ROLE OF HEAT SHOCK PROTEINS IN PROTEIN SYNTHESIS, FOLDING, AND RENATURATION

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PREFACE

The role of heat shock proteins (hsps) in regulating the activity of heme-regulated inhibitor in rabbit reticulocyte lysate of protein synthesis was studied. For these studies, biologically active Hsp 70 and hsp90 were purified from different tissues. These hsps were used for characterizing the refolding of thermally denatured luciferase. I used inhibitors of hsp70 and hsp90 and the classical enzyme inhibition kinetics and Western blot analysis to decipher the mechanism that underly refolding of thermally denatured luciferase. Moreover, the importance of hsp70 and hsp90 in *de novo* folding of luciferase was also studied. Finally, based on the experimental results, a general model for the refolding of denatured luciferase by heat shock proteins in rabbit reticulocyte lysate has been proposed. The research presented here greatly enriches our knowledge in the function of heat shock proteins and strongly supports the prevailing concept that heat shock proteins, working as molecular chaperones, play a fundemental role in living cells.

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ATP	adenosine triphosphate
ADP	adenosine diphosphate
BA	benzoquinonoid ansamycin
BCIP	5-bromo-4-chloro-3-indolyl-phosphate
BSA	bovine serum albumin
CKII	casein kinase II
CHAPS	(3-[(3-cholamidopropyl dimethylammonia]-1-propane sulfonate)
ClA	clofibric acid
CMLA	carboxy methylated lactalbumin
CP	creatine phosphate
CPK	creatine phosphokinase
CsA	cyclosporin A
DMSO	dimethylsulfoxide
DTT	dithiothreitol
EDTA	ethylenediaminetetra acetic acid
eIF-2	eukaryotic initiation factor-2
eIF-2B	eukaryotic initiation factor-2B
FK506	immunosuppressant drug
FKBP	FK506 binding proteins
GA	geldnamycin
GaR-agarose	agarose crosslinked to goat anti-rabbit IgG
GDP	guanosine diphosphate

GTP	guanosine triphosphate
HbA	herbimycin A
HEPES	(N-[2-hydroxyethyl] piperazine-N'- [2-ethanesulfonic]) acid
HRI	heme-regulated eIF-2α kinase or heme-regulated inhibitor
hsc	heat shock cognate protein
HSE	heat shock element
HSF	heat shock transcription factor
hsp	heat shock protein
IL-2	interleukin-2
kDa	kilodaltons
LDL	low density lipoprotein
NBT	nitroblue tetrazolium
NEM	N-ethylmaleimide
NSAD	non steroidal anti-inflammatory drugs
PDI	protein disulfide isomerase
pepF	FYQLALT
pepN	NIVRKKK
PPI	peptidylprolyl cis-trans isomerase
PR	progesterone receptor
RCM-BSA	Reduced carboxymethylated bovine serum albumin
SA	salicylic acid
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SH3	SPPTPKPRPPRPLPVAPGS
SHR	steroid hormone receptors
TPR	tetratricopeptide repeat

CHAPTER I

INTRODUCTION

The process by which protein molecules achieve their native conformations is a subject of fundamental and practical importance. Recent evidence indicates that misfolding of proteins is the molecular basis of a growing list of human diseases (Table 1). Fundamental interest in the "protein folding problem" arises because we do not yet understand how a complex network of noncovalent interactions can specify one particular compact conformation for an intrinsically flexible polypeptide (Dill, 1990), how the polypeptide rapidly finds that compact conformation (Baldwin, 1994, Kuszewski et al., 1994), or why such a purely noncovalent process involves large kinetic barriers (Baker & Agard, 1994). *In vitro*, many unfolded proteins fold to their native conformations spontaneously. This observation, first made by Anfinsen approximately three decades ago, suggested that all the information necessary to specify the three dimensional structure of a protein is contained in its linear amino acid sequence (Anfinsen, 1973). Consequently, it has been assumed that the *de novo* folding of proteins also occurs spontaneously. This view has changed profoundly over the past seven years. The correct folding of proteins *in vivo* is believed to be greatly enhanced by interaction with accessory proteins known as molecular chaperones. Molecular chaperones occur ubiquitously and many of them are classified as heat shock proteins (hsps), although they have essential function under normal growth conditions. Molecular chaperones belong to one of three highly conserved families: hsp90, hsp70 and hsp 60. Hsp60 is commonly referred to as chaperonins. Hsp70 and

Table 1. Some putative protein folding diseases (Thomas et al., 1995).

Disease	Mutant protein/protein involved	Molecular phenotype
Inability to fold		
Cystic fibrosis	CFTR	Misfolding/altered hsp70 & calnexin interactions
Marfan syndrome	Fibrillin	Misfolding
Amytotrophic lateral sclerosis	Superoxide dismutase	Misfolding
Scurvy	Collagen	Misfolding
Maple syrup urine disease	α-Ketoacid dehydrogenase	Misassembly/misfolding
Cancer	p53	Misfolding/altered hsp70 & hsp90 interaction
Osteogenesis imperfecta	Type 1 procollagen pre α	Misassembly/altered BiP expression
Toxic folds		
Scrapie/Creutzfeldt-Jakob/familial insomnia	Prion protein	Aggregation
Alzheimer's disease	β-Amyloid	Aggregation
Familial amyloidosis	Transthyretin/lysozyme	Aggregation
Cataracts	Crystallins	Aggregation
Mislocalization owing to misfolding		
Familial hypercholesterolemia	LDL receptor	Improper trafficking
α_1-Antitrypsin deficiency	α_1-Antitrypsin	Improper trafficking
Tay-Sachs disease	β-Hexosaminidase	Improper trafficking
Retinitis pigmentosa	Rhodopsin	Improper trafficking
Leprechaunism	Insulin receptor	Improper trafficking

hsp60 are involved in the early stages of protein biogenesis. Hsp90 may be involved in the later stages of protein biogenesis where extensive secondary structure has already formed (Melnick et al., 1992, Melnick et al., 1994, Smith, 1993).

Molecular chaperones appear to facilitate protein folding primarily by reducing the number of protein molecules that follow non-productive folding pathways that would lead to aggregation of the protein. The chaperone activity is based on the selective affinity of chaperones for nonnative proteins. A characteristic of nonnative proteins is the exposure of interior hydrophobic residues to the solvent. Chaperones protect these exposed hydrophobic residues from nonproductive hydrophobic inter- and intramolecular aggregation. Transient binding of nonnative proteins by chaperones destabilizes kinetically trapped intermediates that can then resume potentially productive folding pathway. As native proteins are not generally recognized, such cycles of binding, unfolding, and release favor the accumulation of folded native versus nonnative proteins.

Previous work suggests that chaperones function as heteromeric chaperone machines in an ATP dependent manner. The affinity of chaperones for polypeptide substrates is modulated by associated proteins (cohorts) and conformational changes induced by the binding and hydrolysis of ATP (Georgopoulos, 1992, Hartl, 1995). Chaperone associated cohorts modulate chaperone function by a number of mechanisms: they may interact with chaperone-bound substrates directly; they may modulate the interactions of hsps with their protein substrates; or they may modulate the rate of ATP hydrolysis or nucleotide exchange.

Disulfide isomerases and PPIases

Two rate limiting events in the folding of polypeptides are catalyzed by two enzyme families: protein disulfide isomerases and peptidylprolyl cis-trans isomerases. *In vitro* protein folding is limited by the sampling and attainment of the proper configuration of

disulfide bonds and the cis-trans isomerisation of the polypeptide backbone around the peptide bond adjacent to proline residues. Both reactions *in vivo* are characterized by two enzymes families: (i) Protein disulfide isomerases catalyze the making and breaking of disulfide bonds. This reaction occurs in the oxidizing environment of the endoplasmic reticulum. (ii) Peptidylprolyl cis-trans isomerases (PPIases) catalyses the cis-trans isomerization of peptide bonds preceding proline residues. Cis-trans isomerization occurs in the cytoplasm and in most subcellular compartments (Stamnes et al., 1991).

PPIases fall into two structurally unrelated families. These families are named after the clinically important immunosuppressive agents that inhibit their isomerase activity. The eukaryotic cyclophilins bind cyclosporin A (CsA) with high affinity, whereas the FK506-binding proteins (FKBPs) bind the structurally distinct compounds FK506 and rapamycin. Both CsA and FK506 act as immunosuppressants by preventing the transcription of genes involved in activation of T lymphocytes, whereas rapamycin potentially inhibits the response of T cells to the lymphokine IL-2 (Schreiber, 1990). These immunosuppressive drugs do not act through inhibition of the PPIase activity of T cells as they are effective at concentrations far below those of the PPIase enzymes, and they inhibit distinct signalling pathway.

Immunophilins that interact with the hsp90 chaperone machine, display similar structural organization with two functional domains: an N-terminal region with overlapping isomerase and ligand binding domains; and a conserved C-terminal segment that consists of a 3-unit tetratricopeptide repeat (TPR) domain followed by a potential site for calmodulin binding (Ratajczak et al., 1993). The immunophilins bind to hsp90 through their TPR motifs (Radanyi et al., 1994, Ratajczak & Carrello, 1996).

TCP-1(t-complex polypeptide 1) ring complex (TRiC)

The Chaperonin family of chaperones (*Escherichia coli* GroEL, mitochondrial hsp60, and chloroplast rubisco binding protein) have a characteristic oligomeric structure consisting of two stacked heptameric rings (Hendrix, 1979, Hohn et al., 1979, Puskin et al., 1982). The bacterial chaperonin GroEL and the eukaryotic homologs in the mitochondria and chloroplast are homo-oligomeric, whereas the eukaryotic cytoplasmic homolog of GroEL, TRiC, is composed of eight different but homologous subunits (Frydman et al., 1992, Lewis et al., 1992). GroES and hsp10 are the cohorts of GroEL and hsp60, respectively. GroES is a single seven membered ring of 10 kD subunits (Chandrashekar et al., 1986). The eukaryotic equivalent of GroES in the cytoplasm has not yet been identified.

GroEL in conjunction with the cochaperonin GroES facilitates the folding of many *E.coli* proteins in a controlled ATP dependent fashion. GroES binds to GroEL and coordinates its ATPase activity (Langer et al., 1992, Todd et al., 1993). TRiC was analyzed as a potential chaperonin (GroEL/hsp60) equivalent of the eukaryotic cytosol. The main function of TRiC appears to be in chaperoning monomeric protein folding. TRiC binds unfolded polypeptides, thereby preventing their aggregation, and mediates ATP dependent renaturation of small molecular weight proteins.

Heat shock protein 70 (hsp70)

Hsp70 has widely different functions in eukaryotic cells, both under normal growth conditions and under cellular stress (Craig et al., 1994, Hendrick & Hartl, 1993). In cooperation with other chaperones, they stabilize preexistent proteins against aggregation and mediate the folding of newly translated polypeptides in the cytosol as well as within organelles. The hsp70 in mitochondria and endoplasmic reticulum may play an additional role by providing a driving force for protein translocation (Hohfeld & Hartl, 1994, Stuart et al., 1994). A link between the function of the hsp70 class of chaperones and cellular

proteolysis has also been demonstrated (Craig et al., 1994). Finally, hsp70 is suggested to be involved in signal transduction pathways in cooperation with hsp90 (Bohen & Yamamoto, 1993, Rutherford & Zuker, 1994).

Hsp70 participates in all the above mentioned processes through its ability to recognize nonnative conformations of other proteins. Hsp70 binds to extended peptide segments of seven or eight residues with a net hydrophobic character. Extended hydrophobic residues are exposed by polypeptides during translation and membrane translocation, or following stress-induced damage (Blond-Elguindi et al., 1993, Flynn et al., 1991, Landry et al., 1992, Palleros et al., 1991). Peptide binding is mediated by a carboxyl-terminal ~25kD domain of hsp70 (Freeman et al., 1995, Wang, 1993) and is regulated by the binding of ATP to the ~44kD amino-terminal domain (Chappell et al., 1987, Flaherty et al., 1990). Nucleotide-induced conformational changes of the conserved ATPase core are transmitted to the carboxyl-terminal region, modulating its intrinsic peptide affinity. The ATP-bound form of hsp70 binds and releases peptide rapidly, whereas the ADP-bound form binds and releases slowly (Palleros et al., 1991, Schmid et al., 1994, Szabo et al., 1994). Cycling of hsp70 between these nucleotide states depends on its interaction with its cohorts such as DnaJ and GrpE of *E.coli*. GrpE functions as a nucleotide exchange factor (Liberek et al., 1991, Szabo et al., 1994), allowing rebinding of ATP that in turn promotes substrate dissociation (Palleros et al., 1991, Szabo et al., 1994). Another cytosolic cohort of hsc70 called hsc70-interacting protein Hip (also known as p48), has been identified in the eukaryotes (Hohfeld et al., 1995). Hip is not a GrpE homolog, but stabilizes the ADP state of hsc70 that has a high affinity for substrate.

DnaJ (hsp40)

DnaJ activates the ATPase of the bacterial hsp70 homolog DnaK (Langer et al., 1992, Liberek et al., 1991). Hsp40, an eukaryotic DnaJ homolog have been identified in

all compartments of eukaryotic cells that contain hsp70 (Cyr et al., 1994). All DnaJ-like proteins contain an N-terminal J-domain, which is proposed to mediate interactions with hsp70 that regulate ATPase activity. The C-terminal domain of DnaJ is proposed to mediate interactions with polypeptides, but is much less conserved.

DnaJ-dependent activation of DnaK depends on ATP hydrolysis and the conformation of the protein substrates. Unlike hsp70, which recognizes short stretches of amino acids that assume an extended conformation, DnaJ prefers protein substrates exhibiting secondary and tertiary conformation (Wawrzynow et al., 1995, Wawrzynow & Zylicz, 1995). The ability of DnaJ to stimulate DnaK's substrate binding properties correlates with DnaJ's affinity for the various protein substrates. Specifically, DnaJ will activate DnaK to associate to a substrate for which DnaJ itself has a very low affinity, albeit to a lesser extent than for substrates to which DnaJ has a high affinity (Wawrzynow et al., 1995). However, YDJ-1, the yeast homolog of DnaJ, stimulates the ATP-dependent dissociation of complexes between SSA1 (the yeast homolog of DnaK) and CMLA, a protein for which YDJ-1 has a low binding affinity (Cyr et al., 1994). In contrast, YDJ-1 was observed to stabilize complexes between SSA1 and the substrates for which YDJ-1 has high affinity (Cyr et al., 1994). This screening by DnaJ for the conformation of polypeptides that bind to hsp70 is suggested to be the basis for the ability of DnaJ to facilitate both the dissociation and the stablilization of complexes between hsp70 and polypeptides.

P60

P60 is suggested to be the eukaryotic homolog of GrpE. P60 promotes the recycling of hsp70 by increasing the rate of dissociation of hsp70-ADP in the presence of ATP (Gross & Hessefort, 1996, Gross et al., 1996). P60 plays an important role at an intermediate stage of PR assembly (Smith et al., 1995). It binds to the progesterone

receptor in conjunction with hsp70 (Smith et al., 1993b). P60 is thought to pass the protein substrate from the hsp70 chaperone complex to the hsp90-immunophilin chaperone complex (Chen et al., 1996).

P60 contains six to eight TPR domains (Honore et al., 1992). The N-terminal region is necessary for hsp70 binding and a central region containing TPR motifs is necessary for binding to both hsp90 and hsp70 (Chen et al., 1996). P60 can bind either hsp70 or hsp90 independently and in an ATP independent manner.

