 15 minutes). After 24 hours recovery, rats were subjected to one of the following protocols: 1) heart isolation, cryosection and staining for HSP 72 by immunohistochemistry, 2) heart isolation and determination of functional recovery after 25 minutes global ischemia and 40 minutes reperfusion and 3) administration of LPS (500 μg/kg/IP) and evaluation of isolated heart function by Langendorff technique 6 hours later. **Results:** HSP 72 was induced in the myocardium by both LPS and hyperthermia and mainly localized in the nuclei of myocytes and interstitial cells. LPS pretreatment induced a cardiac resistant state not only to I/R injury but also to myocardial depression by subsequent LPS.

	LVDP (mmHg)		
Group	Baseline	Post-ischemia	Post-LPS
Control	101 ± 1.3	44.0 ± 2.8 (17)	58.2 ± 7.4 (9)
LPS 24hrs	100 ± 2.8	67.6 ± 2.7 (8)*	97.0 ± 3.5 (6)*
Hyperthermia 24hrs	103 ± 2.0	59.8 ± 6.3 (9)*	80.1 ± 5.0 (8)*

Mean ± S. E. M., *=P<0.05 vs control

A similar cardiac cross-resistant state was also induced by hyperthermia. The results suggest that LPS induces a cardiac cross-resistant state in the rat heart and that the cardiac cross-resistant state is associated, at least in part, with heat shock response.

B6-320 STABLE EXPRESSION OF A HUMAN HSP70 GENE IN A RAT MYOGENIC CELL LINE CONFERS PROTECTION AGAINST ENDOTOXIN, Ruben Mestril, Shun-Hua Chi and Wolfgang H. Dillmann, Dept. of Medicine, Univ of Calif, San Diego, CA 92103.

We have recently found that a mild heat shock pretreatment in the rat myogenic cell line, H9c2(2-1), confers resistance to a subsequent exposure to endotoxins or lipopolysaccharides. Several recent reports have also shown that a pre-heat shock has a protective effect against endotoxins "*in vivo*" both in rats (Ryan et al, J Appl Physiol 73:1517-1522) and in mice (Hotchkiss et al, Am J Physiol 265:R1447-R1457). In all these mentioned cases it is unclear what actually confers the protection against endotoxins. Obviously, good candidates for this protective effect are the heat shock proteins (hsps) and, in particular, the hsp70 which is the most abundant among them. In addition, several studies have demonstrated that hsp70 is responsible at least partially for protection against a lethal heat shock, metabolic stress and simulated ischemia. We then propose that hsp70 may be also involved in protection against endotoxins. We have now tested this hypothesis and have found that myogenic cells stably transfected with the human hsp70 shows an increased survival rate as compared to cells stably transfected solely with the selectable neomycin marker gene or the parental cell line H9c2(2-1) when exposed to endotoxins (100μg/ml) as measured by trypan blue exclusion (108 ± 17 vs. 80 ± 9 and 79 ± 11; $p < 0.01$, $n=9$) and lactate dehydrogenase (LDH) release (122 ± 21 vs. 179 ± 63 and 147 ± 122; $p < 0.05$, $n=9$) immediately after endotoxin treatment. We then conclude that hsp70 must also be involved in protecting the cell against the deleterious effects of endotoxins.

B6-321 CD3 NEGATIVE LARGE GRANULAR LYMPHOCYTES RECOGNIZE A HEAT INDUCIBLE IMMUNOGENIC DETERMINANT ASSOCIATED WITH THE 72 KD HEAT SHOCK PROTEIN (HSP) ON HUMAN SARCOMA CELLS, Gabriele Multhoff*, Claus Botzler*, Marion Wiesnet*, Wolfgang Wilmanns*# and Rolf Dieter Issels*#, * Institut für Klinische Hämatologie der GSF, Marchioninistr. 25 and # Klinikum Großhadern, Medizinische Klinik III der Ludwig-Maximilians-Universität München, Marchioninistr. 15, D-81377 München, FRG

Traditionally, heat shock proteins (HSP) are believed to be located intracellularly, where they perform a variety of chaperoning functions. However, recent publications have demonstrated that under certain circumstances malignant cell types express HSP on the cell surface. Our studies confirm this finding and correlate HSP72 cell surface expression, induced by nonlethal heat shock, with increased tumorigenicity against $CD3^-$ natural killer cells (NK). A monoclonal antibody (mAb, RPN1197) directed against the major heat inducible 72 kD heat shock protein (HSP72) binds to the cell surface of tumor cells (i.e. human Ewing's sarcoma cells or osteosarcoma cells), but not to normal cells (i.e. PBL, fibroblasts, PHA blasts, B-LCL) after single nonlethal heat shock (41.8°C, 200 min) followed by a recovery period at 37°C (4h). Despite a decrease in the MHC class I cell surface expression after heat shock a marked increase (2-fold) in tumorigenicity as compared to untreated tumor cells was found. Analysis of cytotoxic activity of $CD3^-$ large granular lymphocytes (NK cells), $CD3^+$ MHC restricted CTL and unseparated effector cells in a cell mediated lympholysis assay (CML), demonstrated that the $CD3^-$ NK effector cell population and not the $CD3^+$ CTL population, is responsible for the recognition of heat shocked tumor cells. By antibody inhibition (using this HSP72 specific mAb, RPN1197) an immunogenic HSP72 determinant, which is expressed only on the cell surface of tumor cells after nonlethal heat shock could be determined as the relevant recognition structure for $CD3^-$ NK cells. As a control, blocking of MHC class I restricted recognition (using either MHC class I specific mAb W6/32 on the target cells or a/ß TCR WT31 on effector cells) had no inhibitory effect on the lysis of heat shocked tumor cells. In summary, our data indicate that $CD3^-$ NK cells recognize a heat inducible HSP72 related immunogenic epitope, on the cell surface of sarcoma cells, but not on normal cells.