Heat shock protein 90 (hsp90)

Several lines of evidence indicate that the 90-kDa heat shock proteins (hsp90) facilitate the folding of "substrate" proteins. Hsp90 is essential for cell viability, and is expressed both constitutively and during cell stress [reviewed in (Craig et al., 1994, Gething & Sambrook, 1992, Jacob & Buchner, 1994)]. Hsp90 prevents the aggregation of proteins *in vitro* (Jacob et al., 1995, Miyata & Yahara, 1992, Wiech et al., 1992) and stimulates the recovery of activity from denatured or improperly folded proteins (Jacob et al., 1995, Miyata & Yahara, 1992, Schumacher et al., 1994, Shaknovich et al., 1992, Shue & Kohtz, 1994, Wiech et al., 1992). The activities or conformations of hsp90-bound substrate proteins change upon dissociation from cytoplasmic hsp90 (Bresnick et al., 1989, Brugge et al., 1983, Courtneidge & Bishop, 1982, Hartson & Matts, 1994, Kost et al., 1989, Sanchez et al., 1985, Ziemiecki, 1986). Also consistent with a role for hsp90 in protein folding, genetic evidence indicates that hsp90 supplies an essential positive function for certain signal transduction proteins (Aligue et al., 1994, Carver et al., 1994, Cutforth & Rubin, 1994, Picard et al., 1990, Xu & Lindquist, 1993). Recently it has been shown that hsp90 is required for the initial *de novo* folding and the reiterative support of $p56^{lck}$ structure in rabbit reticulocyte lysate (Hartson et al., 1996, Hartson & Matts, 1994). However, hsp90 does not appear to play a generic role in folding of all nascent

polypeptides (Frydman et al., 1994), but rather appears to act on specific target proteins (Xu & Lindquist, 1993). Thus, hsp90 may function as a "signal transduction chaperone", participating in the folding of certain proteins in concert with regulatory stimuli [reviewed in (Bohen et al., 1995, Craig et al., 1994, Rutherford & Zuker, 1994)]. In addition to the role of hsp90 in folding, hsp90 is speculated to be involved in protein targeting (Brugge, 1986, Owens-Grillo et al., 1996, Pratt, 1992).

Hsp90 functions as a chaperone machine with a number of noncovalently associated cohorts-hsc70, p60, p48, p23 and an assortment of immunophilins (Hutchinson et al., 1994b, Hutchinson et al., 1995, Jackson et al., 1983, Jacob & Buchner, 1994, Schumacher et al., 1994, Smith et al., 1993a, Smith et al., 1990a, Smith et al., 1992, Smith et al., 1995). Hsp90 interacts with a variety of proteins such as steroid receptors (Bohen & Yamamoto, 1993, Joab et al., 1984, Picard et al., 1990), the aryl hydrocarbon receptor (Carver et al., 1994), serine/threonine protein kinases (Matts et al., 1992, Miyata & Yahara, 1992), cytoskeletal proteins (Koyasu et al., 1986, Minami et al., 1993).and the v-src (Xu & Lindquist, 1993), Wee-1 (Aligue et al., 1994) and sevenless receptor (Cutforth & Rubin, 1994) tyrosine kinases.

Much of what is known about the hsp90 chaperone machine comes from studies examining the factors required to reconstitute the hormone binding activity of salt-stripped glucocorticoid and progesterone steroid hormone receptors (SHR) in rabbit reticulocyte lysate (RRL) (Hutchinson et al., 1994a, Hutchinson et al., 1994b, Hutchinson et al., 1995, Johnson & Toft, 1995, Smith et al., 1992, Smith et al., 1993b, Smith et al., 1995, Smith et al., 1990b). ATP-dependent binding of hsc70 to SHR is required for the subsequent interaction of hsp90 and its associated cohorts with SHR (Hutchinson et al., 1994b, Smith et al., 1993a). Hsp90, hsc70, p60 and p48 are present in chaperone complexes at intermediate times during SHR reconstitution, while hsp90, p23 and individual immunophilins are present in later complexes formed just prior to or at the point of SHR reconstitution (Johnson et al., 1994, Johnson & Toft, 1995, Smith et al., 1993b,

Smith et al., 1995). The SHR in this mature complex is competent to bind hormone and is maintained in this state via reiterative, ATP-dependent interactions with the RRL chaperone machinery.

Originally it was thought that hsp90 played a passive role in keeping steroid receptor inactive. In the inactive form, the steroid receptors are associated with large (9S-10S) particles composed in part of chaperones and PPIases. Upon activation, the steroid receptors dissociate from hsp90 and become competent to bind DNA. The tight relation between receptor activity and dissociation from hsp90 led to the initial interpretation that the function of hsp90 is purely inhibitory, playing a passive role in keeping receptor inactive. However, using the yeast genetic system it was established that an hsp90-dependent function is essential for the function of steroid hormone receptors *in vivo* (Bohen & Yamamoto, 1994, Picard et al., 1990).

Protein 23 (p23)

The specific function of the hsp90 cohort p23 is unknown. p23 binds to hsp90 in an ATP-dependent manner, but this binding does not require ATP hydrolysis (Johnson et al., 1994, Johnson & Toft, 1994). Besides hsp90, p23 containing complexes are found to have three different immunophilins - cyclophilin40, FKBP52, and FKBP54, in rabbit reticulocyte lysate. It is proposed that the p23 complex seen is a collection of different complexes, each with hsp90, p23, and one type of immunophilin.

p23 is present in progesterone receptor (PR) complexes under specific conditions and absent under others. Depletion of p23 from lysate prevents the assembly of PR complexes, in which the PR is competent to bind hormone. Addition of purified p23 restores the hormone binding activity, indicating that PR chaperone complexes containing p23 are an essential precursor to the formation of active PR (Hutchinson et al., 1995,

Johnson & Toft, 1994). ATP hydrolysis is required for the association of p23 with PR/hsp90 chaperone machine complexes (Johnson & Toft, 1994).

Role of chaperones in regulating protein synthesis

In this dissertation, RRL is used as the model system because of its unique capabilities for *in vitro* protein synthesis. Moreover RRL is a rich source of molecular chaperones that are involved in regulating protein synthesis, folding, and renaturation. In RRL, protein synthesis is regulated at the initiation level by heme. Ninety percent of the protein produced in RRL is hemoglobin. Without heme, newly synthesized globin is converted to apohemoglobin, which readily denatures. In heme deficiency, protein synthesis in RRL is inhibited at the initiation level due to activation of the heme regulated inhibitor (HRI). HRI on activation, phosphorylates the 38kDa α-subunit of eIF-2. Phosphporylation of eIF-2 sequesters the factor responsible for eIF2 recycling (eIF-2B). eIF-2B is required for catalyzing the exchange of GDP for GTP bound to eIF-2, and hence for the formation of the [eIF-2•GTP•met-tRNA$_i$] ternary complex. Thus phosphorylation of eIF-2 leads to inhibition of protein synthesis at the initiation level. The physiological role of the phosphorylation of eIF-2 in reticulocytes is to coordinate the synthesis of globin with the availability of heme.

In addition to heme deficiency HRI is activated under oxidative stress, heat stress and heavy metal ion treatment in hemin supplemented lysates. HRI has been demonstrated to interact with the hsps and hsp cohorts, hsp90, hsc70, FKBP52 and p23*in situ* in RRL (Matts & Hurst, 1989, Matts et al., 1992, Xu et al., In preparation). Correlative data suggest that hsps regulate the activation of HRI; HRI is less sensitive to activation by heat and oxidative stress in lysates containing high levels of hsc70. The notion that the binding of hsp70 to HRI inhibits its activation is supported by the observation that denatured

proteins bind to hsp70, block the interaction of hsp70 with HRI, activate HRI and inhibit protein synthesis (Matts et al., 1993).

The ability of RRL to renature protein appears to be regulated by the amount of hsc70 present. The rate of luciferase renaturation in the reticulocyte lysate is proportional to the amount of hsp70 present (Schumacher et al., 1994). Heat shock, which is suggested to lower the amount of active pool of hsp70, inhibited the rate of luciferase renaturation. From these observations, we propose that activation of HRI is regulated by a competition for hsp70 between HRI and nascent or denatured proteins (Matts et al., 1993). Physiological conditions that lead to the accumulation of denatured or unfolded nascent polypeptides would lead to the activation of HRI and the cessation of protein synthesis. Such a mechanism would coordinate the rate of protein synthesis with the ability of the cell to properly fold nascent polypeptides or to renature damaged proteins. This dissertation addresses the relationship of hsp function between protein folding, protein renaturation and regulation of protein synthesis. This is accomplished by using different pharmacological drugs directed against hsps.

CHAPTER II

ROLE OF HSP90 IN LUCIFERASE RENATURATION

Introduction

Protein folding and renaturation *in vivo* are facilitated by a group of proteins referred to heat shock proteins (hsp) or molecular chaperones [reviewed in (Becker & Craig, 1994, Craig et al., 1994, Georgopoulos, 1992, Gething & Sambrook, 1992, Hartl, 1995). Molecular chaperones appear to function in complexes referred to as chaperone machines (Georgopoulos, 1992) Chaperones primarily interact with hydrophobic regions of newly synthesized or denatured protein substrates to prevent illicit interactions between the bound protein and other cellular components. The affinity of chaperones for polypeptide substrates is modulated by associated proteins (cohorts) and conformational changes induced by the binding and hydrolysis of ATP (Becker & Craig, 1994, Georgopoulos, 1992, Hartl, 1995). Chaperone-associated cohorts modulate chaperone function by a number of mechanisms: they may interact with chaperone-bound substrates directly; they may modulate the interactions of hsps with their protein substrates; or they may modulate the rate of ATP hydrolysis or nucleotide exchange. Chaperones appear to facilitate protein folding primarily by reducing the number of molecules that follow non-productive folding pathways that would lead to aggregation of the protein.

Hsp90 is an extremely abundant constitutively expressed heat shock protein [reviewed in (Jacob & Buchner, 1994)]. Genetic (Bohen & Yamamoto, 1993, Picard et al., 1990, Xu & Lindquist, 1993) and biochemical (Jacob et al., 1995, Pratt & Welsh,

1994b, Schumacher et al., 1994, Wiech et al., 1993, Wiech et al., 1992) studies indicate that members of the hsp90-gene family are involved in protein folding and renaturation under normal and adverse physiological conditions. Little is known, however, about the mechanism of action of hsp90 in the folding and renaturation of proteins, but it appears to act at later stages in protein folding pathways where extensive secondary structure has already formed (Melnick et al., 1992, Melnick et al., 1994, Shue & Kohtz, 1994, Smith, 1993). Hsp90 functions as chaperone machine with a number of noncovalently associated cohorts [hsc70, p60; p48; p23; and an assortment of immunophilins (Chang & Lindquist, 1994, Hutchinson et al., 1994a, Hutchinson et al., 1994b, Hutchinson et al., 1995, Jacob & Buchner, 1994, Johnson & Toft, 1995, Schumacher et al., 1994, Smith et al., 1992, Smith et al., 1993b, Smith et al., 1995, Smith et al., 1990b)]. Much of what is known about the hsp90 chaperone machine comes from studies examining the factors required to reconstitute the hormone binding activity of salt-stripped glucocorticoid and progesterone hormone steroid hormone receptors (SHR) in rabbit reticulocyte lysate (RRL) (Hutchinson et al., 1994a, Hutchinson et al., 1994b, Hutchinson et al., 1995, Johnson & Toft, 1995, Smith, 1993, Smith et al., 1992, Smith et al., 1995, Smith et al., 1990b). ATP-dependent binding of hsc70 to SHR is required for the subsequent interaction of hsp90 and its associated cohorts with SHR (Hutchinson et al., 1994b, Smith et al., 1992). Hsp90, hsc70, p60 and p48 are present in chaperone complexes at intermediate times during SHR reconstitution, while hsp90, p23 and individual immunophilins are present in later complexes formed just prior to or at the point of SHR reconstitution (Johnson & Toft, 1994, Johnson & Toft, 1995, Smith, 1993, Smith et al., 1995). These studies indicate that these complexes represent dynamic, reiterative, ATP-dependent cycles of association of the SHRs with the hsp90 chaperone machine that maintain these SHRs in a conformation competent to bind hormone.

Benzoquinonoid ansamycins are antibiotics of fungal origin that revert cell transformation mediated by $p60^{v\text{-}src}$ and several other tyrosine kinases (Uehara et al.,

1986, Uehara et al., 1988). Benzoquinonoid ansamycins such as geldanamycin (GA) and herbimycin A (HbA) were traditionally thought to be direct inhibitors of tyrosine kinases (Uehara et al., 1989). However, recent work suggests that benzoquinonoid ansamycins inhibit the tyrosine kinase activity of $p60^{v\text{-}src}$ through an indirect mechanism (Whitesell et al., 1994). In extracts of cells transformed by $p60^{v\text{-}src}$, hsp90 was the predominant protein bound by immobilized GA (Whitesell et al., 1994). Binding of hsp90 to immobilized GA was specifically blocked by soluble HbA and soluble GA. Immobilized GA did not bind $p60^{v\text{-}src}$. GA treatment disrupted the interaction of hsp90 with $p60^{v\text{-}src}$ *in vivo*, concomitant with reversion of $p60^{v\text{-}src}$ transformation. Since genetic studies have previously demonstrated that hsp90 function is required for maturation or support of $p60^{v\text{-}src}$ function (Xu & Lindquist, 1993), these results strongly argue that the mechanism of GA and HbA action involves the disruption of hsp90 function. Consistent with this putative mechanism, GA induces loss of function for the *erb-B2* gene product (Chavany et al., 1996), the progesterone receptor (Johnson & Toft, 1995, Smith et al., 1995), the glucocorticoid receptor (Whitesell et al., 1995), and the raf kinase (Schulte et al., 1995) and disrupts the normal interaction of these proteins with hsp90 or with the hsp90 homolog GRP94. Two studies address the mechanism of GA inhibition of hsp90 chaperone machinery (Johnson & Toft, 1995, Smith et al., 1995). These studies indicate that GA decreases the recovery of progesterone receptors in complexes with hsp90 that containing the p23 and immunophilin cohorts. Instead, receptor accumulates in complexes containing hsp90, hsp70, and two hsp cohorts, p60 and p48.

To characterize further the mechanism of action of BAs, we have examined the effects of GA on the steady-state kinetics of the renaturation of denatured luciferase in RRL. Previously, we have demonstrated that chaperone-mediated renaturation of thermally denatured firefly luciferase requires hsp90, hsc70, and other as yet unidentified components present in RRL (Schumacher et al., 1994). The RRL model system was selected for these studies because luciferase renaturation mediated by mixing partially

purified preparations of hsc70 and hsp90 was previously observed to be noncatalytic, requiring the addition of a large excess of the chaperone components relative to amount of luciferase present (Schumacher et al., 1994). In this report, we demonstrate that luciferase renaturation in RRL obeyed Michaelis-Menten kinetics. The Eadie-Hofstee plot of the rates of luciferase renaturation determined while varying both ATP and luciferase concentrations was a series of nearly parallel lines that is characteristic of sequential reaction mechanisms. Kinetic analysis of inhibition of luciferase renaturation by GA reveals the probable mechanism and targets of GA. Our findings also support the notion that BAs are specific inhibitors of hsp90, and indicate that the hsp90 chaperone machinery associated with denatured luciferase is identical to that described above for the progesterone receptor. Similarly, these associations are modulated by GA in a fashion identical to that described for the progesterone receptor. These results imply that the action of hsp90 on well documented hsp90-bound proteins is related to that occurring during protein renaturation, i.e., chaperoning of protein folding.

Experimental procedures

Materials

Bovine Creatine phosphokinase (Type I), serum albumin (acetylated), herbimycin A, firefly luciferase and luciferin were purchased from Sigma. Geldanamycin was provided by the Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute. Geldampicin was provided by Dr. Kenneth L. Rinehart (U. of Illinois, Urbana). The F5 anti-p60 and the JJ3 anti-p23 monoclonal antibodies were provided by Dr. David Smith (U. of Nebraska, Omaha) and Dr. David Toft (Mayo Medical School, Rochester, MN), respectively. The 4322 anti-hsp70/90 and anti-hsp90 (84/86) antiserum was provided by Dr. Stephen Ullrich (NCI)

and the anti-hsp40 antiserum was provided by Dr. William Welch (UCSF). Anti-luciferase affinity purified polyclonal antibodies was purchased from Promega.