B6-322 INHIBITION OF AUTOLOGOUS, TUMOR SPECIFIC, γ/δ, CYTOTOXIC T-LYMPHOCYTE (CTL) MEDIATED CYTOLYSIS BY ANTI-GRP75 ANTISERA AND CHARACTERIZATION OF A NOVEL 69 kD CELL SURFACE PROTEIN, Edward L. Nelson, Clarissa Naftzger, William J. Welch, Carol Clayberger, Alan M. Krensky, Lung Biology Center University of California at San Francisco, Departments of Medicine and Pediatrics, Stanford University, School of Medicine, Stanford, CA 94305

We have previously characterized an autologous, tumor specific, γ/δ T cell receptor bearing, CTL line that recognizes the Burkitt's lymphoma (BL) target in an antigen specific and non-MHC restricted fashion. Furthermore, non-classical MHC molecules were excluded as antigen presenting molecules. In an effort to identify and characterize the antigen presenting molecule, antibodies directed against multiple heat shock proteins were evaluated. Anti-GRP75 antisera, anti-HSP60 antisera, and monoclonal antibodies directed against members of the HSP70 family (W27 & 1B5) demonstrated positive staining of the BL by FACS analysis. Only anti-GRP75 antisera inhibited (>70%) the cytolytic response of autologous CTL and this inhibition was not evident in allogeneic CTL mediated cytolysis. Immunoprecipitation of cell surface ^{125}I radiolabeled BL with the anti-GRP75 antisera identified a 69kD protein distinct from GRP75. Screening of an eukaryotic expression library has yielded a 1.8 kb cDNA encoding a novel protein recognized by this antisera. Preparation of monoclonal antibodies recognizing this protein are in progress. These findings provide further support for the role of heat shock (stress) proteins in antigen presentation and anti-tumor immune responses.

B6-323 INDUCTION OF HSP 70 BY PHENANTHROLINE DURING ACIDOSIS AND SALICYLATE EXPOSURE IN CULTURED RAT ASTROCYTES. Robert .N. Nishimura and Barney E. Dwyer, Department of Neurology, UCLA School of Medicine, Los Angeles, CA 90024.

The induction of HSP 70 is associated with thermotolerance and survival after many types of stress in cultured mammalian cells. This investigation studied the induction of HSP 70 in cultured rat astrocytes after cells were exposed to acidosis and salicylate which primarily activate heat shock transcription factor 1 and binding to the heat shock element but do not lead to transcription. In this study the induction of HSP 70 mRNA and translation of HSP 70 are noted when cells were simultaneously exposed to acidosis or salicylate with 1,10 phenanthroline. HSP 70 mRNA was transiently induced under these conditions which indicated that a continuous stress of the phenanthroline was not apparently the cause of induction. Phenanthroline alone did not induce the synthesis of HSP 70. The mechanism of this induction is unknown. These findings seem to support the multi-step induction of HSP 70. It may also be possible to selectively induce HSP 70 by utilizing conditions in this study.

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B6-324 SYNTHETIC PEPTIDES DERIVED FROM CLASS I MHC MOLECULES INHIBIT T CELL FUNCTION AND BIND TO MEMBERS OF THE HSP70 STRESS PROTEIN FAMILY.

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Peptides corresponding to the $\alpha 1$ helix of class I MHC molecules are capable of inhibiting T lymphocyte function *in vitro* and organ transplant rejection *in vivo*. These peptides may be useful in clinical applications to prevent graft rejection, graft versus host disease and T cell mediated autoimmune diseases. The mechanism by which these peptides mediate their immune modulatory effects is under investigation. Peptides found to inhibit T cell mediated cytotoxicity also induce an increase in intracellular calcium levels in T cells. Peptide induced calcium flux is seen in both the T cell tumor cell line Jurkat and its T cell receptor negative derivative, indicating that the peptide effect is mediated through a T cell receptor independent pathway. Biochemical analysis performed to identify peptide ligands demonstrate high affinity binding of T cell inhibitory peptides to two proteins with apparent molecular weight of 74 kDa and 70 kDa. These peptide binding proteins appear to be expressed at the cell surface of T cells, but not EBV transformed B cells, fibroblasts or pre-erythroid cell lines. They are stress inducible and reactive with anti-HSP70 monoclonal antibodies suggesting, that they are members of the HSP70 family. Analysis of peptides with single amino acid substitutions revealed that an isoleucine is a critical residue required for the inhibitory effect, the calcium flux, and the binding to the heat shock proteins. This striking correlation is highly indicative that the identified binding proteins play a role in the observed immune modulatory effect.

B6-325 HEAT SHOCK PROTECTS KIDNEY TRANSPLANTS FROM ISCHEMIC INJURY AND ENHANCES HSP 72 PRODUCTION

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All transplanted tissue is exposed to multiple stresses as a result of conventional preservation techniques. Warm and cold ischemic injury contribute to organ damage and detract from the already limited supply of transplantable organs and tissues. We tested the hypothesis that heat shock (42.5°Cx15') and recovery (37°Cx6-8 hrs) of donor animals prior to organ retrieval will protect kidney transplants from ischemic injury. Yorkshire pigs (15-20 kg) received 90 min of warm ischemia (WI) prior to kidney removal. Three groups were divided according to their treatment, prior to warm ischemic injury. Group 1, controls received no pretreatment. Group 2 were sham pretreated by receiving an arterio-venous shunt under general anesthesia while core body temperature was maintained at 37-38°C for 4-6 hrs prior to WI. Group 3 (heat shock) received hyperthermic perfusion by extracorporeal heating to obtain a core body temperature of 42.5°C for 15 min followed by 4-6 hrs of recovery at 37-38°C prior to WI. After WI, all kidneys were stored 20 hours, and then transplanted using immunosuppressive therapy. Daily serum creatinine levels were tested for 10 days, at which time all animals were sacrificed. Heat shock protein (HSP 72) from renal tissues was measured at the time of transplantation by Western Blot (Amersham, inducible HSP 72). Differences in serum creatinine were significant ($p < 0.001$) between the Group 1 (11.2 ± 4.3) and Group 3 (3.5 ± 2.4) on day 7. Western blot analysis showed an increase in HSP-72 in 3 kidneys alone. Survival was significantly better in Group 3 vs Group 1 ($p \leq 0.04$). HSR induction protects kidneys against WI and enhances HSP 72 production.

Group	Pre-treatment	7 Day Serum Creatinine	10 Day Survival
1	None	11.2 ± 4.3	4/8 (50%)
2	Sham	9.3 ± 5.7	4/6 (66%)
3	Heat-Shock	3.5 ± 2.4	8/8 (100%)

B6-326 EFFECT OF MYCOBACTERIAL HSP65 ON EXPERIMENTAL ARTHRITIS IN TWO RAT STRAINS

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Introduction: The 65kDa heat shock protein (hsp65) of *Mycobacterium tuberculosis* (Mtb) has been implicated in both the experimental arthritis of rodents and human rheumatoid arthritis (RA). Previously, we showed increased expression of an hsp65 homologue in the human rheumatoid synovial membrane. Reasoning that ongoing inflammation in the joint may result in the release of this highly immunogenic hsp, we questioned the outcome of an intra-articular presentation of hsp65 using a modified model of antigen-induced arthritis. Since Lewis rats are particularly susceptible to arthritis induction, we compared the response in both Lewis and Wistar rats.

Aim: To assess the potential arthritogenic properties of hsp65.

Methods: Wistar or Lewis rats were sensitised in the scruff by injection of 1mg heat-killed powdered Mtb (strains C, DT, PN) in light paraffin oil and, after 7 days, challenged intra-articularly (i-a) in one knee with either PPD or the recombinant hsp65 from *M Leprae* or the vehicle (PBS) alone. Sensitised non-injected animals were also included. Knee swelling was monitored for up to 6 weeks and radiological and histological assessments carried out at various time points.

Results: Both strains displayed knee joint swelling, maximal 2-3 days after injection of either PPD or hsp65. Histologically, the early response was similar with marginal pannus and $\alpha\beta$T-cell infiltration of the synovium. In the Wistar strain, the inflammation settled and joints appeared normal within 2-4 weeks. However, Lewis rats suffered recurrent joint swelling at approximately 10 day intervals. By 4-6 weeks, marginal erosive pannus was seen overlying articular cartilage and peripheral new bone formation was apparent. Control groups were histologically normal.

Conclusion: Intra-articular presentation of hsp65 can induce synovial inflammation but its arthritogenic properties are strain dependent, implying a degree of MHC restriction. The chronic response with recurrent flares in Lewis rats shows similarities with human RA.

B6-327 CIRCULATING SERUM ANTIBODY IN PATIENTS WITH IDIOPATHIC PROGRESSIVE BILATERAL SENSORINEURAL HEARING LOSS (IPBSNHL) TARGETS HEAT SHOCK PROTEIN 70 (HSP70),

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Idiopathic, progressive, bilateral, sensorineural hearing loss (IPBSNHL) is one of the few medically remediable forms of nerve deafness. Immunosuppressive therapy can improve or restore hearing in many cases. Symptoms of vertigo or imbalance are also found in more than half of patients and often the presentation cannot be distinguished from Ménière's disease, suggesting they are separate but overlapping entities. We have shown previously that nearly 60% of patients with IPBSNHL have circulating antibodies against a 68 kD protein of bovine inner ear (IE) or renal extract (RE). Furthermore, we have shown that presence of the anti-68 kD Ab is highly correlated with activity of disease and steroid responsiveness. The 68 kD target protein from bovine RE was resolved into a single band on PAGE and transferred to nitrocellulose membrane. The narrow region of membrane containing this protein was digested with trypsin and the resulting peptides separated by HPLC. One fraction contained a peptide of 2776 mw with the sequence of a 22 amino acid segment corresponded 100% with amino acids 213-234 of bovine and 424-445 of human HSP70. Identity of the 68 kD protein as HSP was confirmed by Western blot; purified RE Ag developed with monoclonal Ab to HSP70, commercial HSP70 as Ag developed with human Ab to the 68 kD protein, and commercial HSP70 as Ag developed with mouse monoclonal anti-HSP70 Ab all formed a single precipitin line. These findings suggest that HSP70 is the target antigen in IPBSNHL. Whether Ab to HSP70 forms in response to an infectious agent bearing HSP70 sequences or in response to HSP70 production by inner ear tissue undergoing infection or other stress remains to be determined.

B6-328 BINDING OF PROTEIN ANTIGEN TO CONSTITUTIVELY EXPRESSED STRESS PROTEIN BYPASSES A PEPTIDE TRANSPORTER DEFECT IN THE MHC CLASS I PROCESSING. Jörg Reimann and Reinhold Schirmbeck. *Institute for Medical Microbiology, University of Ulm, Ulm, Albert-Einstein-Allee 11, D-89069 ULM, FRG.*

We investigated the role of stress proteins in alternative processing pathways of cytosolic protein antigens for MHC class I-restricted epitope presentation. Cytotoxic $CD8^+$ T lymphocytes (CTL) usually recognize peptides derived from intracellularly synthesized and processed 'endogenous' protein antigens that are presented on the cell surface in association with major histocompatibility complex (MHC) class I molecules. Murine RMA-S cells are deficient in translocating peptides from the cytosol into the lumen of the endoplasmic reticulum (TAP^-), and therefore fail to present many intracellular protein antigens to CTL. Two MHC class I (D^b)-restricted epitopes of simian virus 40 large T-antigen (T-Ag) were presented to CTL by wild-type (wt) T-Ag-transfected RBL5 or RMA cells but not by transfected RMA-S cells, demonstating that the endogenous MHC class I presentation pathway of this protein is TAP-dependent. These two T-Ag epitopes were however efficiently presented to CTL clones by RMA-S cells expressing a truncated cytoplasmic cT-Ag or an N-terminal T-Ag fragment. The cT-Ag protein and the N-terminal T-Ag fragment, but not the wtT-Ag protein were tightly bound to the constitutively expressed heat shock protein 73 (hsp73). Class I-restricted presentation of T-Ag epitopes processed in RMA-S cells from hsp73-associated cT-Ag was blocked by ammonium chloride, chloroquine, and leupeptin but not by brefeldin A or cycloheximide. Efficient presentation of CTL-defined epitopes by TAP-deficient RMA-S cells hence correlated with the association of these protein antigens to hsp73 indicating an alternative, peptide transporter-independent pathway for class I-restricted presentation of intracellular proteins in which hsp play a central role. In current experiments, the role of bacterial hsp60 and hsp70 proteins expressed after transfection in TAP-deficient cell lines on processing and class I-restricted presentation of 'endogenous' protein antigens is investigated. (Part of this work was published in *Eur.J.Immunol.* **24:** 1478; *1994*)

B6-329 DIFFERENTIAL EXPRESSION OF HSP27 IN CULTURED HUMAN NEURONS AND GLIAL CELLS UNDER UNSTRESSED AND HEAT-STRESSED CONDITIONS, Jun-ichi Satoh and Seung U. Kim, Division of Neurology, Department of Medicine, University of British Columbia, Vancouver, BC V6T 2B5, Canada