Buffers and reagents

The buffers used were as follows: assay buffer (AB) consisting of 25 mM Tricine-HCl (pH 7.8), 8 mM $MgSO_4$, 0.1 mM EDTA, 33 mM dithiothreitol, 470 μM D-luciferin, 240 μM coenzyme A, and 0.5 mM ATP; stability buffer (SB) consisting of 25 mM Tricine-HCl (pH 7.8), 8 mM $MgSO_4$, 0.1 mM EDTA, 10 mg/ml bovine serum albumin, 10% glycerol, and 1% Triton X-100; dialysis buffer (DB) consisting of 10 mM Tris-HCl (pH 7.4), 3 mM $Mg(OAc)_2$, 100 mM KCl, and 2 mM dithiothreitol; tris buffer (TB) consisting of 10 mM Tris-HCl (pH 7.4); tris buffered saline (TBS) consisting of TB and 150 mM NaCl; TB/500 consisting TB and 500 mM NaCl; and TB/50 consisting of TB and 50 mM KCl.

Luciferase renaturation assay

Reticulocyte lysate was prepared as described (Matts et al., 1991). Dialyzed RRL was prepared by dialysis of 2.5 ml of RRL against 500 ml of DB buffer for six h at 4 °C with buffer changes every 1.5 h. The endogenous ATP concentration in undialyzed lysate and the ATP and ADP concentrations remaining in the dialyzed lysate were analyzed as described (BioOrbit, 1991).

Firefly luciferase (2 mg/ml) in SB was denatured by incubation at 41° C for 10 min (Schumacher et al., 1994) unless otherwise specified. After being cooled on ice, aliquots were diluted 20-fold into heme-deficient protein synthesis mixes (Matts et al., 1991) containing 10 μM edeine or dialyzed reticulocyte lysate containing 10 mM creatine phosphate, 20 U/ml creatine phosphokinase and varying concentrations of ATP. Samples

were incubated at 28 °C and at various time points (usually 10 min) the luciferase activity present in 2 µl aliquots (unless otherwise stated) was determined by dilution into 50 µl AB. Light production was measured for 10 s in a Lumac (3M) Bioluminometer (model 2010A). The rate of luciferase renaturation was determined to be linear over the time period for all assays and the rate of luciferase renaturation is expressed in the figures and table as light producing units renatured per 10 min interval (light units/10 min). For the kinetic analysis, less than 1% of the denatured luciferase was renatured over the time course of the assay, so the concentration of denatured luciferase effectively remained constant. Curve fitting was done with the nonlinear least squares program STEPT (Chandler, 1988) using weighting factors equal to the reciprocal of the variances of each data point. As described previously (Schumacher et al., 1994), (i) luciferase renaturation was ATP-dependent; (ii) no significant spontaneous (chaperone-independent) renaturation of the luciferase was observed when the denatured luciferase stock was incubated at 28 °C in the absence of RRL; and (iii) at very limiting luciferase concentrations (0.03-0.3 nM) 60 to 100% of the denatured luciferase could be renatured upon prolonged incubation (75-90 min) in RRL

Several factors were found to affect the rate of luciferase renaturation in the assay. As previously reported, different lysate preparations renatured luciferase at different rates because the preparations contain different quantities of hsps (Matts & Hurst, 1992, Schumacher et al., 1994). Because of the expense of the enzyme, stock solutions of native luciferase were stored at 4 °C and used over the period of a week. Aliquots of the stock solution were clarified by centrifugation prior to use. The rate of luciferase renaturation was observed to decrease with time of storage of the stock solution. This correlated with the accumulation of some insoluble material in the stock solution over the storage period. Ongoing protein synthesis in RRL was also observed to inhibit luciferase renaturation. Undialyzed lysate was thawed rapidly at 30 °C, followed by the addition of ediene to insure that translation was fully inhibited. All data points reported in the figures represent

measurements determined in a single experiment using a single lysate preparation and luciferase stock. All experiments were carried out at least three times.

Purification of hsps and hsp cohorts

Hsc70 was purified to apparent homogeneity from the post ribosomal supernatant of rabbit reticulocyte lysate as described for the purification of supernatant factor (Gross, 1976, Gross et al., 1994). The initial steps in the purification of the hsp90 were similar to the steps for the purification of supernatant factor: precipitation at pH 5.4; reprecipitation at 40-80% saturated ammonium sulfate; and chromatography on DEAE-cellulose. These steps were followed by chromatography on Superose 12/HR and monoQ columns as previously described (Denis, 1988). p60 copurified with hsc70 up to the ATP-agarose affinity chromatography step, where p60 was present in the fraction of proteins that did not bind to the column in buffer containing 0.1 M KCl (Gross et al., 1994). The p60 was approximately 67% pure at this stage with hsc70 being the primary contaminating protein (p60:70 ~5:1). Chicken p23 (Johnson & Toft, 1994) was generously provided by Dr. David Toft (Mayo Medical School). Western blotting with an anti-hsp40 antiserum indicated that none of these preparations were contaminated with hsp40.

Immunoadsorptions of proteins

Goat anti-rabbit IgG was cross-linked to agarose (GaR-agarose) as previously described (Matts et al., 1992). Affinity pure anti-luciferase antibody (10 µl) was adsorbed to 20 µl of GaR-agarose (diluted 1:1 in TBS) in the presence or absence (control) of luciferase (40 µg) for 2 hr on ice. In the absence of stability buffer, purified luciferase spontaneously denatures. The GaR-agarose was then washed with 500 µl each of TBS, TB/500, TBS, and TB/50. RRL (35 µl), diluted 1:1 to contain 10 mM creatine

phosphate,20 U/ml creatine phosphokinase, 75 mM KCl and either DMSO or GM (1 μg/ml), were then added to GaR-agarose. The mixtures were incubated for 5 min at 30 °C with periodic mixing to prevent settling of the agarose. The unadsorbed proteins were separated from adsorbed proteins (pellets) by centrifugation. Pellets were washed four times with TB/50.

Western blot analysis

Samples were prepared for SDS-PAGE, separated in 10% gels, and transferred to PVDF membrane as previously described (Matts & Hurst, 1992, Matts et al., 1992). Hsp70 and hsp90 were detected by western blotting with the 4322 anti-hsp70/hsp90 antiserum (Ehrhart et al., 1988) or an anti-hsp90 (anti-mouse hsp84/86 antipeptide) antiserum provided by Dr. Stephen Ullrich (NCI) as previously described (Matts & Hurst, 1992, Matts et al., 1992). p60, p48 and p23 were detected by western blotting with the F5 anti-p60 (Smith et al., 1993b) 3G6 anti-p48 (Smith et al., 1995) and the JJ3 anti-p23 (Johnson & Toft, 1994)mAb, respectively.

Results

GA and HbA inhibit luciferase refolding.

Recent studies suggest that benzoquinonoid ansamycins bind hsp90 and inhibit its function (Whitesell et al., 1994). Since it is previously demonstrated that hsp90 plays a role in chaperone mediated renaturation of denatured luciferase in RRL (Schumacher et al., 1994), we examined the effect of GA and HbA on the renaturation of luciferase. While neither GA nor HbA had any effect on the activity of native luciferase (not shown), they inhibited luciferase renaturation in a dose-dependent manner (Fig. 1). The concentrations of GA and HbA that inhibited luciferase renaturation by 50% of the maximally induced inhibition (IC_{50}) were estimated to be 0.2 and 1.3 μM, respectively. Maximum inhibition

of luciferase renaturation was observed at a GA concentration of 0.7 μM. Addition of the DMSO vehicle at concentrations up to 0.2% (v/v) had no effect on the rate of luciferase renaturation. The pharmacologically inactive BA, geldampicin (Whitesell et al., 1994) did not inhibit luciferase renaturation when added at concentrations up to 15 μM (not shown).

The extent of GA-induced inhibition of the rate of luciferase renaturation was observed to be dependent on the temperature at which the luciferase was denatured (Table II). GA decreased the initial velocity of luciferase renaturation by 50% when the luciferase was denatured at 38 °C and by 80% when the luciferase was denatured at 41 °C. These observations suggest that the more severely luciferase was denatured, the more dependent luciferase became on a GA-inhibitable event for rapid renaturation.

Kinetic analysis of chaperone-mediated luciferase renaturation.

We next examined the kinetics of luciferase renaturation in reticulocyte lysate to determine whether the mechanism by which BAs inhibit the renaturation of luciferase could be analyzed using steady state kinetics. At saturating ATP concentrations, the rate of luciferase renaturation was saturable with respect to the concentration of denatured luciferase present, and followed a rectangular hyperbolic curve that is characteristic of enzymes following Michaelis-Menten kinetics (Fig 2A). The K_{app} and V_{app} for the reaction were calculated to be 1.0 ± 0.17 μM and 1.0 ± 0.097 10^5 Light Units/10 min, respectively. The K_{app} was calculated assuming that the luciferase preparation was homogeneous, and that all the denatured luciferase (MW=65,000) present represented soluble monomeric substrate with a single chaperone binding site. Therefore, this estimated K_{app} represented a maximum value, since some insoluble material was noted to be present in the luciferase stock.

The renaturation of luciferase in RRL requires the presence of an optimum concentration of K^+, Mg^{2+} and ATP (Schumacher et al., 1994). To allow the kinetic

analysis of the effects of varying ATP concentrations on the rate of luciferase renaturation, RRL was dialyzed to remove endogenous ATP. The ATP remaining in the lysate was quantified and was found to be 5 μM and 20 μM in the absence and presence of a CP/CPK ATP regenerating system, respectively. The rate of luciferase renaturation was determined at a near-saturating concentration of denatured luciferase and varying ATP concentrations (Fig 2B). The K_{app} and V_{app} for ATP in the reaction were estimated to be 61 ± 8.0 μM and 4.2 ± 0.25 x 10^4 Light Units/10 min, respectively. Renaturation rate was next determined while varying both ATP and luciferase concentrations. An Eadie-Hofstee plot of the data generated a series of nearly parallel lines that is characteristic of sequential reaction mechanisms (Fig. 3).

Kinetic analysis of GA-induced inhibition of luciferase renaturation.

We next examined the kinetics of luciferase renaturation in the presence of GA to gain insight into the mechanism by which GA inhibited chaperone-mediated luciferase renaturation. Luciferase renaturation rates were determined at saturating ATP and varying luciferase and GA concentrations. Eadie-Hofstee plots of the data indicated that GA inhibited luciferase renaturation noncompetitively, affecting both the V_{app} and the V_{app}/K_{app} (Fig. 4A). Replots of V_{app}/K_{app} *versus* [GA] (Fig. 4B) and $1/V_{app}$ *versus* [GA] were hyperbolic (Fig. 4C), indicating that luciferase renaturation could not be completely inhibited even at saturating concentrations of GA. Concentrations of GA up to 3.5 μM inhibited luciferase renaturation no more than that observed with 0.7 μM GA.

We next examined the effect of GA on the kinetics of luciferase renaturation at near saturating luciferase and varying ATP concentrations in a dialyzed RRL as described above (Fig 5A). Eadie-Hofstee plots of the data generated a series of straight lines that intersected at a single point; a pattern which is characteristic of uncompetitive inhibition. However, the lines intersected to the right of the y-axis, indicating that GA should theoretically stimulate

luciferase renaturation in a competitive manner with respect to ATP, at very low ATP concentrations. The replots of K_{app}/V_{app} *versus* [GA] (Fig 5B) and $1/V_{app}$ *versus* [GA] (Fig. 5C) appeared nonlinear. The best-fit of the $1/V_{app}$ *versus* [GA] replot was parabolic, but a rigorous exclusion of a linear fit would require more data. While the stimulatory effect of GA on luciferase renaturation that is predicted to occur at very low ATP concentrations is curious, it is physiologically irrelevant, since the parabolic shape of the [GA] *versus* $1/V_{app}$ replot indicates that this phenomenon does not occur at higher ATP concentrations where GA inhibits luciferase renaturation.

Effects of GA on the interaction of components of the hsp90-chaperone machine with denatured luciferase.

To further elucidate the mechanism by which GA inhibits luciferase renaturation, the components of the hsp90 chaperone machine that were co-immunoprecipitable with denatured luciferase in RRL were characterized. Hsp90, hsc70 and the cohorts p60, p48 and p23 were observed to interact with luciferase (Fig. 6). The interaction of hsc70 with luciferase was indicated by the increase in amount hsc70 present in the immune pellet relative to the nonspecific binding apparent in the minus luciferase control (Fig. 6, lane 1 *vs* lanes 2 & 3). GA treatment was found to increase the steady state level of p60, while it decreased the steady state level of p23 interacting with luciferase relative to the control (Fig. 6, lane 2 *vs* lane 3).

Hsp90 specifically reverses inhibition of luciferase renaturation induced by geldanamycin.

To further test the hypothesis that GA binds hsp90 and inhibits its function, we examined the effect of purified hsps and hsp cohorts on luciferase renaturation in reticulocyte lysate (Fig. 7). In the absence of GA, addition of purified hsp90, p60 or p23

to control RRL had no effect on luciferase renaturation (Fig. 7, closed bars: none *vs* 90, 23, or 60). Luciferase renaturation was stimulated by 70% upon the addition of purified hsc70 (Fig. 7: 70). Addition of both hsp90 and hsc70 stimulated luciferase renaturation by 80% (Fig 7: 90+70). A small (an additional 5%), but reproducible stimulation of luciferase renaturation was observed when p23 was added in conjunction with hsc70 alone, or with hsc70 and hsp90 (Fig. 7: 23+70 & 90+70+23). Addition of other combinations of components had no effect on luciferase renaturation.

The effect of the purified hsps and hsp cohorts on GA-induced inhibition of luciferase renaturation was then examined. Luciferase renaturation was inhibited by 60% in RRL in the presence of 0.7 μM GA (Fig. 7, closed *vs* open bars: none). GA inhibited luciferase renaturation to a similar degree (60 to 70%) in RRL supplemented with purified p23, p60, hsc70 or any combination of these components (Fig. 7) compared to RRL that was supplemented with the purified components and incubated in the absence of GA.

In contrast to the other components, hsp90 was observed to specifically reverse GA-induced inhibition of the rate of luciferase renaturation. In the presence of GA, addition of hsp90 to RRL stimulated luciferase renaturation by 100% relative to the control containing GA but lacking hsp90 (Fig. 7, open bars: none *vs* 90). In hsp90-supplemented RRL containing GA the rate of luciferase renaturation was restored to within 80% of the rate observed in hsp90-supplemented RRL incubated in the absence of GA (Fig. 7, closed *vs* open bars: 90). In RRL supplemented with hsp90 in combination with the other purified components, GA-induced inhibition of the rate of luciferase renaturation ranged from 7 to 20%. In addition, the extent to which hsp90 reversed the inhibitory effect of GA was proportional to the amount of hsp90 added to the lysate (data not shown). The ability of hsp90 to protect against GA-induced inhibition of the rate of luciferase renaturation was not simply an effect of adding protein to the lysate, since addition of BSA showed no protection against GA-induced inhibition of luciferase renaturation.

Discussion

Kinetics of Chaperone Facilitated Protein Renaturation in RRL.