Heat shock proteins (HSPs) are produced when cells are confronted with a variety of stressful insults and may play a protective role against irreversible cell damage. To investigate biological roles of HSPs in the human central nervous system (CNS), HSP27 expression was studied in primary cultures of neurons and glial cells isolated from fetal human brains of 12-20 weeks' gestation using immunoblotting and immunocytochemistry. Under unstressed conditions, HSP27 was identified at a high level (>99%) in astrocytes, at a low level (7%) in neurons, at a minimally detectable level (<1%) in microglia, and none in oligodendrocytes. Under unstressed conditions, HSP27 was located in the cytoplasm, fractionated into the Triton X-100-soluble phase, and composed chiefly of the basic isoform (HSP27a). During the post-recovery period of 8-48 h following heat stress (43°C, 90 min) treatment, the level of HSP27 expression was not altered in astrocytes, but was elevated in neurons (11-21%) and microglia (4-7%), while it remained undetectable in oligodendrocytes. Following heat stress (45°C, 30 min), granular HSP27 aggregates were observed in the cytoplasm and most of the HSP27 protein was distributed within the Triton X-100-insoluble fraction associated with an increase in two acidic isoforms (HSP27b,c). HSP27 and αB-crystallin were coexpressed in astrocytes under unstressed and heat-stressed conditions. When astrocytes were exposed to known HSP27 inducers, H_2O_2 and cysteamine reduced the synthesis of HSP27, while estradiol showed no effects. The differential patterns of constitutive and heat-induced HSP27 expression in cultured human neurons and glial cells suggest that the cellular mechanisms by which HSP27 expression is regulated are different among various cell types in the human CNS.

B6-330 REGULATION OF HSP70 GENE EXPRESSION DURING THE LIFE CYCLE OF SCHISTOSOME AND SPECIFIC INTERACTION OF THE HEAT SHOCK FACTOR WITH A UNIQUE HSE VARIANT. Israel Schechter, Sylvia Neumann and Rivi Levy-Holtzman, Dept. Chemical Immunology, The Weizmann Inst. of Science, Rehovot, Israel.

Schistosomes are parasitic helminths with a complex life cycle in which they are exposed to different environments and temperatures. Therefore it is of interest to study the hsp70 of schistosome. We have shown that hsp70 mRNA of *S. mansoni* reveal stage specific expression (miracidia$^+$, sporocyst$^+$, cercaria$^-$, schistosomula$^+$, adult worm$^+$), hsp70 mRNA levels are regulated by a developmental program and by stress, temperature is an important factor but not a sufficient factor for strong induction of hsp70 mRNA, and the cercarial tail may produce inhibitory signals that suppress hsp70 gene expression. A heat shock element (HSEI) that differs from the consensus sequence by one base pair is at appropriate location from the cap site of the hsp70 gene. Schistosome extracts with active transcription factors revealed by the gel retardation assay were prepared. Parasite extracts from different developmental stages showed heat shock factor (HSF) binding activity that correlates with the pattern of hsp70 mRNA expression. Cercarial extracts did not bind ^{32}P-HSEI. Extracts of schistosomula and of adult worms kept at 37°C or 42°C bound HSEI. The HSEI-HSF interaction is specific because it is inhibited by cold HSEI and not by other DNA fragments. Analyses of HSEI-HSF complexes by gels estimating the size of the native DNA-protein complex and of the monomeric HSF (U.V.-crosslinking) indicate that: 1) schistosome express two (maybe three) forms of HSF, 2) different forms of HSF are expressed at different developmental stages, 3) the native HSEI-HSF complex of schistosome contains a single HSF molecule and not multimers of HSF as found in other organisms. Binding and inhibition studies using series of HSE mutants and HSF of *S. mansoni* (SmHSF) and *D. melanogaster* (DmHSF) demonstrate a major difference between DmHSF and SmHSF. An array of three nGAAn inverted repeats according to the ideal consensus sequence is recognized by DmHSF but not by SmHSF. In schistosome binding is attained only when the third nGAAn is a variant composed of nGTAn, suggesting coevolution of the variant sequence in the promoter together with SmHSF that interacts with the variant but not with the ideal HSE. Additional differences between SmHSF and DmHSF in DNA-sequence recognition were defined. The implication of these findings on the DNA binding domain of HSF and on hsp70 induction by various types of stresses will be discussed.

B6-331 FREQUENCIES AND PHENOTYPES OF T CELLS RESPONDING TO *M. LEPRAE* HSP65 IN TRANSGENE-VACCINATED MICE. Celio L. Silva, Rosemeire C.L.R. Pietro, Marcelo F. Silva & Douglas B. Lowrie*, School of Medicine of Ribeirão Preto, University of São Paulo, Brazil, *National Intitute for Medical Research, Mill Hill, London NW7 1AA, UK.

We have shown that *Mycobacterium leprae* hsp65, when expressed from a transgene in J774 tumour cells in BALB/c mice can generate a high degree of protective immunity against both mycobacterial infection and tumour challenge (C.L. Silva & D.B. Lowrie, Immunology 1994 82 244; K.V. Lukacs et al., J. Exp. Med. 1993 178 343). We now report that this system of vaccinating with hsp65 as an endogenous antigen is a highly efficient means of generating hsp65 responsive splenic T cells, with $CD8^+$ cells featuring strongly (Table).

Frequency of T cells proliferating to MLhsp65

Immunization	$CD4^+CD8^-$	$CD4^-CD8^+$
J774-hsp65	1:128	1:64
J774-vector + rMLhsp65	1:8950	1:48970
rMLhsp65 in IFA	1:2670	1:25950
Live BCG	1:2370	1:2960
Dead BCG	1:19800	1:38500
J774-vector	<1:100000	<1:100000
Listeria monocytogenes	<1:100000	<1:100000
Saline	<1:100000	<1:100000

Twelve $CD4^+CD8^-$ and 12 $CD8^+CD4^-$ antigen-responsive T cell clones derived from J774-hsp65-vaccinated mice were characterized for lymphokine secretion and antigen-specific cytotoxicity. IFN-gamma, IL-4 and cytotoxicity were largely independent properties among $CD4^+CD8^-$ clones and among $CD8^+CD4^-$ clones. Of the $CD4^+CD8^-$ clones, 75% produced IFN-gamma, 58% were cytotoxic and 25% produced IL-4 (none produced both IFN-gamma and IL-4). Of the $CD8^+\delta4^-$ clones, 83% produced IFN-gamma, 83% were highly cytotoxic and 33% produced IL-4 (16% produced both lymphokines). The clones are being tested for ability to confer protection to recipient mice.

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B6-332 ALTERED EXPRESSION OF hsp27 IN EXPERIMENTAL NEPHROTIC SYNDROME, William E. Smoyer, Tricia A. Ellis, John Strahler, Roger C. Wiggins, Michael J. Welsh, Departments of Pediatrics, Medicine and Anatomy and Cell Biology, University of Michigan, Ann Arbor, MI 48109

Heat shock proteins (hsp) comprise several families of proteins whose expression are increased after exposure to heat or various metabolic insults. These proteins have been reported to be involved in the assembly, intracellular translocation, function, and degradation of intracellular proteins. The low molecular weight hsp, hsp27, has been described as an actin-associated protein, and has been shown to inhibit actin polymerization *in vitro*. We have identified hsp27 in normal Sprague-Dawley rat kidneys where immunofluorescence (IF) is specific for glomerular capillary loops. The loops are comprised in part by glomerular epithelial cells (GEC) whose foot processes are extremely actin-rich. Treatment of rats with a single dose of puromycin aminonucleoside (PAN) results in development of disease which resembles human nephrotic syndrome. The nephrotic syndrome is characterized by massive proteinuria and edema associated with marked alterations in the GEC cytoskeleton. Using this model we observed an increase in capillary loop hsp27 expression by IF in sham vs. PAN-treated rats. Specificity of the mAb was confirmed by elimination of glomerular IF staining in the presence of the immunizing hsp27 peptide. Comparison by 2-D immunoblots of proteins from glomeruli isolated from disease vs. control animals revealed an apparent increase in total (all isoforms) hsp27 expression and alteration of the phosphorylation pattern associated with disease. We conclude that hsp27 is present in glomerular capillary loops of normal rats and that both its expression and phosphorylation pattern are altered during development of experimental nephrotic syndrome. Studies are now underway to define the intracellular location of glomerular hsp27 by immunoelectron microscopy and to correlate the temporal relationship between development of clinical disease and hsp27 expression.

B6-333 SCRAPIE PRIONS SELECTIVELY MODIFY THE CELLULAR STRESS RESPONSE. Jörg Tatzelt[1], Jianru Zuo[2], Richard Voellmy[2], William J. Welch[1] and Stanley B. Prusiner[1], [1]University of California, San Francisco, CA 94143-0518 and [2]University of Miami School of Medicine, Miami, FL

The fundamental event underlying scrapie infection seems to be a conformational change in the prion protein (PrP). To investigate proteins that might feature in the conversion of the cellular prion protein (PrP^C) into the infectious scrapie prion protein (PrP^{Sc}) we examined the expression and cellular distribution of molecular chaperones in scrapie infected neuroblastoma (ScN2a) cells. In contrast to uninfected cells, heat shocked ScN2a cells did not respond with increased expression of Hsp 72 and Hsp 28 as determined by immunoblotting. Transcription of the Hsp 72 gene in ScN2a cells was also unchanged by heat shock. In contrast, the induction of Grp 94 and Grp 78 (BIP) after exposure to azetidine was not impaired, thereby arguing for a selective suppression of Hsp 72 and Hsp 28. Upon heat shock in the uninfected cells the constitutively expressed Hsp 73 was translocated from the cytoplasm into the nucleus and nucleolus. Although the levels of Hsp 73 were unchanged in ScN2a cells, Hsp 73 failed to redistribute after heat shock. Furthermore, in ScN2a cells Hsp 73 was found to be associated with Triton X-100 insoluble structures. The alterations in the expression and cellular distribution of specific heat shock proteins in scrapie infected neuroblastoma cells may reflect cellular consequences due to the formation of PrP^{Sc} and contribute to the pathogenesis of prion diseases. How alterations in the stress response lead to vacuolation of neurons and attendant astrocytic gliosis which are the morphologic hallmarks of scrapie remains to be established.

B6-334 HYPOXIA LEADS TO INCREASED EXPRESSION OF HSP70 AND RESISTANCE TO SUBSEQUENT HYPOXIC INJURY IN HUMAN PROXIMAL TUBULAR EPITHELIAL CELLS, Martin A. Turman, Annie Mathews, and Daniel A. Kahn, Department of Pediatrics, Ohio State University, Columbus, OH 43205

Preconditioning of heart or brain with brief periods of sublethal ischemia induces tolerance for subsequent ischemia. However, results with ischemic preconditioning in kidneys have been variable, perhaps because of post-ischemic tubular obstruction and other mechanical factors. To determine if human proximal tubular epithelial cells (PTEC) acquire resistance for hypoxia at the cellular level, cultured PTEC were incubated in an anaerobic chamber in glucose-free buffer (combined glucose oxygen deprivation; COGD) for 4 to 16 h, then allowed to recover (R) in complete medium in an aerobic incubator (95% air/5% CO_2) for 24 h and then re-exposed to COGD for 20 h. Cell injury was assessed by LDH efflux. PTEC preconditioned with 4, 8, 12, or 16 h of COGD had 22±2.9%, 15±1.2%, 15±2.6%, and 27±4.0% LDH efflux, respectively, compared to 36±4.1% LDH efflux in control PTEC exposed to 20 h COGD (mean±SEM, n=18, $p<0.05$ for all values). By phase contrast microscopy, the surviving preconditioned PTEC had normal morphology 72 h after the second hypoxic insult, whereas all control cells were crenated and dysmorphic, suggesting irreversible injury. Thus, hypoxic preconditioning results in long-term survival of PTEC exposed to COGD. To determine if heat shock protein (HSP) expression correlated with the development of tolerance for hypoxia, PTEC were exposed to COGD for 12 h. Protein extracts were then isolated after 0 h to 62 h R and subjected to Western blot analysis. HSC70 and HSP90 protein content did not change substantially after COGD, whereas HSP70 protein expression began to increase by 16 h R, peaked at 3.4-fold above baseline by 27 h R and at 62 h R was still 2.1-fold above baseline. These results indicate that, at the cellular level, human PTEC can develop tolerance for hypoxia and HSP70 may contribute to the ability of PTEC to tolerate hypoxic injury.

B6-335 INVESTIGATION OF THE NEUROPROTECTIVE EFFECTS OF HSP70I USING TRANSGENIC AND PRIMARY CELL CULTURE TECHNOLOGIES. James B.Uney. Department of Medicine, University of Bristol. Bristol. BS2 8HW. U.K.

Heat shock proteins have been shown to be induced rapidly in the mammalian brain following exposure to excitotoxins and ischaemia and correlative evidence from several recent studies suggests that hsp70i may play a role in enhancing neuronal survival during and after stress. The hsc70 family have been shown to be clathrin uncoating enzymes and protein chaperones, mediating the transport of precursor proteins across intracellular membranes. It is therefore possible that cells expressing high concentrations of hsp70i are more resistant to toxic insults due to the rapid replacement of any proteins damaged during the stress. To test this hypothesis transgenic and primary culture transfection techniques have been used.