While the mechanism by which protein renaturation occurs in RRL is undoubtedly complex, the data presented here indicate that the problem is at least partially tractable to analysis using steady state kinetics. Luciferase renaturation showed substrate saturation with respect to both ATP and luciferase concentrations and followed Michaelis-Menten kinetics. Eadie-Hofstee plots of the initial velocity data (Fig. 3) were a family of nearly parallel lines characteristic of sequential mechanisms. The parallel pattern observed for the chaperone-mediated luciferase renaturation of this study could be explained by either of the abbreviated alternatives presented in Fig. 8. Specifically, addition of denatured luciferase could immediately follow addition of ATP to the chaperone machine (Fig. 8A). In this scheme the hsp90 chaperone machine would be a single large complex of proteins [e.g., a foldasome (Hutchinson et al. 1995, Hutchinson et al., 1994b). The data is also consistent with models proposed by the laboratories of Smith and Toft (Johnson & Toft, 1995, Prapapanich et al., 1996, Smith et al., 1995) in which there is an ATP-dependent assembly of an hsp90-p23-immunophilin complex which subsequently binds to SHR/hsp90/hsc70/p60/p48 complexes and from which the previously bound chaperones dissociate (Fig. 8B).

The association of hsp90, hsc70, and cohorts p60, p48, and p23 with denatured luciferase suggests that renaturation of luciferase occurs by a mechanism similar to that involved in restoring the hormone binding activity of SHRs (Hutchinson et al., 1994a, Hutchinson et al., 1994b, Hutchinson et al., 1995, Johnson & Toft, 1995, Smith, 1993, Somma et al., 1995). This observation implies that the function of the hsp90 chaperone machine is not specific to its previously described interactions with signal transduction proteins, and that it is capable of carrying out a more generalized protein folding function *in*

vivo. This folding activity appears to target proteins containing partially unfolded structure. This notion is consistent with previous reports that indicate hsp90 interacts with late folding intermediates of proteins prior to their conversion to the native state (Hartson & Matts, 1994, Jacob et al., 1995, Melnick et al., 1992, Melnick et al., 1994, Smith, 1993).

Effect of geldanamycin on the interactions of hsps and associated cohorts with denatured luciferase.

The data presented here support the hypothesis that GA is a specific inhibitor of hsp90 function. GA inhibited luciferase renaturation in RRL, but had no direct effect on luciferase activity *in vitro* or in RRL after its renaturation. The difference observed between the IC_{50} values for GA and HbA-induced inhibition of luciferase renaturation is similar to differences observed between the potency of the two drugs in *in vivo* assays of their tumoricidal activity (Whitesell et al., 1992). GA-induced inhibition of luciferase renaturation was specifically reversed by the addition of hsp90 to RRL, but not by the addition of hsc70, p60, or p23.

The binding of GA to hsp90 appears to inhibit the normal sequential interaction of components of the hsp90-chaperone machinery with luciferase. GA increased the amount of p60 and decreased the amount of p23 associated with the hsp90-chaperone machinery containing bound luciferase. GA has similar effects on the association of p23 and p60 with progesterone receptor/hsp90 complexes reconstituted in RRL (Johnson & Toft, 1995, Smith et al., 1995). Recent results indicate that hsp40 also binds to denatured luciferase. We have not yet examined whether immunophilins interact with hsp90-p23 bound luciferase or whether GA blocks this interaction as has been reported for the progesterone receptor. Thus, as for the progesterone receptor, GA appears to prevent the transition of hsp90/hsc70/p60/p48 complexes that contain bound substrate to hsp90 complexes containing p23 and bound substrate. Previous work indicates that GA does not simply act

by stabilizing hsp90/hsc70/p60/p48 complexes containing bound progesterone receptor (Smith et al., 1995). Therefore, the binding of GA to hsp90 appears to block the ability of p23 or hsp90-p23 complexes to interact stably with luciferase present in hsp90/hsc70/p60/p48 complexes. These observations imply that a GA-sensitive event is a hsp90-p23 dependent event.

An alternate pathway for luciferase renaturation exists in RRL.

Kinetic analyses of GA-induced inhibition of luciferase renaturation at varying luciferase concentrations indicate that luciferase can renature by more than one pathway. GA inhibited luciferase renaturation noncompetitively with respect to luciferase concentration, indicating that at saturating ATP concentrations GA binds to both the hsp90-chaperone machine/ATP complex and the hsp90-chaperone machine/ATP/luciferase complex. The hyperbolic replots of GA concentration *versus* $1/V_{app}$ and K_{app}/V_{app} indicate that GA can not completely inhibit luciferase renaturation even at saturation. This indicates that luciferase can renature by a slower alternate reaction pathway in the presence of GA. The existence of an alternate pathway is consistent with the observations that: (i) folding of newly synthesized luciferase in RRL requires hsc70, hsp40 and additional lysate chaperone components (e.g., TRiC), but does not appear to involve hsp90 (Frydman et al., 1994); and (ii) refolding of luciferase that has been denatured by treatment with guanidinium•HCl can be chaperoned by hsc70, hsp40 and Hip (p48) *in vitro* (Freeman et al., 1995, Hohfeld et al., 1995).

The observations that the extent to which GA inhibits the rate of luciferase renaturation depends on the age of the luciferase stock and the temperature at which it was denatured also suggest that denatured luciferase is a mixed population of molecules whose rate of renaturation varies in their sensitivity to inhibition by GA. Evidence for the existence of multiple unfolding intermediates of thermally denatured citrate synthase that

renatured at different rates in the presence of hsp90 *in vitro* has been previously reported (Jacob et al., 1995).

The alternate pathway that is followed in the presence of GA probably involves dissociation of luciferase from the intermediate hsp90-chaperone machine complex containing hsc70, p60 and p48 (Prapapanich et al., 1996, Smith et al., 1995), and likely involves hsp40 (Hohfeld et al., 1995). The GA-insensitive folding pathway may be slower for a number of related reasons. The interaction of the folding intermediate with hsp90-p23 might facilitate the acquisition of a folded structure (e.g., the binding of the folding intermediate to the hsp90-p23 chaperone machine may increase the rate constant of the forward reaction). In addition, more nonproductive folding pathways might be accessible to the folding intermediate in the absence of an interaction with hsp90 containing bound p23 (e.g., the binding of the folding intermediate to the hsp90-p23 chaperone machine may decrease the rate constant for the back reaction). As a result, luciferase would require more reiterative cycles of association with the chaperone machinery to fold. Furthermore, since denatured luciferase is likely a mixed population of substrate, the folding of a portion of the denatured luciferase population may be accelerated to a greater extent or completely dependent upon association with the hsp90 chaperone machinery containing p23. Reiterative cycles of association of this population of luciferase molecules with the hsp90-chaperone machine complex containing hsc70, p60 and p48 would slow the rate of folding of the luciferase population capable of following the GA-insensitive folding pathway.

Other observations reported here suggest that the GA-sensitive folding pathway is normally preferred. GA inhibited the rate of luciferase renaturation by 70% in RRL lysate supplemented with hsc70. However, a 35% inhibition would have been expected if hsc70 was stimulating luciferase renaturation solely through a GA-insensitive (hsp90-p23 independent) pathway. In addition, hsp90 reversed GA-induced inhibition of luciferase renaturation in hsc70 supplemented lysate nearly completely. Thus, the exogenous hsc70

appeared to be preferentially stimulating luciferase renaturation through the GA-sensitive (hsp90-p23 dependent) pathway in RRL.

Mechanism of action of geldanamycin.

Kinetic analyses of GA-induced inhibition of luciferase renaturation with respect to varying ATP concentration suggests a possible mechanism by which the binding of GA to hsp90 inhibits hsp90 function. Inhibition of luciferase renaturation by GA was uncompetitive with respect to ATP (Fig. 5A). This shows that the inhibition by GA is either not reversibly connected with the addition of ATP or that GA inhibits an enzyme form downstream from that with which ATP combines. These possiblities are consistent with the schemes presented in Fig. 8. The best-fit curve of the replot of [GA] *versus* $1/V_{app}$ was parabolic, suggesting that two molecules of GA are binding to each hsp90-chaperone machine complex. This is consistent with the notion that hsp90 is present in the chaperone complex as a dimer (6), if the conformation of each hsp90 present in the dimer is not equivalent. Alternatively, two GA binding sites may be present in each hsp90 monomer. Since no amount of ATP relieves the inhibitory effect of GA, GA and ATP are clearly binding to different sites.

The data suggest that GA acts by inducing changes in the binding or hydrolysis of ATP by the hsp90 chaperone machinery. GA caused a significant reduction in the K_{app} of the hsp90-chaperone machine for ATP from ~60 to ~12 μM. This observation indicates that the binding of GA to the hsp90 chaperone machine complex either decreases the rate of ATP dissociation (e.g., increases the affinity of the hsp90-chaperone machine for ATP), or slows the rate at which ATP is converted to product (e.g., inhibits the rate of ATP hydrolysis). The effects of GA on the K_{app} for ATP and the interaction of p23 with the hsp90-chaperone machine containing bound luciferase are consistent with previous reports that (a) p23 interacts with hsp90 in the presence of either ATP or nonhydrolyzable ATP

analogs; but (b) the presence of hydrolyzable ATP is required for the stable association of p23 with hsp90/progesterone receptor complexes (Johnson & Toft, 1995); and (c) the hsp90 cohort p23 does not interact with the hsp90 chaperone machinery containing bound substrate (SHR) in the presence of GA [(Johnson & Toft, 1995, Smith et al., 1995). & Fig. 6].

Based on the correlation between GA's effects on p23 binding to the hsp90 chaperone machinery and GA's effects on ATP affinity or hydrolysis, we speculate that p23 plays a role in regulating either ATP hydrolysis or nucleotide exchange during the action of the hsp90 chaperone machine. The binding of GA to hsp90 could be directly affecting the ATP binding site that has been proposed to be present on hsp90 (Fig. 8A) (Csermely et al., 1993, Johnson & Toft, 1995, Nadeau et al., 1993) However, since the existence of the hsp90 ATP binding site has recently been questioned (Jacob et al., 1996), the binding of GA to hsp90 may affect the ATP binding site present on some other some component of the hsp90 chaperone machinery, such as hsc70 through allosteric interactions.

Figure 1. Concentration dependence for benzoquinonoid ansamycin-induced inhibition of luciferase renaturation in RRL.

Heme-deficient protein synthesis mixes (100 µl) containing 10 µM edeine were preincubated for 20 min at 30 °C in the presence of 0.1 µl of DMSO (vehicle control), or 0.1µl of DMSO containing varying concentrations of GA(O) or HbA(□). Five µl of denatured luciferase (10 µg/ml) was then diluted into 100 µl of lysate. After 10 min, the amount of luciferase activity present in a 20 µl aliquot of each reaction was measured as described under "Materials and Methods". Light forming units renatured per 10 min of incubation is plotted versus the final concentration of GA or HbA.

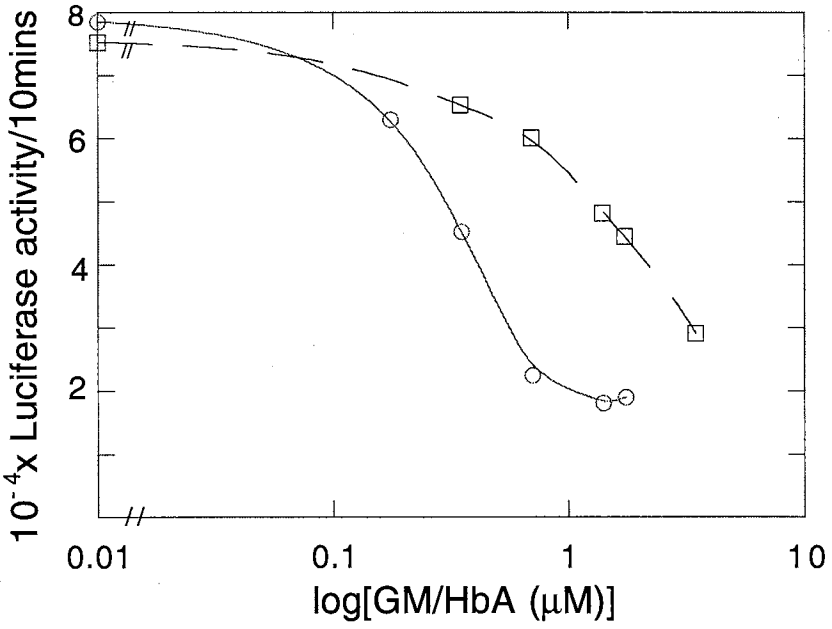
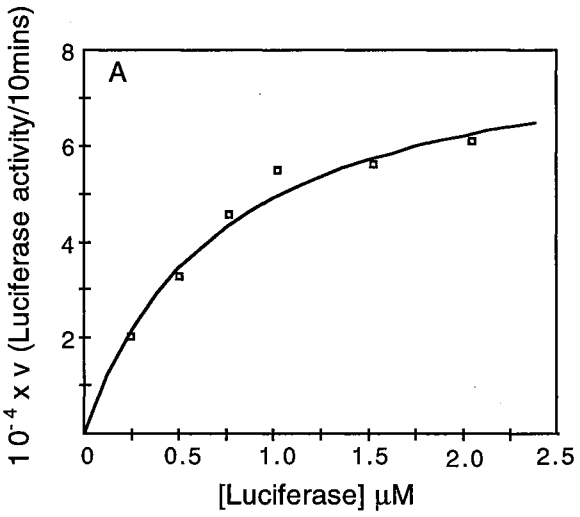

8
6
4
2
10^{-4}x Luciferase activity/10mins
0.01
0.1
1
10
log[GM/HbA (μM)]

Figure 2. Luciferase renaturation shows Michaelis-Menten kinetics in RRL.

(A) The rate of luciferase renaturation was measured at saturating ATP and varying luciferase concentrations in heme-deficient protein synthesis mixes containing 10 μM edeine as described under "Materials and Methods". Light forming units renatured per 10 min of incubation is plotted versus luciferase concentration. (B) The rate of luciferase renaturation was measured at 2.1 μM luciferase and varying ATP concentrations in a dialyzed lysate as described under "Materials and Methods".


A
8
6
4
2
0
0.5
1.0
1.5
2.0
2.5
10^{-4} x v (Luciferase activity/10mins)
[Luciferase] μM

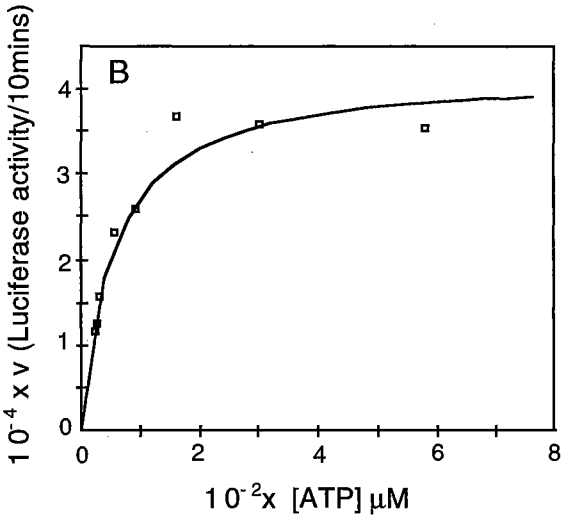

B
4
3
2
1
0
2
4
6
8
10^{-4} x v (Luciferase activity/10mins)
10^{-2}x [ATP] μM

Figure 3. Eadie-Hofstee plot of kinetics of luciferase renaturation.

The rate of luciferase renaturation was measured in dialyzed RRL in the presence of varying ATP and luciferase concentrations as described under "Materials and Methods" and Fig. 2. Concentration of luciferase present in the renaturation assay was 1.03 μM (O), 0.77 μM (◇), 0.51 μM (●), and 0.26 μM (◆), respectively. ATP concentrations were varied between 0.02 and 0.1 mM. The experiment was repeated three times with similar results. Lines were analyzed as described under "Materials and Methods ".