Transgenic animals have been generated and express hsp70i mRNA and protein. The rhombotin gene promoter was used to drive expression of hsp70i to the hippocampal CA1 and CA2 but not CA3 neurons in the adult mouse brain. This precise expression pattern is ideal as it enables the neuroprotective effects of hsp70i to be assessed by: a, stereotaxically injecting glutamate agonists into the hippocampus of the transgenic animals and comparing the excitotoxin-induced damage of the CA1 and CA2 regions of the hippocampus (which express the hsp70i transgene) with the CA3 region (which do not express the hsp70i transgene); b, comparing the vulnerability of primary cultures made from the hippocampi of transgenic. Further to these experiments I have also shown that transfection of dorsal root ganglian primary cultures with an EF-1 alpha-hsp70i expression vector protects neurones and glia against exposure to heat stress. If this strategy proves successful it may help us understand the mechanism by which hsp70i mediates its neuroprotective effects and also provide impetus to the strategy of ameliorating neuronal injury by elevating intra-neuronal hsp levels.

B6-336 ***MYCOBACTERIUM GORDONAE* HEAT SHOCK PROTEINS AND PRIMARY BILIARY CIRRHOSIS**

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Primary biliary cirrhosis (PBC) is a chronic cholestatic liver disease associated with autoimmune disorders. Although the etiology is unknown, it has been suggested that the disease may result from exposure of susceptible subjects to a particular infectious agent. Previous studies revealed that sera from patients with PBC reacted against *M. gordonae* protein extract. This reactivity was characterized by a recognition of two polypeptides of 70-65 and 55 kDa, which share epitopes with the two major mitochondrial autoantigens in PBC. Since the most immunogenic components of mycobacteria are the heat shock proteins (hsp), which have been associated with autoimmunity, the current study has been addressed to characterize these reacting polypeptides. Cultures of *M. gordonae* were incubated at 37 °C or 46 °C before sonication, protein extraction and separation by SDS-PAGE. The intensity of a 65 kDa band and other bands of lower molecular weight were increased after incubation at 46 °C. To confirm a heat shock-induced over-expression, two mouse monoclonal antibodies (MoAb) recognizing the 65 kDa hsp from mycobacteria were tested. Immunoprecipitations of the heat shock-induced protein extract with these MoAb and sera from patients with PBC showed bands of similar molecular weight (65 and 55 kDa). Moreover, a similar pattern of reactivity to that obtained with sera from patients with PBC, was found when the *M. gordonae* protein extract was tested with the 65 kDa hsp MoAb. In conclusion, these results suggest that stress protein antigens of *M. gordonae* may play a pathogenic role in PBC.

B6-337 IMMUNOLOGY OF ATHEROSCLEROSIS: CYTOTOXICITY OF ANTI-HEAT SHOCK PROTEIN 65/60 ANTIBODIES FOR ENDOTHELIAL CELLS, Georg Wick*°, Georg Schett*, Albert Amberger* and Roman Kleindienst*, *Institute for Biomedical Aging Research, Austrian Academy of Sciences, and °Institute for General and Experimental Pathology, University of Innsbruck, Medical School, Innsbruck, Austria

Previous studies in our laboratory have led to the establishment of a new concept for an autoimmune pathogenesis of atherosclerosis, based on the demonstration of both, humoral and cellular immune reactions against heat shock protein 65/60 (HSP 65/60) in the earliest stages of the disease. Thus, activated HSP 65/60 reactive T cells were the first cellular elements that emerge in very early intimal lesions at sites of major haemodynamic stress. Furthermore, high titers of anti-HSP 65/60 antibodies were demonstrated in the sera of clinically healthy persons with sonographically demonstrable atherosclerotic lesions in their carotid arteries. We now report the expression of HSP 60, the mammalian analogue of mycobacterial HSP 65, on the surface of stressed (temperature, H_2O_2, tumor necrosis factor α) human venous and arterial endothelial cells. In addition, we show on the mRNA and protein level that the intercellular adhesion molecule-1 (ICAM-1) and HSP 60 are expressed simultaneously in a coordinated fashion by endothelial cells upon application of various forms of stress. Affinity chromatograhpy purified anti-HSP 65/60 human antibodies derived from the above mentioned study were shown to be cytotoxic for stressed (42° C, 30 min) but not unstressed human endothelial cells. This cytotoxicity involved both, complement-mediated and antibody-dependent cellular cytotoxicity (ADCC). In conclusion, anti-HSP 65/60 antibodies are not only of diagnostic significance but also have a clearcut pathogenetic potential. Similar investigations with HSP 65/60 specific T cell clones are now under way in our laboratory.

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B6-338 **LACK OF H_2O_2 -INDUCED HO-1 EXPRESSION BY ARTHRITIC SYNOVIAL ENDOTHELIAL CELLS**

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Introduction: Redox imbalance specifically induces a 32kDa stress protein, the enzyme haem oxygenase-1 (HO-1). In rheumatoid arthritis, the microenvironment of the inflamed joint is both hypoxic and oxidatively stressed. Since the endothelium plays a central role in redox control, we examined the ability of isolated synovial microvascular endothelial cells (SMEC) to respond to the HO-1 inducers hydrogen peroxide (H_2O_2), cadmium chloride (Cd^{2+}) and disodium aurothiomalate (AuTM); endothelial cells derived from human umbilical veins (HUVEC) or subcutaneous adipose tissue (ADMEC) were used for comparison. Previously, we demonstrated serum-dependent transcription of HO-1 in HUVEC [1]. Since serum-dependency is characteristic of H_2O_2-mediated activation of the early response genes, serum effects were considered.

Methods: EC were isolated using combinations of enzymic, mechanical and immunoaffinity techniques. At passage 2 or 3, EC were treated with increasing concentrations of H_2O_2, Cd^{2+} or AuTM in the presence or absence of 10% foetal calf serum for 8 hours, radiolabelled methionine being added 2 hours prior to harvest. Cell lysates were separated on 12.5% polyacrylamide gels in the presence of SDS and 32kDa protein synthesis assessed by autoradiography.

Results: All EC expressed the 32kDa protein in response to AuTM and Cd^{2+} and this was serum independent. However, differential responses to H_2O_2 were noted. Both HUVEC and ADMEC showed serum-dependent synthesis of a 32kDa protein at H_2O_2 concentrations as low as 5μM but, in SMEC, this response was impaired as the 32kDa protein band was barely detectable, even at high H_2O_2 levels (100μM).

Conclusion: EC are able to synthesise the 32kDa protein and two mechanisms of induction are possible: Oxidants, unlike other stressors, require serum. Moreover, SMEC show an impairment in their response to oxidants. Whether this reflects an adaptive response or a defect in transcriptional control remains to be elucidated.