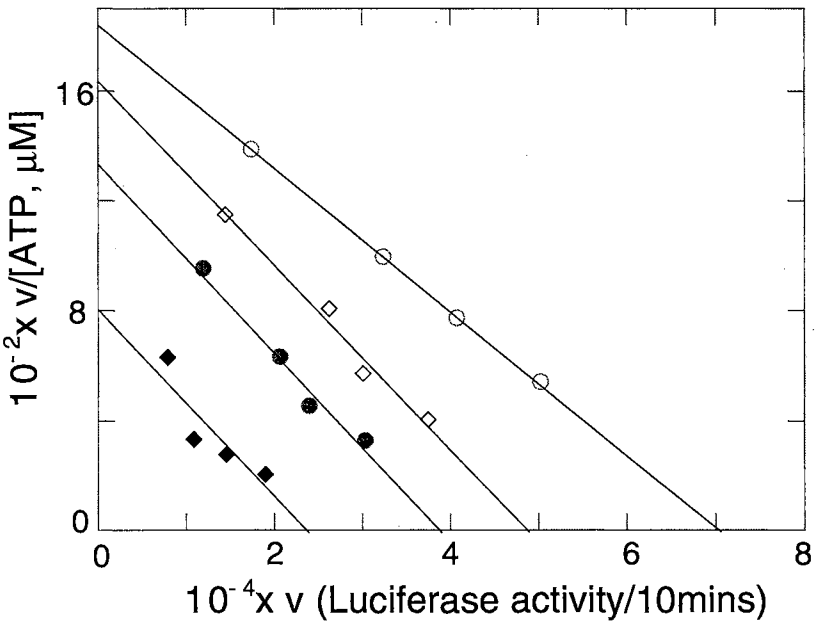
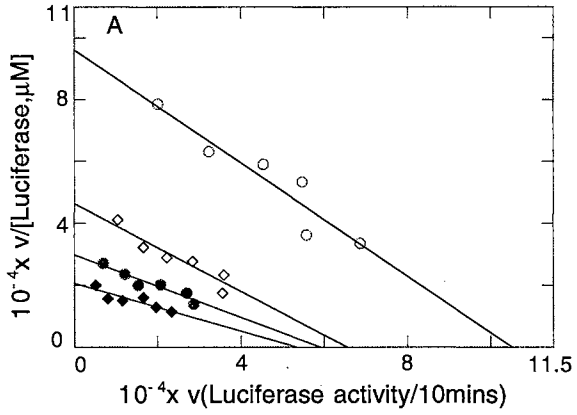
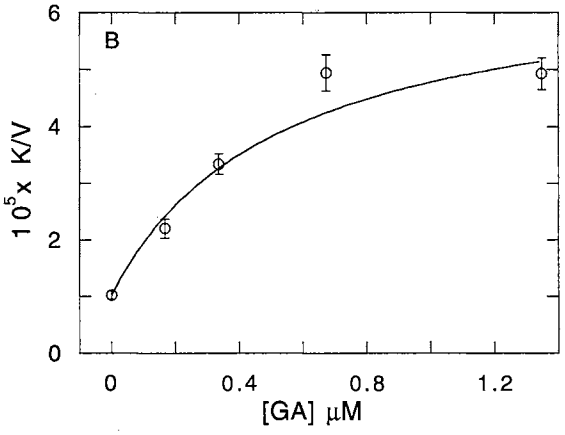

16
8
0
0
2
4
6
8
10-2x v/[ATP, µM]
10-4x v (Luciferase activity/10mins)

Figure 4. Effect of GA on the rate of luciferase renaturation in the presence of saturating ATP and varying luciferase concentrations.

(A) Eadie-Hofstee plot of the kinetics of luciferase renaturation measured at saturating ATP (1.7 mM endogenous) and varying luciferase and GA concentrations. The rate of luciferase renaturation was measured in 100 µl of heme-deficient protein synthesis mixes containing 10 µM edeine and 0.1 µl DMSO (○), or 0.17 µM (◇), 0.34 µM (●), and 0.67 µM (◆) GA as described under "Materials and Methods" and Fig. 2 and 3. (B) Replot of K_{app}/V_{app} versus [GA]. (C) Replot of $1/V_{app}$ versus [GA]. The experiment was repeated five times with similar results. The data were analyzed as described under "Material and Methods". The smooth curves (B & C) represents the best-fit hyperbola. A linear fit can be excluded by a chi-square probability > 0.9995 for the K_{app}/V_{app} curve, and ~0.90 for the $1/V_{app}$ curve.


A
11
8
4
0
0
4
8
11.5
10^{-4}x v/[Luciferase,μM]
10^{-4}x v(Luciferase activity/10mins)


B
6
4
2
0
0
0.4
0.8
1.2
10^5x K/V
[GA] μM

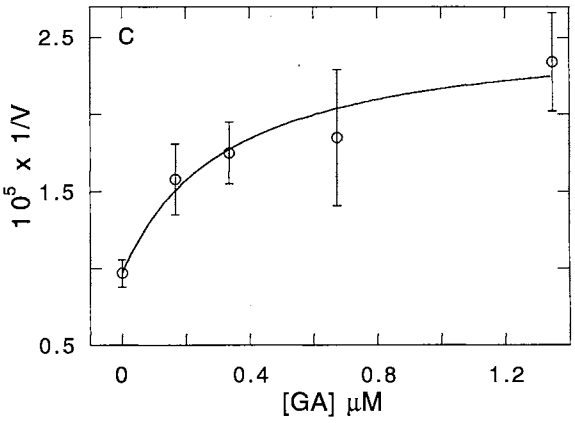
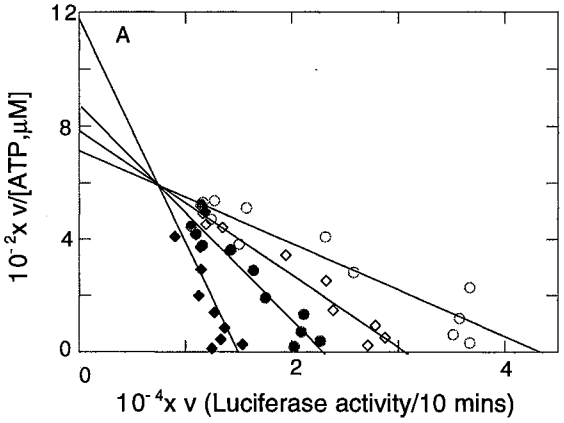
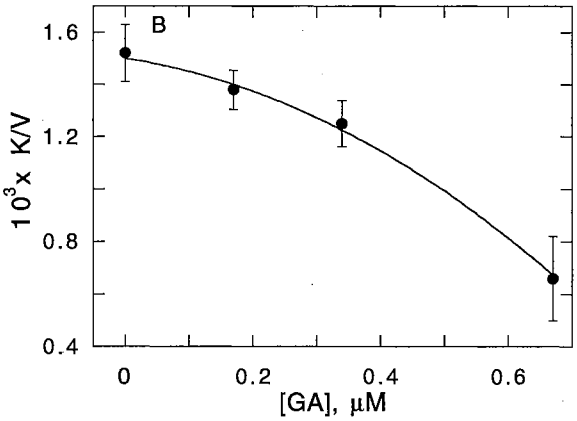

C
2.5
1.5
0.5
0
0.4
0.8
1.2
10^5 x 1/V
[GA] μM

Figure 5. Effect of GA on the rate of luciferase renaturation in the presence of saturating luciferase and varying ATP concentrations.

(A) Eadie-Hofstee plot of the kinetics of luciferase renaturation measured at saturating luciferase and varying ATP and GA concentrations. The rate of luciferase renaturation was measured in 100 µl of dialyzed lysate containing 2.1 µM luciferase and varying ATP concentrations in the presence of 0.1 µl DMSO (O), or 0.17 µM (◇), 0.34 µM (●), and 0.67 µM (◆) GA as described under "Materials and Methods". (B) Replot of K_{app}/V_{app} versus [GA]. (C) Replot of $1/V_{app}$ versus [GA]. The data were analyzed as described under "Material and Methods". The smooth curves (B & C) represents the best-fit parabola.


A
12
8
4
0
0
2
4
10^{-2}x v/[ATP,μM]
10^{-4}x v (Luciferase activity/10 mins)


B
1.6
1.2
0.8
0.4
0
0.2
0.4
0.6
10^{3}x K/V
[GA], μM

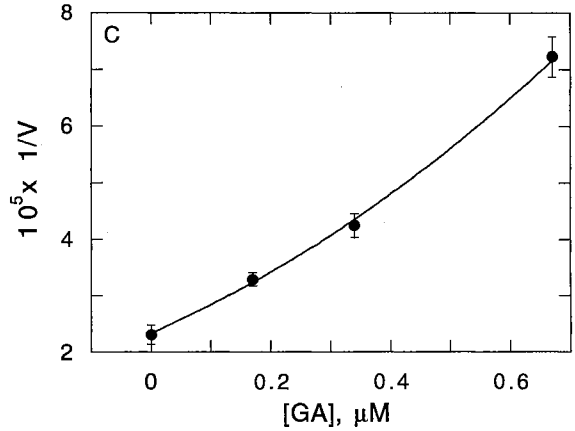

C
8
6
4
2
0
0.2
0.4
0.6
10^{5}x 1/V
[GA], μM

Figure 6. Effect of GA on the interaction of denatured luciferase with heat shock proteins.

Luciferase was adsorbed to affinity-purified anti-luciferase antibody bound to GaR-agarose as described under "Materials and Methods". The immobilized luciferase was incubated for 5 min at 30 °C in heme-deficient protein synthesis mixes in the presence or absence of GA (1μg/ml). A control incubation containing anti-luciferase antibody adsorbed to GaR-agarose, but no luciferase.

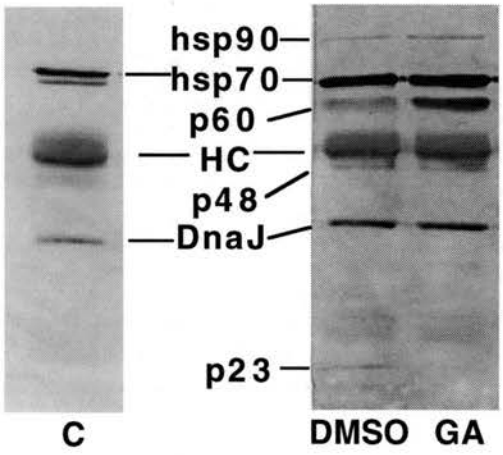

hsp90
hsp70
p60
HC
p48
DnaJ
p23
C
DMSO
GA

Figure 7. Effects of purified hsps and hsp cohorts on GA-induced inhibition of luciferase renaturation in RRL.

Rabbit reticulocyte lysate containing 10 mM creatine phosphate, 20 U/ml creatine phosphokinase, 75 mM KCl, 1 mM $Mg(OAc)_2$ and 1 µl/ml DMSO (closed bars) or 0.7 µM GA (open bars) was supplemented with: buffer (con); 20 µg/ml of p23 (23); 160 µg/ml of hsp90 (90); 80 µg/ml of hsc70 (70); 25 µg/ml of p60 (60); or combinations of these quantities of proteins. Denatured luciferase was diluted 20 fold into the reaction mixes and the

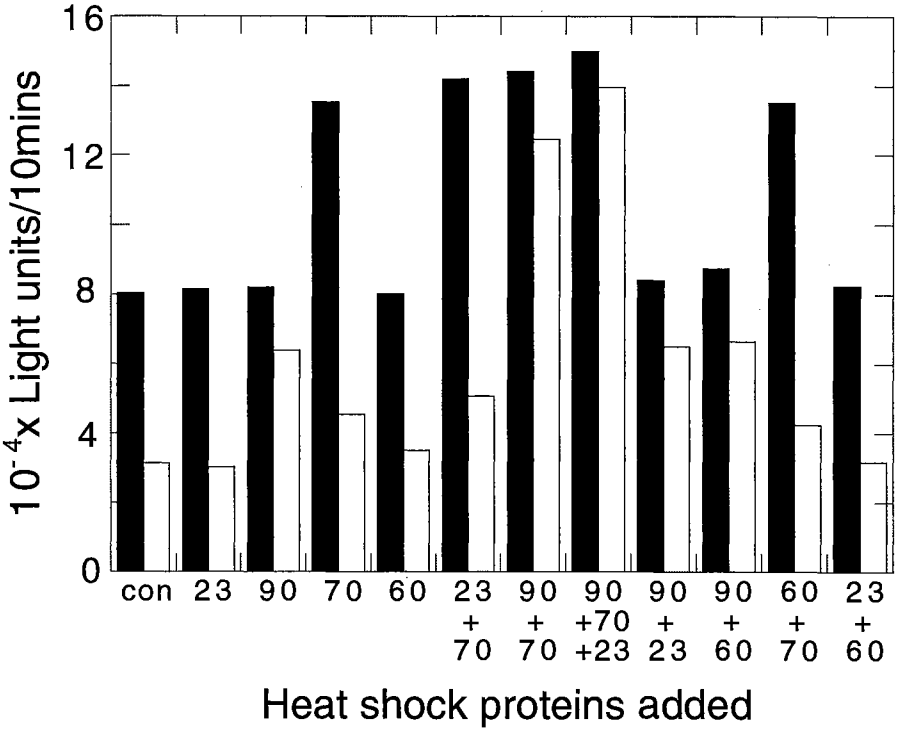
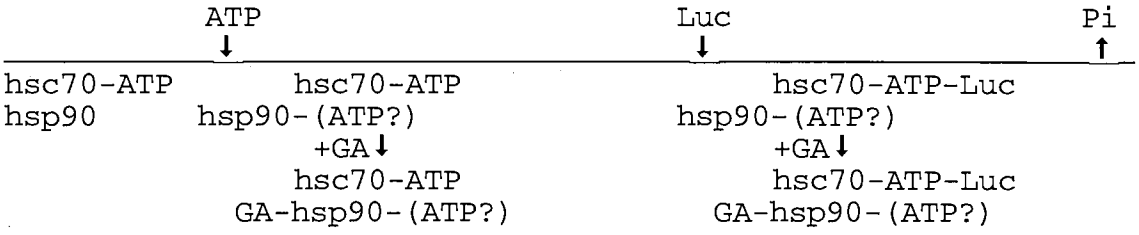

16
12
8
4
0
10^{-4}x Light units/10mins
con
23
90
70
60
23 + 70
90 + 70
90 +70 +23
90 + 23
90 + 60
60 + 70
23 + 60
Heat shock proteins added

Figure 8. Possible schemes for GA-induced inhibition of luciferase renaturation.

(A) A possible reaction sequence for the hsp90 chaperone machinery. The hsp90 chaperone machinery is assumed to be a large complex (foldasome) containing p60, p48, p23 and the immunophilins. Only hsp90 and hsc70 are shown. (B). An alternate scheme in which there is an ATP-dependent assembly of chaperones X (hsp90, p23 and immunophilins) to form complex Y which then adds to the hsp90/hsc70 complex containing p60, p48 and bound luciferase. The ? after ATP associated with hsp90 denotes the fact that the binding of ATP to hsp90 has recently been questioned (Jacob et al., 1996).

A.


ATP
Luc
Pi
hsc70-ATP
hsp90
hsc70-ATP
hsp90-(ATP?)
+GA
hsc70-ATP
GA-hsp90-(ATP?)
hsc70-ATP-Luc
hsp90-(ATP?)
+GA
hsc70-ATP-Luc
GA-hsp90-(ATP?)

B.