1. Winrow VR, Watson A, Harley S, Blake DR. In: *Oxidative Stress, Cell Activation and Viral Infection.* Basel: Birkhauser Verlag, 1994: 91-100.

B6-339 THE ROLE OF HSP47 DURING NEURAL PLATE AND EARLY BRAIN FORMATION, Li Zhe and David Walsh, Mammalian Development B19, University of Sydney, NSW 2006, Australia.

A novel heat shock protein HSP47 was identified during neural plate formation in early mammalian brain development and is constitutively expressed in neuroectoderm and mesoderm. HSP47 is located on the ER and binds to collagens I-IV and may function as a specific molecular chaperone to these structural proteins. We have investigated the induction, kinetics and inhibition of the hsp47 gene during gastrulation and its role in collagen synthesis. This is the start of craniofacial and CNS development, a critical stage in embryogenesis. Collagen a major structural component of the extracellular matrix and basal laminae is dependant on HSP47 synthesis during early forebrain formation and initial expression of these two genes is closely linked. Northern analysis, whole mount in situ hybridisation and confocal microscopy were used to examined this relationship. HSP47 mRNA transcripts were detected at days 9.5, 10.5, 11.5 and 14.5 with hsp47 expression highest at 9.5 & 10.5 days. Two transcripts of 2.1 and 1.8 Kb were detected constitutively at all stages. Two additional bands at 0.6 & 0.5Kb were observed following heat shock and at day 11-14 suggesting procollagen mRNA splicing. Exposure to heat shock at mild, lethal and thermotolerant regimes showed a rapid 4-10 fold increase of hsp47 mRNA in neuroectoderm. Hsp47 expression and synthesis appears normaly in mid S phase though to M in the cell cycle as determined by FAScan analysis. Alteration of hsp47 expression following heat shock was followed with a thermotolerant heat shock resulting in down-regulation of the gene and HSP47 synthesis in late S phase. HSP47 may regulate collagen transcription and translation during early formation of the brain and eye following heat shock.

Late Abstracts

ACTIVATION OF HEAT SHOCK FACTOR-1 IN G_1; Stuart K. Calderwood*, Jackie A. Bruce and Mary Ann Stevenson, Dana Farber Cancer Institute and Harvard Joint Center for Radiation Therapy, Boston MA 02115

The heat shock transcription factor (HSF) genes are a multigene family that encode transcription factors active during exposure to physical stress and during cellular differentiation. In this study, we have demonstrated a potential role for one member of the HSF family during the mammalian cell cycle. We observed HSF activation and association with its binding element (HSE) during early G_1 in synchronous populations of human cells. Analysis using antibodies specific for two members of the HSF family (HSF-1 and HSF-2) indicates that the HSF-1 isoform previously shown to be inducible by heat shock was the species activated during G_1. The activation of HSF-1 occurred simultaneously with the burst in cellular protein synthesis that occurs early during G_1. The observed HSF-1 activation was extremely sensitive to inhibitors of translation such as cycloheximide (CHX), anisomycin and emitine and inhibition of HSF-1 activity correlated closely with cell cycle arrest by these agents. These properties of HSF-1 activation in G_1 closely resemble those described for the *restriction point*, a CHX sensitive period in G_1 which marks the commitment point for replication and cell cycle progression. The data thus suggest that HSF-1, previously identified as a transcriptional activator under stress conditions, may also play a role in cell cycle regulation during G_1.

UNCOUPLING OF HUMAN HEAT SHOCK FACTOR 1 PHOSPHORYLATION FROM MULTIMERIZATION BY SPHINGOLIPIDS, Donald A. Jurivich & Joseph Welk, Department of Medicine, Northwestern University Medical School, Chicago, IL 60611

Sphingolipids function as potent negative regulators of intracellular signaling in addition to specifically activating certain protein kinases. Although this class of lipids appears to modulate cellular signal transduction, accumulation can lead to chronic disease states associated with progressive cellular injury and death. These observations suggest sphingolipids affect the molecular response to stress. To test this relationship, heat shocked cells were analyzed for accumulation of sphingolipids. After 60 minutes at 42°C, murine fibroblasts demonstrated a 2 fold accumulation in the mass levels of ceramide. Based on this observation, cells were examined for their response to cell-permeable, exogenous lysosphingolipids. Cells exposed to 20-40 μM erythro-dihydrosphingosine at 37°C induced HSF-DNA binding that was comparable to a 42°C heat shock as determined by scanning densitometry of electromobility shift assays (EMSA). Supershift EMSA employed with antibodies to HSF1 and HSF2 indicated that sphingosine induced HSF1. Western blot analysis of HSF1 revealed that sphingosine-induced HSF1 exhibited little shift in its mobility relative to heat-induced HSF1 in growing Hela S3 cells, and there was no shift in plateau-phase cells. Thus, sphingosine uncouples the multimerization signal of HSF1 from its phosphorylation. Nuclear run-on analysis demonstrated less than 2 fold, 10 fold, and 100 fold increases in heat shock 70 gene expression in quiescent/sphingosine-treated cells, growing/sphingosine-treated cells and heat shocked cells respectively.

We conclude that HSF1 can be partially phosphorylated by sphingosine-triggered signals in growing cells, thus suggesting a relationship between stress- and growth-activated protein kinase activity. Furthermore, sphingosine uncouples HSF1 multimerization from phosphorylation in quiescent cells, thus suggesting important implications for sphingosine-mediated stress responses in post-mitotic cells.

MOLECULAR CHAPERONE FUNCTION IN PROTEIN FOLDING OF DNA INITIATOR REPA
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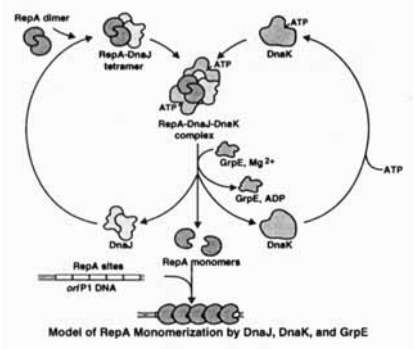
The DnaK molecular chaperone system activates site-specific DNA binding by several DNA replication initiator proteins, including the Rep proteins of plasmids P1 and F and *E. coli* DnaA. The best characterized example is the activation of the P1 plasmid initiator protein, RepA. RepA is an origin-specific DNA binding protein that recognizes five direct repeats of a 19 bp sequence in the P1 origin, *ori*P1. RepA protein exists in solution as a dimer that is unable to bind DNA specifically. It forms a stable complex with DnaJ containing a dimer each of RepA and DnaJ. DnaK, in a reaction dependent on DnaJ and ATP but independent of DNA, activates the specific *ori*P1 DNA binding function of RepA by about 100-fold. DnaJ and DnaK are not part of the RepA-DNA complex. The activated form of RepA is a monomer. We have used biochemical and genetic techniques to elucidate the mechanism of activation and have used the experimental results to develop the model diagrammed below. We have found that mutations that weaken the RepA dimer result in more active RepA in absence of DnaK and DnaJ activation. We have assembled multiple single mutations into a single RepA gene and expressed a RepA protein that is greater than 95% monomeric in the absence of DnaK and DnaJ. The multiple mutants are functionally equivalent to wild type RepA activated by DnaK and DnaJ.



Model of RepA Monomerization by DnaJ, DnaK, and GrpE

CLONING, SEQUENCE ANALYSIS AND EXPRESSION OF A CDNA CLONE ENCODING HEAT SHOCK PROTEIN 98 [HSP98] FROM MAIZE, Jorge Nieto-Sotelo[1] & T.-H. David Ho[2]. [1]Instituto de Biotecnología, Universidad Nacional Autónoma de México, Cuernavaca, Mor. 62271, México; [2]Dept. of Biology, Washington University, St. Louis, MO 63130

We isolated several heat inducible cDNA's by screening a λgt10 cDNA library, prepared from heat-shocked (40°C) maize [Zea mays] etiolated seedlings, by differential hybridization. Cell free translation of polyA$^+$ RNA obtained from heat shocked seedlings hybrid-selected by clone p11, shows a protein with an apparent molecular mass of 98 kDa. This protein comigrates with a heat inducible protein obtained by *in vitro* translation of total polyA$^+$ RNA isolated from heat shocked maize seedlings. In RNA blot experiments clone p11 detects a single mRNA species, that is inducible by heat shock. No mRNA from control seedlings hybridized to clone p11. These results indicate that clone p11 encodes maize hsp98. Expression of hsp98 mRNA was detected as early as 10 minutes after heat shock, reaching a maximum after 1 hour of induction. A gradual decrease in hsp98 mRNA levels was detected between 1 and 4 hours after heat shock treatment. The insert of the p11 cDNA clone was used for rescreening, under high stringency conditions, the same cDNA library prepared from heat shocked seedlings. Clone p31, with an insert of 2.1 kbp, contained the largest cDNA insert of all positive clones analyzed. Partial DNA sequence analysis of clone p31 shows an open reading frame that predicts a protein with 80% similarity to yeast hsp104 (Parsell, *et al.*, 1991, Nature 353, 270-273). Yeast hsp104 is so far the only hsp shown to be involved in the phenomena of 'induced thermotolerance'. Our results seem to indicate that maize hsp98 is the homologue of yeast hsp104. By using clones p11 and p31 we should be able to ask if hsp98 is involved in the phenomena of 'induced thermotolerance' in vascular plants.

STRUCTURE-FUNCTION OF THE *ESCHERICHIA COLI* GrpE HEAT SHOCK PROTEIN: BIOCHEMICAL PROPERTIES OF MUTANT GrpE PROTEINS, Bin Wu[1], Alicia Wawrzynow[2], Maciej Zylicz[2], and Costa Georgopoulos[1,3]. [1]Department of Cellular, Viral and Molecular Biology, University of Utah Medical Center, Salt Lake City, Utah 84132, [2]Division of Biophysics, Department of Molecular Biology, University of Gdansk, 80-822 Kladki, Gdansk, Poland and [3]Département de Biochimie Médicale, Centre Médical Universitaire, 1 rue Michel-Servet, 1211 Genève 4, Switzerland.

We have isolated two groups of missense mutations in the essential *grpE* gene of *Escherichia coli* based on their inability to propagate bacteriophage λ at 30° or 42°C. To better understand the biochemical mechanism of GrpE action in various biological processes, six mutant proteins were overexpressed and purified. GrpE2/280(G122D) and GrpE17(L127P) are resistant to λ at both temperatures, and GrpE103(E53G), GrpE66(L102P), GrpE13a(H154R), and GrpE25(G177S) are temperature-sensitive for λ growth. The biochemical defect(s) of each mutant GrpE protein was identified by examining their abilities to (a) support *in vitro* λ DNA replication, (b) stimulate the weak ATPase activity of DnaK at 30° or 43°C, (c) help release λP substrate protein from DnaK, (d) dimerize/oligomerize, and (e) physically interact with wild type DnaK. Based on the results obtained, we propose that GrpE forms hexamers by oligomerization through its N-terminal region, presumably mediated by a predicted coiled-coil region. GrpE hexamer is the most active form that interacts with DnaK, and the interaction is vital for GrpE's biological function. ATP-binding, but not hydrolysis, is sufficient to disrupt the DnaK-GrpE complex. The C-terminal half of GrpE contains the DnaK binding site and is critical for GrpE's stimulatory effect on DnaK's ATPase activity.

KINETICS OF PEPTIDE RELEASE OF DNAK, Jundong Zhang and Graham C. Walker, Department of Biology, Massachusetts Institute of Technology, Cambridge, MA 02139

It is generally believed that the coupling of the cycles between the ATP-bound DnaK state and ADP-bound DnaK state with the substrate binding and release plays a central role in DnaK's biological function. We synthesized a fluorescence labeled peptide (NRLLLCG-ANBD, PepH-ANBD)to investigate the kinetics of peptide release by ATP and the effect of GrpE and DnaJ on peptide release. After PepH-ANBD bound to DnaK, its fluorescence intensity at 540 nm increased 210%. The dissociation constant of PepH-ANBD was measured at 30 nM. ADP does not affect its dissociation constant. The ATP-bound DnaK has a Kd of 650 nM, which is 22 times lower than ADP-bound state and the nucleotide free state.

The kinetics of the PepH-ANBD release from DnaK by ATP was studied by stop-flow equipment with a dead time of 1ms. The experiment was carried out in such a way that 4 mM DnaK and 10 mM PepH-ANBD solution was first incubated at room temperature for an hour and then mixed with equal volume of ATP solution by the stop-flow equipment. The release of PepH-ANBD from DnaK follows single-exponential reaction. When 10 mM ATP was mixed with the DnaK-peptide solution, the peptide was released with a $T_{1/2}$=4.3s. The mixing of either 10 mM ATP and 10 mMADP or 10 mM ATP and 20 mM ADP with DnaK-peptide solution did not change the peptide release rate, indicating ADP can't compete with ATP to in such a short time. The ATP analog, ATP-γ-S, can not release peptide from DnaK at all. GrpE increased the peptide release rate and DnaJ has no significant effect on the peptide release.