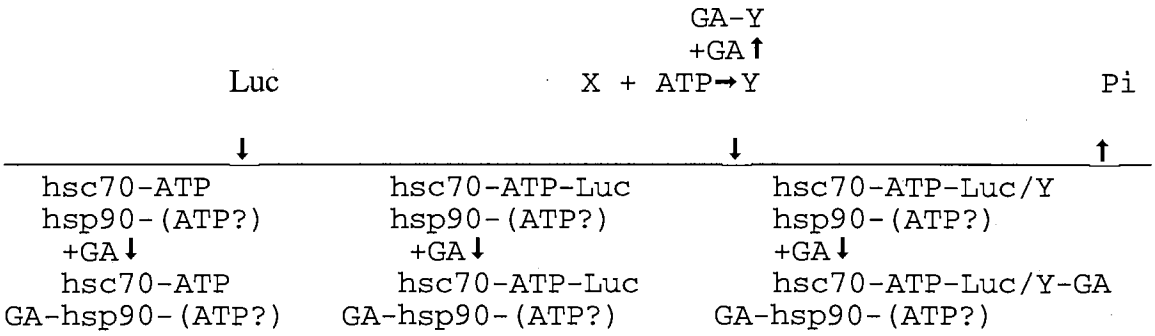

GA-Y
+GA
Luc
X + ATP→Y
Pi
hsc70-ATP
hsp90-(ATP?)
+GA
hsc70-ATP
GA-hsp90-(ATP?)
hsc70-ATP-Luc
hsp90-(ATP?)
+GA
hsc70-ATP-Luc
GA-hsp90-(ATP?)
hsc70-ATP-Luc/Y
hsp90-(ATP?)
+GA
hsc70-ATP-Luc/Y-GA
GA-hsp90-(ATP?)

Table II. The degree of GA-induced inhibition of luciferase renaturation depends on the temperature of denaturation.

Luciferase (0.7 μM), denatured at 38 or 41 °C for 10 min, was diluted 20 fold into heme-deficient protein synthesis mixes supplemented with edeine and containing either GA (1.8 μM) or DMSO (1μl/ml). The amount of luciferase renatured after incubation for 10 min at 28 °C was measured after 10 min as described under "Materials and Methods". The rate of luciferase renaturation is reported as light units renatured/10 min.

Temperature of denaturation	Additions		
	DMSO	GA	
	Light Units Renatured/10min		Ratio (DMSO/GA)
38°C	40176	20505	1.96
41°C	39357	9883	3.98

CHAPTER III

HSP90 AND PROTEIN FOLDING PATHWAYS

Introduction

Organisms have evolved molecular chaperones to facilitate protein folding events occurring in the concentrated environments of the cellular cytosol and organelle interiors [for review see (Gething & Sambrook, 1992)]. Chaperones are thought to interact primarily with hydrophobic regions of protein substrates to prevent illicit interactions that would lead to protein aggregation [see (Ellis, 1993)]. Additionally, certain members of chaperone families can catalyze the isomerization of peptidyl-prolyl bonds or of disulfide bonds [see (Gething & Sambrook, 1992)]. Chaperones are thought to both facilitate the *de novo* folding of newly synthesized proteins and to allow renaturation of partially denatured proteins [see (Becker & Craig, 1994, Hartl, 1996)]. Although chaperones participate in both *de novo* folding and protein renaturation, the relationships between these two folding processes are poorly understood.

The biogenesis of certain kinases appears to require one or more members of the 90-kDa family of heat-inducible phosphoproteins (hsp90). Newly synthesized molecules of the viral tyrosine kinase $p60^{src}$ occur in cytoplasmic complexes with hsp90 and the p50 cohort of hsp90 (Brugge et al., 1983, Courtneidge & Bishop, 1982). Viral $p60^{src}$ molecules within this complex are deficient in kinase activity and are hypophosphorylated. During subsequent maturation, these kinase molecules dissociate from hsp90 and p50, acquire greater specific activity and further posttranslational phosphorylations, and

associate with the plasma membrane [for review, see (Brugge, 1986)]. Hsp90 thus plays an essential positive role in viral $p60^{src}$ function (Xu & Lindquist, 1993). Consistent with hsp90's role in the biogenesis of viral $p60^{src}$, modeling of protein biogenesis in rabbit reticulocyte lysate (RRL) indicates that hsp90-bound intermediates exist for most if not all molecules of the cellular tyrosine kinase $p56^{lck}$ (Hartson et al., 1996). These interactions are necessary for $p56^{lck}$ molecules to demonstrate protease-resistant conformations (Hartson et al., 1996). More recently, co-translational interactions between hsp90 and the heme-regulated eIF-2α kinase have been observed during the hsp90-dependent maturation of this kinase (S. Uma, S. Hartson, J.-J. Chen, and R. L. Matts, in preparation). Thus, chaperone machinery containing hsp90 appears to be necessary for the *de novo* folding of certain kinases.

In addition to its apparent role in *de novo* folding of these kinases, hsp90 also maintains active protein conformations. In the RRL model of chaperone function, reiterative interactions with chaperone machinery containing hsp90 are required to maintain certain steroid hormone receptors in conformations that are competent to bind hormone (Scherrer et al., 1990, Smith, 1993, Smith et al., 1990a). Consistent with this model, treatment of whole cells with the hsp90-binding drug geldanamycin causes glucocorticoid receptors to rapidly lose hormone binding competence and accelerates turnover of glucocorticoid receptor molecules (Whitesell & Cook, 1996). Hsp90 may similarly maintain the structure of other regulatory proteins [e.g. see (Blagosklonny et al., 1995, Hartson et al., 1996, Mimnaugh et al., 1996, Stancato et al., 1993, Stepanova et al., 1996, Wartmann & Davis, 1994)]. Also consistent with this role in maintenance of protein structure, the active conformation of heat-denatured firefly luciferase can be restored via the ATP-dependent action of hsp90 and other chaperones present in RRL (Schumacher et al., 1994, Thulasiraman & Matts, 1996).

Although hsp90 facilitates the efficient renaturation of firefly luciferase, physical interactions between hsp90 and newly synthesized molecules of luciferase have not been

observed (Frydman et al., 1994, Hartson & Matts, 1994). These results imply that hsp90 may be involved in some, but not other, *de novo* folding pathways. In this report, we have utilized three chaperone antagonists, geldanamycin, clofibric acid, and denatured protein [reduced carboxymethylated-BSA (RCM-BSA)] to dissect the pathways for chaperone-mediated *de novo* folding and for chaperone-mediated maintenance of protein function in RRL. Data thus obtained indicate that: (1) distinct chaperone-mediated pathways for *de novo* folding can exist for unrelated proteins; (2) efficient protein renaturation may utilize chaperone machinery that is not involved in *de novo* protein folding pathways; and (3) for a given protein, pathways for chaperone-mediated maintenance or renaturation may differ in conjunction with the degree of denaturation. We also present data demonstrating that the hsp90 inhibitor geldanamycin changes the mode by which hsp90 binds to its substrates.

Experimental Procedures

Materials

Purified crystalline firefly (*Photinus pyralis*) luciferase was obtained from Sigma. Plasmids bearing the full cDNA coding sequence of firefly luciferase or of $p56^{lck}$ were constructed to allow efficient synthesis of these proteins in RRL (Hartson & Matts, 1994). RRL for protein synthesis reactions was obtained from Promega Corporation (TNT nuclease-treated RRL kits). RRL used to study the maintenance of active conformations of purified luciferase was prepared as previously described (Matts et al., 1991). Geldanamycin (obtained from the Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute) was prepared as a 1.0 mg/ml stock in DMSO. Clofibric acid (Sigma) was prepared as a 0.15 M stock in water. RCM-BSA was prepared as previously described (Matts et al., 1993).

De novo synthesis and folding in RRL.

Firefly luciferase and p56^{lck} were synthesized following the manufacturer's suggestions. Protein synthesis was assessed by determination of [^{35}S]Met incorporation into TCA-insoluble protein. The folding of p56^{lck} was assessed as described previously (Hartson et al., 1996) via protease nicking assays of native [^{35}S]kinase molecules followed by SDS-PAGE and autoradiography. Luciferase folding was assayed via determination of luciferase activity present in aliquots of protein synthesis reactions as described elsewhere (Thulasiraman & Matts, 1996). The specific enzymatic activity of luciferase was calculated assuming an endogenous methionine content of 3.5 μM for rabbit reticulocyte lysate. The effects of individual chaperone antagonists were assessed in translations synchronized using one of two approaches described below.

Geldanamycin and reduced carboxymethylated-bovine serum albumin (RCM-BSA) as antagonists of *de novo* protein folding.

Although geldanamycin had no effect on protein synthesis at levels used in work described here, RCM-BSA inhibited re-initiation of protein synthesis via activation of the heme-regulated inhibitor of protein synthesis (Matts et al., 1993). Neither compound affected the rates of luciferase elongation during protein synthesis. Thus, to assay the effects of these compounds on luciferase folding, luciferase was first synthesized at 30 °C for 6 min in the absence of drug treatment. Following this loading of polyribosomes, a mixture of two inhibitors of translation initiation, edeineA (20 μM final concentration) and aurin tricarboxylic acid (ATA, 130 μM final concentration) were added. After addition of initiation inhibitors and chaperone antagonists, polyribosomes were allowed to run off and rates of luciferase's acquisition of activity (folding) were assayed. The chaperone antagonists RCM-BSA (44 μM final concentration), geldanamycin (5 μg/ml final), or the

appropriate drug vehicle control were added prior to onset of protein synthesis, after arrest of initiation of protein synthesis, or after polyribosome runoff, as indicated.

Clofibric acid as an antagonist of luciferase *de novo* folding.

Because clofibric acid inhibited polypeptide elongation during protein synthesis, polyribosomes programmed with luciferase message were first loaded for 10 min as described above and then ATA was added (130 μM final concentration) to arrest reinitiation of protein synthesis. Polyribosomes were then allowed to run off for 6 min, clofibric acid (15 mM final concentration) was then added as indicated, and rates of luciferase folding after translation were assayed as described above.

Assays of chaperone maintenance of luciferase structure in RRL.

Luciferase stock (0.1mg/ml) was diluted 300-fold into heme-deficient protein synthesis mix containing DMSO (0.5 %), geldanamycin/DMSO (5 μg/ml and 0.5 %, respectively), or clofibric acid (15mM). These reactions were incubated at either 37 °C or 42 °C as indicated. Luciferase activity present in 1.5 μl of lysate was measured following incubation for 1 hr. For assessment of ATP-dependence, the ATP-regenerating system (creatine kinase and phosphocreatine) normally added to protein synthesis reactions was omitted.

Assays of chaperone maintenance of $p56^{lck}$ structure in RRL.

To assess maintenance of $p56^{lck}$ protease resistance by geldanamycin-inhibitable hsp90 function, $[^{35}S]p56^{lck}$ was synthesized at 30 °C in the absence of geldanamycin, synthesis was arrested via treatment with ATA, and $[^{35}S]p56^{lck}$ molecules allowed to

mature for 1 hr at 30 °C. Subsequently, geldanamycin and/or DMSO vehicle were added to 5 μg/ml and 0.5% final concentrations, respectively and the reactions incubated for an additional hour at 30 °C. Following incubation, resistance to protease nicking was assayed as previously described (Hartson et al., 1996, Hartson & Matts, 1994).

Effects of geldanamycin on the hsp90-p56^{lck} complex.

p56^{lck} was synthesized for 30 min in RRL containing geldanamycin and/or DMSO vehicle (5 μg/ml and 0.5% final concentrations, respectively). Complexes between p56^{lck} and hsp90 were co-immunoadsorbed with rabbit anti-p56^{lck} antibodies (Amrein et al., 1992) as previously described (Hartson et al., 1996, Hartson & Matts, 1994). Immunoadsorptions from 70 μl of protein synthesis reaction were washed twice with 10 mM TrisHCl (pH=7.4), once with either 10 mM TrisHCl pH7.4 or with 10 mM TrisHCl pH7.4, 0.5 M NaCl, and finally with 10 mM TrisHCl pH7.4. Immunoadsorbed proteins were analyzed by western blotting with anti-hsp90 antiserum [rabbit 4322 antiserum at 1:2000 (Ehrhart et al., 1988) and rabbit antibodies directed against the N-terminus of mouse hsp86 at 0.5 μg/ml (Affinity BioReagents)]. Immunoreactive materials (rabbit anti-hsp90 and heavy chain of immunoadsorbing rabbit anti-p56^{lck} antibodies) were visualized with alkaline phosphatase-conjugated anti-rabbit IgG as previously described (Matts et al., 1992).

Results

The nature of geldanamycin-sensitive binding of hsp90 to substrates.

The effects of geldanamycin on hsp90-substrate interactions appears to vary among published studies. To address these apparent discrepancies regarding the effects of

geldanamycin on hsp90-substrate complexes, we examined the various experimental protocols that have been used to assess interactions between hsp90 and substrate proteins. We observed that the ionic strength used for the co-immunoadsorption of hsp90 complexes varied among studies.

Therefore, we examined the effect of salt concentration on the hsp90-p56^{lck} complex produced in RRL in the presence or absence of geldanamycin. As previously described (Hartson et al., 1996, Hartson & Matts, 1994), complexes containing hsp90 were co-immunoadsorbed by anti-p56^{lck} antibodies in an p56^{lck} -specific fashion (Figure 9). These complexes could be detected following washing under either low or high ionic-strength conditions (Figure 9, lane 3 and lane 4, respectively). However, in translation reactions treated with 5 μg/ml geldanamycin, interactions between hsp90 and p56^{lck} were no longer detected following washing with 0.5 M NaCl (Figure 9, lane 6). In contrast, some interaction between hsp90 and p56^{lck} could still be detected when hsp90-p56^{lck} complexes from geldanamycin-treated translations were washed in low-salt buffer (Figure 9, lane 5). Thus, geldanamycin disrupted the normally salt-stable interaction between hsp90 and p56^{lck}, but did not prevent the detection of interactions between these proteins in the absence of high-ionic-strength washes.

Geldanamycin inhibits the *de novo* folding of the p56^{lck} tyrosine kinase.

Previous work in the RRL model system has shown that the hsp90-binding drug geldanamycin inhibits the hsp90-dependent folding of p56^{lck}, resulting in the failure of the kinase to demonstrate a protease-resistant conformation in subsequent nicking assays of native p56^{lck} structure (Hartson et al., 1996). Inhibition of hsp90 function by geldanamycin could have either: (1) prevented the kinase from acquiring a protease-resistant conformation; (2) prevented the kinase from maintaining a protease-resistant conformation; or (3) both 1 and 2 above. In this previous study, pulse-chase analyses of

p56^{lck} biogenesis indicated that hsp90-bound intermediates existed for most if not all p56^{lck} molecules synthesized in RRL (Hartson et al., 1996). Thus, we concluded that hsp90 participated in the *de novo* folding of p56^{lck}.

Protease nicking assays of [^{35}S]p56^{lck} molecules produced in RRL were used to confirm the involvement of hsp90 in *de novo* folding of p56^{lck}. As previously reported, [^{35}S]p56^{lck} produced and matured for 1 hr in geldanamycin-treated RRL was dramatically hypersensitive to proteolysis relative to the kinase produced in the absence of this drug (Figure 10, upper panel). To confirm that this hypersensitivity reflected a role for hsp90 in the *de novo* folding of p56^{lck}, we tested the counter hypothesis, that an ongoing requirement for geldanamycin-inhibitable hsp90 was necessary to maintain p56^{lck} in a protease-resistant conformation. To test this counter hypothesis, [^{35}S]p56^{lck} was synthesized and allowed to mature to a protease-resistant conformation in the absence of geldanamycin. Following this maturation, hsp90 function was inhibited by addition of geldanamycin and the reaction was further incubated in the presence of geldanamycin for 1 hr prior to proteolytic fingerprinting of [^{35}S]p56^{lck}. The fingerprint of the kinase thus produced was indistinguishable from that of the kinase produced, matured, and incubated in the absence of geldanamycin (Figure 10, lower panel). Thus, newly synthesized p56^{lck} molecules required geldanamycin-inhibitable hsp90 function to achieve protease-resistant conformations, while older p56^{lck} molecules did not demonstrate a similar requirement for maintenance of protease resistance. These results indicated that the counter hypothesis was false: the protease sensitivity of the kinase produced in the presence of geldanamycin did not result from inhibition of hsp90's maintenance of protease resistance. Instead, this result clearly demonstrated a role for geldanamycin-inhibitable hsp90-function in the *de novo* folding of p56^{lck}.

Geldanamycin does not inhibit the *de novo* folding of luciferase.

Previous studies have failed to demonstrate stable physical interactions between hsp90 and nascent or newly synthesized firefly luciferase (Frydman et al., 1994, Hartson & Matts, 1994). Failure to detect such interactions could have two explanations: (1) complexes between hsp90 and luciferase are inherently more difficult to detect than those formed between hsp90 and certain other proteins; or (2) hsp90 does not participate in the *de novo* folding of luciferase. To discriminate between these two possibilities, we examined the effect of geldanamycin on the *de novo* folding of luciferase into an enzymatically active conformation. Although luciferase synthesis began at approximately 8 min following assembly of the protein synthesis reaction, accumulation of luciferase activity did not begin until approximately 16 min following assembly of the protein synthesis reaction (Figure 11). This lag reflected the time required for chaperone-mediated luciferase folding (Frydman et al., 1994). In the presence of concentrations of geldanamycin that inhibit the *de novo* folding of $p56^{lck}$, neither the rate of luciferase synthesis (not shown), the rate of luciferase acquisition of enzymatic activity (not shown), nor the specific enzymatic activity of luciferase thus produced were inhibited (Figure 11). This concentration of geldanamycin (5 μg/ml) is more than an order of magnitude greater than that which maximally inhibits the renaturation of heat-denatured luciferase (Thulasiraman & Matts, 1996). Thus, geldanamycin's failure to inhibit *de novo* folding of luciferase occurred at concentrations of geldanamycin which maximally inhibited hsp90 function. Additionally, equivalent results were obtained when RRL was treated with geldanamycin (5 μg/ml) prior to the onset of luciferase synthesis. Thus, geldanamycin-inhibitable hsp90-function was not necessary for the efficient *de novo* folding of firefly luciferase.

The *de novo* folding of newly synthesized luciferase requires hsc70 function.

Geldanamycin had no effect on the folding of newly synthesized luciferase in RRL (Figure 11). Thus, the effects of this drug on the folding of $p56^{lck}$ (Figure 10) did not appear to reflect indirect global inhibition of chaperone function in RRL. However, this conclusion assumed that such assays would detect inhibition of chaperone functions essential to the *de novo* folding of luciferase. To ensure that these assays would detect such inhibition, titration of hsc70 function was assessed for its effects on *de novo* folding of luciferase. Hsc70 binds tightly to RCM-BSA, thus blocking or competing for the interaction of hsc70 with other substrates in RRL (Matts et al., 1993). Titration of hsc70 function by the addition of RCM-BSA caused an approximately 5 min delay in the *de novo* folding of luciferase (Figure 12A), indicating that efficient *de novo* folding of luciferase required hsc70 function.

Inhibition of hsc70 function was also examined using clofibric acid. Clofibric acid specifically binds to hsc70 at or near the ATP-binding site (Alvares et al., 1990). Such binding inhibits the interaction of hsc70 with heat-denatured luciferase (V. Thulasiraman and R.L. Matts, in preparation). In control assays, clofibric acid had no direct effect on luciferase activity when added directly to luciferase assays or when RRL reactions containing luciferase were incubated at 30 °C for 10 min (not shown). However, clofibric acid completely inhibited the *de novo* folding of luciferase (Figure 12B). These results confirmed that: (1) assays of *de novo* folding of luciferase were sensitive to inhibition of hsc70 function; (2) *de novo* folding of luciferase required active chaperone machinery, as has been previously demonstrated (Frydman et al., 1994); and (3) the inhibition of $p56^{lck}$ folding by geldanamycin resulted from the direct inhibition of hsp90's role in folding of $p56^{lck}$ rather than a global disruption of chaperone machinery present in RRL.

Chaperones provide ongoing maintenance of protein structure and function in rabbit reticulocyte lysate.

Under pseudo-physiological conditions, geldanamycin-inhibitable hsp90 function is necessary to efficiently maintain the activity of $p56^{lck}$ (Hartson et al., 1996). In contrast to inhibition of *de novo* folding, this failure to maintain function was not accompanied by gross defects in kinase structure as assessed by protease fingerprinting (Figure 10; Hartson and Matts, unpublished). To determine if cellular chaperone machinery might similarly support the function of luciferase at 37 °C, the maintenance of luciferase activity at this temperature was assessed for: (1) ATP dependence; (2) inhibition by the hsc70-binding drug clofibric acid; and (3) inhibition by the hsp90-binding drug geldanamycin.

Normally, RRL is supplemented with an ATP-regenerating system (creatine kinase and phosphocreatine) to maintain ATP concentrations at levels necessary to support protein synthesis and chaperone function. However, when endogenous ATP present in RRL was not regenerated via this ATP regenerating system, the activity of purified luciferase incubated in RRL declined by 43% in the course of one hr (Figure 13). Thus, the efficient maintenance of luciferase in an active conformation required ATP-dependent events.

To determine if this ATP-dependent maintenance function required hsc70, the effect of clofibric acid on luciferase maintenance was determined. At 37 °C, the ATP-dependent maintenance of luciferase in an active conformation was inhibited by 96% in the presence of 15 mM clofibric acid (Figure 13). Thus, efficient maintenance of luciferase activity required clofibrate-inhibitable hsc70 function. These results indicated that thermal denaturation of luciferase occurred in RRL at 37 °C and that a rapid ATP-dependent and hsc70-dependent event maintained the activity of the luciferase under these conditions.

The degree to which hsp90-chaperone machinery was required to maintain luciferase molecules in active conformations was a function of temperature. Geldanamycin caused minor losses in luciferase activity at 37 °C (23% inhibition; Figure 13). Thus, at this temperature, efficient maintenance of luciferase was somewhat dependent on geldanamycin-inhibitable hsp90. However, at 42 °C geldanamycin caused a 86% loss of the specific enzymatic activity of luciferase (Figure 14). Similar temperature-dependent

results have been obtained when purified luciferase is denatured at these temperatures in buffer and subsequently renatured in ATP-supplemented RRL (Thulasiraman & Matts, 1996).

Discussion

Effects of geldanamycin on interactions between hsp90 and substrates.

The effects of geldanamycin on hsp90-substrate interactions appears to vary among published studies. Geldanamycin inhibition of hsp90 function has been reported to disrupt hsp90's interaction with several "substrate" proteins *in vivo* [e.g. viral p60^{src} (Whitesell et al., 1994), *raf* (Schulte et al., 1995), and p185^{erbB2} (Chavany et al., 1996)]. Similar disruption of hsp90-substrate complexes can be modeled in the RRL model system (Hartson et al., 1996). In contrast, during RRL-mediated reconstitution of heteromeric complexes between hsp90 machinery and steroid hormone receptors, geldanamycin causes the accumulation of progesterone hormone receptors in intermediate complexes containing hsp90 and the hsp90-associated chaperones hsc70 and p60 (Johnson & Toft, 1995, Smith et al., 1995). Similarly, interactions between hsp90 and glucocorticoid receptor have been reported following treatment of whole cells with geldanamycin (Whitesell & Cook, 1996). Additionally, we have found that during renaturation assays in geldanamycin-treated RRL, heat-denatured luciferase accumulates in complexes containing hsp90, hsc70, and p60 (Thulasiraman & Matts, 1996).

However, the conditions under which complexes between hsp90 and its substrates are analyzed vary among studies. In the absence of geldanamycin, hsp90-mediated folding of p56^{lck} is accompanied by a tight salt-stable interaction between hsp90 and its protein substrate (Figure 9). Similarly, the complex formed between hsp90 and viral p60^{src} is salt-stable [see (Brugge, 1986)]. Although geldanamycin renders this normally salt-stable association salt-labile, weak interactions between hsp90 and p56^{lck} can still be detected

under conditions of low ionic strength (Figure 9). Thus, geldanamycin-induced disruption of physical associations between hsp90 and its substrate are only apparent when such associations are analyzed under moderate to high ionic strength conditions. In contrast to the normally salt-stable interaction of hsp90 with tyrosine kinases, complexes between hsp90 and steroid hormone receptors are reported to be salt-labile, and are thus typically analyzed under conditions of low ionic strength. Under these conditions, geldanamycin-induced disruption of hsp90-binding to substrate would not be predicted to be readily apparent.

The observation that normal interactions between hsp90 and tyrosine kinases are stable under conditions of high ionic strength suggests that hsp90 binds directly to these kinases via hydrophobic interactions. Thus, certain hsp90-mediated folding events may involve salt-stable hydrophobic binding that does not occur following geldanamycin treatment. However, it does not necessarily follow that all hsp90-mediated folding events are accompanied by similar salt-stable complexes.

Geldanamycin-induced changes in the mode by which hsp90 binds to substrate are consistent with a model for geldanamycin-mediated inhibition of hsp90. Although the exact mechanism of geldanamycin action is poorly understood, kinetic analysis in RRL demonstrates that geldanamycin inhibits hsp90 noncompetitively with respect to substrate during renaturation of heat-denatured luciferase (Thulasiraman & Matts, 1996). Thus, although geldanamycin prevents the normally salt-stable interaction of hsp90 with p56^{lck}, disruption of this interaction probably does not result from direct competition between geldanamycin and p56^{lck} for a binding site on hsp90. Rather, we speculate that geldanamycin inhibits hsp90 by inhibiting the ATP-dependent switching of hsp90 to a hydrophobic "slow-off" conformation. Kinetic analysis of the effects of geldanamycin on hsp90-mediated luciferase renaturation indicates that geldanamycin either increases the binding affinity of hsp90 machinery for ATP or slows the rate of ATP hydrolysis (Thulasiraman & Matts, 1996). ATP has been shown to alter the conformations of hsp90

(Csermely et al., 1993) and other chaperones. For the chaperones hsc70, DnaK, and GroEL/ES, the ATP-bound form of the chaperone is the conformation from which substrates rapidly bind and rapidly dissociate; following ATP hydrolysis, the chaperones switch to a conformation that allows tight hydrophobic binding of the substrate [for review, see (Hartl, 1996) and references therein]. Additionally, inhibition of hsp90 by geldanamycin is correlated to the absence of the hsp90 cohort p23 from heteromeric hsp90 chaperone machinery (Johnson & Toft, 1995, Smith et al., 1995, Thulasiraman & Matts, 1996). Together, these observations suggest that p23 may regulate ATP hydrolysis or exchange during folding events mediated by hsp90 machinery (Thulasiraman & Matts, 1996), and thus, changes in the conformation of hsp90 that allow tight hydrophobic binding to substrates (Figure 9).

Following geldanamycin treatment, hsp90 interacts with $p56^{lck}$ via weak electrostatic forces (salt-labile ionic interactions). These weak interactions may be indirect. This speculation postulates that in the presence of geldanamycin, hsp90 is not associated with the substrate per se, but is instead electrostatically associated with other chaperone machinery directly bound to the substrate. Thus, in the presence of geldanamycin, hsp90 might co-purify with folding intermediates due to these indirect interactions. Consistent with this speculation, the association of hsp90 with certain of its cohorts (e.g. immunophilins) does not require concomitant binding to partially folded "substrates" [for review, see (Pratt & Welsh, 1994a)] and many of these interactions are not inhibited by geldanamycin (Smith et al., 1995).

Hsp90 and protein folding pathways.

Regardless of the exact mechanism by which geldanamycin inhibits certain hsp90 functions, the data presented in this report demonstrate three significant features of chaperone-mediated protein folding in the RRL model of the eukaryotic cytosol: (1) the

specific chaperone activities utilized during *de novo* folding pathways may differ among different proteins; (2) for a given protein, pathways of chaperone-mediated *de novo* folding may differ from those utilized for renaturation folding; and (3) for a given protein, pathways for chaperone-mediated maintenance or renaturation may differ in conjunction with the degree of heat denaturation.

Our conclusion that specific chaperone activities utilized during *de novo* folding pathways may differ among different proteins is based on pharmacological characterization of the *de novo* folding pathways of the mammalian cellular tyrosine kinase $p56^{lck}$ and the firefly enzyme luciferase. Three lines of evidence indicate that hsp90 is involved in the *de novo* folding of $p56^{lck}$: (1) newly synthesized molecules of $p56^{lck}$ interact with hsp90 within 3 min of their synthesis at 30 °C in RRL, with the proportion of $p56^{lck}$ molecules interacting with hsp90 declining during subsequent chase incubations (Hartson et al., 1996); (2) disruption of this normally salt-stable interaction by geldanamycin prevents the kinase from acquiring a protease-resistant conformation, but does not cause a loss of protease resistance once such resistance is acquired (Figure 10); and (3) the mechanism of geldanamycin inhibition of $p56^{lck}$ folding does not involve the global or indirect inhibition of other chaperone functions, since geldanamycin does not inhibit luciferase's acquisition of an active conformation (Figure 11). Thus, we conclude that geldanamycin-inhibitable hsp90 function is necessary for the *de novo* folding of $p56^{lck}$ molecules. Consistent with this conclusion, complexes between hsp90 and $p56^{lck}$ are detected in cytoplasmic, but not membrane, fractions of LSTRA cells (Hartson et al., 1996). Also consistent with this conclusion, the *de novo* folding of the heme-regulated eIF-2α kinase involves the co-translational association of hsp90 with nascent kinase molecules (S. Uma, S. Hartson, J.-J. Chen, and R.L.Matts, in preparation). However, in contrast to geldanamycin's inhibition of the *de novo* folding of $p56^{lck}$, geldanamycin has no effects on the rate nor magnitude of the *de novo* folding of firefly luciferase (Figure 11). Thus, geldanamycin-inhibitable hsp90 function is not essential for the efficient *de novo* folding of luciferase.

Together, these data indicate that the chaperone-mediated *de novo* folding pathways of two unrelated proteins differ in the involvement of geldanamycin-inhibitable hsp90 function. By extension, chaperone-mediated folding pathways may differ among other proteins or protein families. This possibility is of interest in light of the complex nature of chaperone machinery, including apparent differences in substrate specificity, the various homologs reported for individual members of conserved chaperone families, and the observation that many chaperones are phosphorylated with unknown consequences.

Pharmacological characterizations presented here also indicate that for a given protein, pathways of *de novo* folding may differ from those utilized for renaturation folding. This conclusion is based on three lines of evidence obtained following geldanamycin treatment of ATP-supplemented RRL: (1) geldanamycin-inhibitable hsp90 function is not necessary for the *de novo* folding of firefly luciferase (Figure 11); (2) geldanamycin-inhibitable hsp90 function is necessary for the efficient renaturation when heat-denatured luciferase is renatured in RRL, although alternative less-efficient pathways of folding can function to renature luciferase in the absence of geldanamycin-inhibitable hsp90 function (Thulasiraman & Matts, 1996); and (3) ongoing support of luciferase structure at heat-shock temperatures is most efficient when geldanamycin-inhibitable hsp90 function is present (Figure 14). Thus, the renaturation of heat-denatured luciferase populations can occur most efficiently via a chaperone-mediated protein folding pathway that does not normally function during the *de novo* folding of this protein, namely, a renaturation pathway utilizing geldanamycin-inhibitable hsp90 function. Similarly, proteins denatured by exposure to concentrated guanidinium-HCl refold by chaperoned-mediated mechanisms that differ somewhat from those utilized during *de novo* folding (Frydman & Hartl, 1996). These results imply that during heat shock *in vivo*, denatured proteins can renature via chaperone-mediated processes that are distinct from those utilized during *de novo* folding. Thus, renaturation of protein structure is not necessarily the simple recapitulation of *de novo* folding.

Our results also indicate that at physiological temperatures, given protein populations may experience ongoing support of structure and function via rapid transient reiterative cycles of chaperone mediated folding. At 37 °C, luciferase requires ongoing chaperone support, as evidenced by the loss of luciferase activity observed upon treatment with the hsc70 binding drug clofibric acid or by similar losses observed in ATP-deficient RRL (Figure 13). At this temperature, geldanamycin-inhibitable hsp90 function makes minor contributions to the efficient maintenance of luciferase activity. For $p56^{lck}$F505 at 37 °C (Hartson et al., 1996) or for firefly luciferase at 42 °C (Figure 14), geldanamycin-inhibitable hsp90 function makes major contributions to the maintenance of the active conformations of these proteins in RRL. These results demonstrate that an active protein population may require ongoing chaperone support that only becomes evident when chaperone machinery is inhibited. Thus, although a protein population as a whole may show neither defects in structure nor defects in function, individual molecules within this population may be experiencing chaperone-mediated folding at any given moment. We speculate that cryptic chaperone support becomes more evident when certain mutations compromise the kinetics of *de novo* folding or the inherent thermodynamic stability of a protein; this possibility may be relevant to riddles regarding differences in the detection of interactions between hsp90-chaperone machinery and cellular versus viral $p60^{src}$ [see (Brugge, 1986)] and wild-type versus mutant p53 (Blagosklonny et al., 1996, Blagosklonny et al., 1995, Hainaut & Milner, 1992, Sepehrnia et al., 1996).

Geldanamycin-inhibitable hsp90 function is not intrinsic to all chaperone-mediated folding pathways occurring in RRL for thermally denatured luciferase (Thulasiraman & Matts, 1996). Rather, the degree to which hsp90 is involved in the renaturation pathway of luciferase is dependent on the temperature at which luciferase is denatured (Figure 13 and 14). Thus, hsp90 machinery may recognize a specific temperature-dependent protein folding defect or may recognize structures with significant perturbations. Regulatory

proteins whose function is supported by hsp90 may share with heat-denatured luciferase either these specific folding defects or degrees of misfolding.

Why does $p56^{lck}$ require geldanamycin-inhibitable hsp90 machinery under non-heat-shock conditions? The obligate role for hsp90 in *de novo* folding of $p56^{lck}$, the heme-regulated eIF-2α kinase, and perhaps other signal transduction proteins suggests that for these proteins, folding defects are inherent to their *de novo* folding pathways. Additionally, hsp90 machinery appears to supply ongoing maintenance of protein structure for these and other proteins (see Introduction). Thus, kinetic (*de novo*) and thermodynamic ("disactivation") folding defects may have evolved to ensure that the activity of signal transduction proteins is not expressed inappropriately in the absence of ligands (Smith, 1993), during biogenesis (Hartson et al., 1996), or prior to other specific stabilization events.

Alternatively, kinetic and thermodynamic folding "defects" may be inherent for the function of $p56^{lck}$ and other regulatory proteins. These multi-conformational proteins usually have small differences between the free energies of their individual conformations [e.g. see (Cooper & Howell, 1993)]. These small differences may result in a nearly continuous free-energy spectrum of folding intermediates, with no deep energy minima. Theoretically, such a spectrum would slow rates of *de novo* folding and would decrease the stability of the folded proteins [for discussion, see (Baldwin, 1994) and references therein]. Thus, the involvement of hsp90 machinery in both *de novo* folding of $p56^{lck}$ (Figure 10) and in maintaining $p56^{lck}$ function (Hartson et al., 1996) might reflect specialized chaperone support for a slow-folding, inherently unstable protein with a nearly continuous free-energy spectrum.

Figure 9. Geldanamycin inhibits the salt-stable interaction of hsp90 with p56^{lck} (Courtesy of Steve Hartson).

The drug vehicle DMSO (lanes 2-4) or geldanamycin (lanes 5+6) was added to *in vitro* translation reactions that were (lanes 3-6) or were not (lane 2) programmed with *lck* template. Reactions were then incubated at 30 °C for 45 min, chilled on ice, and immunoadsorbed with rabbit anti-p56^{lck} antibodies. Immunopellets were washed with low salt buffer and with one stringency wash of either low salt buffer (lanes 2,3,+5) or with buffer containing 0.5 M NaCl (lanes 4+6) as described in Materials and Methods. Aliquots of each immunoadsorption were analyzed by SDS-PAGE and autoradiography to assess the efficiency of immunoadsorption of p56^{lck} (top panel). Bands representing [^{35}S]p56^{lck} are indicated. The balance of each immunoadsorption was analyzed by SDS-PAGE and western blotting with rabbit anti-(hsp90/hsp70) antisera to assess co-adsorption of the hsp90-p56^{lck} complex. Additionally, rabbit reticulocyte lysate was applied to the gel (lane 1) to supply a standard for the detection of hsp90 and hsp70. Hsp90, hsp70, and the heavy chain of the immunoadsorbing antibody (ab hc) are indicated. Migrations of size standards are indicated along the right side of each panel (kDa).

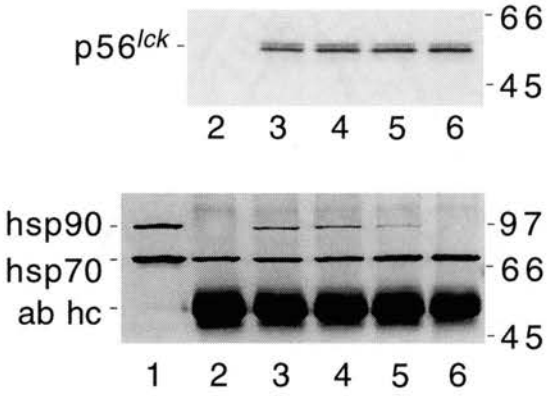
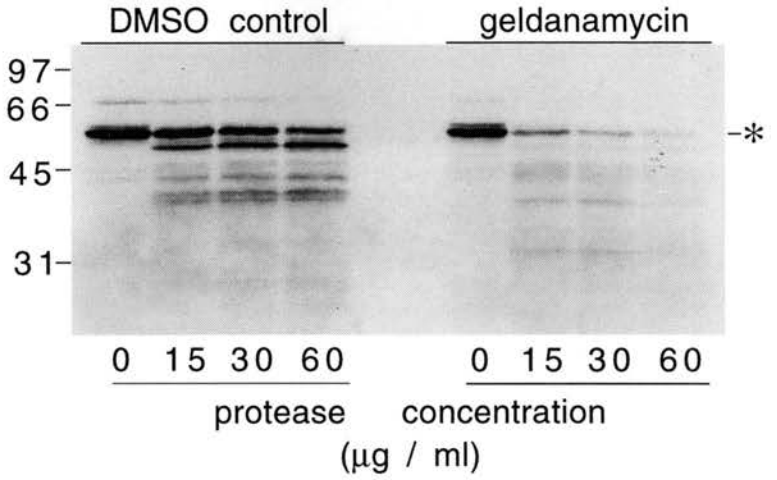

p56lck
66
45
2 3 4 5 6
hsp90
hsp70
ab hc
97
66
45
1 2 3 4 5 6

Figure 10. Geldanamycin prevents, but does not reverse, the acquisition of protease resistance by p56^{lck} (Courtesy of Steve Hartson).

[^{35}S]p56^{lck} was produced in rabbit reticulocyte lysate to which either DMSO or geldanamycin were added before synthesis of p56^{lck} as indicated (top panel). Alternatively, p56^{lck} was synthesized and matured in rabbit reticulocyte lysate lacking geldanamycin and subsequently incubated for 1 hr in rabbit reticulocyte lysate to which either drug vehicle DMSO or geldanamycin had been added as indicated (lower panel). In both cases, translation reactions were chilled on ice and treated with the indicated amounts of chymotrypsin for 6 min. Proteolyzed samples were analyzed by SDS-PAGE and autoradiography. The full-length *lck* translation product is indicated (*). Migrations and molecular masses of standards (kDa) are indicated along the left side of the panel.


DMSO control
geldanamycin
97
66
45
31
*
0 15 30 60
0 15 30 60
protease concentration
(μg / ml)

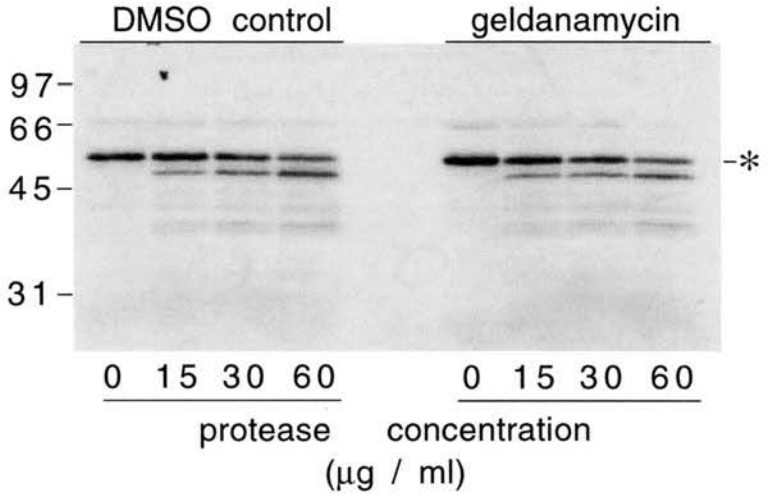

DMSO control
geldanamycin
97
66
45
31
*
0 15 30 60
0 15 30 60
protease concentration
(μg / ml)

Figure 11. Geldanamycin does not inhibit the *de novo* folding of luciferase.

After 10 min of luciferase synthesis in rabbit reticulocyte lysate, inhibitors of translation initiation were added and polyribosomes were allowed to run off for another 6 min. Following this 6 min runoff, either the drug vehicle DMSO (◇) or geldanamycin (◆) was added as indicated. Aliquots were assayed at indicated times to determine [^{35}S]met incorporation into acid precipitable protein and to determine luciferase activity. Individual points represent luciferase specific enzymatic activity synthesized in the absence or presence of geldanamycin assuming an endogenous methionine content of 3.5 μM for rabbit reticulocyte lysate.

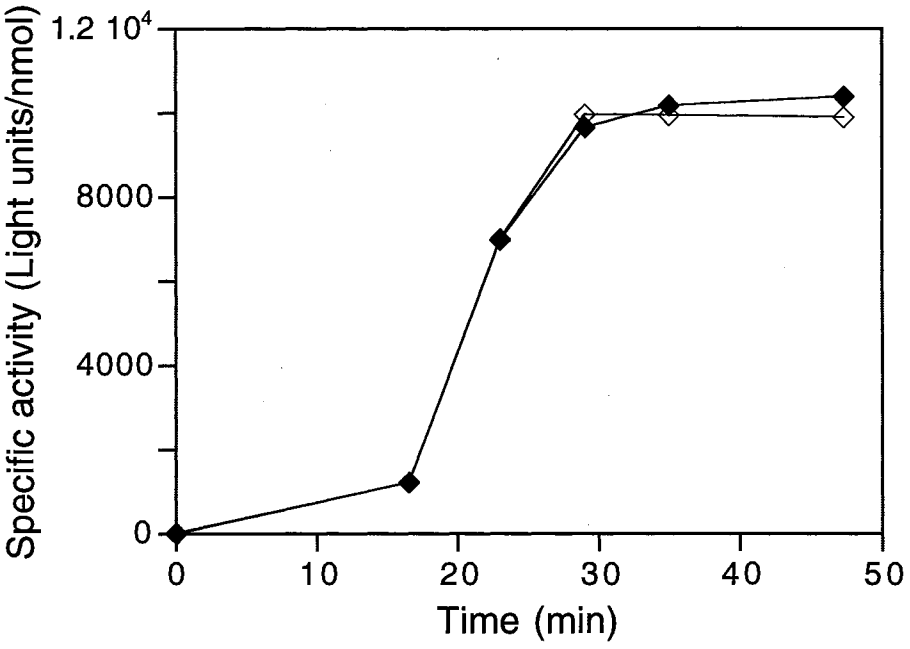
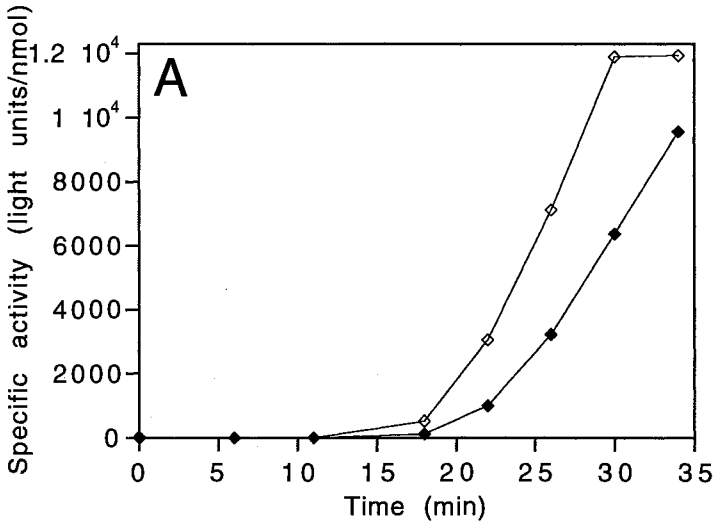

Specific activity (Light units/nmol)
1.2 10^4
8000
4000
0
0
10
20
30
40
50
Time (min)

Figure 12. Antagonists of hsc70 inhibit the *de novo* folding of luciferase.

Panel A: After 6 min of luciferase synthesis in rabbit reticulocyte lysate, inhibitors of translation initiation and either BSA (◇) or RCM-BSA (◆) were added. The specific enzymatic activity of luciferase was determined at the indicated times as described in Figure 11.

Panel B: After 10 min of luciferase synthesis in rabbit reticulocyte lysate, inhibitors of translation initiation were added and polyribosomes were allowed to run off for another 6 min. At this time, either the drug vehicle water (◇) or clofibric acid (◆) was added as indicated and the specific enzymatic activity of luciferase was determined at the indicated times as described in Figure 11.


A
Specific activity (light units/nmol)
1.2 10^4
1 10^4
8000
6000
4000
2000
0
0
5
10
15
20
25
30
35
Time (min)

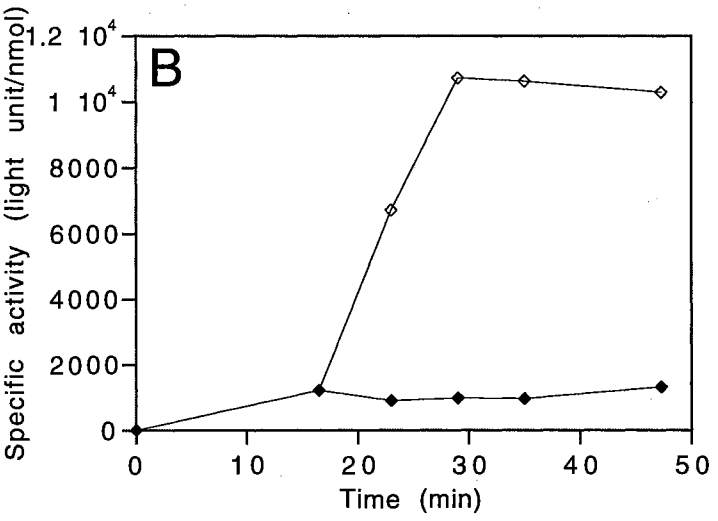

B
Specific activity (light unit/nmol)
1.2 10^4
1 10^4
8000
6000
4000
2000
0
0
10
20
30
40
50
Time (min)

Figure 13. Maintenance of luciferase at 37 °C requires ongoing chaperone support.

Purified luciferase was added to rabbit reticulocyte lysate lacking or containing an ATP-regenerating system, clofibric acid (CIA), DMSO (DMSO), or geldanamycin (GA) as indicated. Reactions were incubated for 1 hr at 37 °C and luciferase activity assayed. Luciferase activity is expressed as a percentage of that recovered from control incubations in lysate containing an ATP-regenerating system but no drugs.

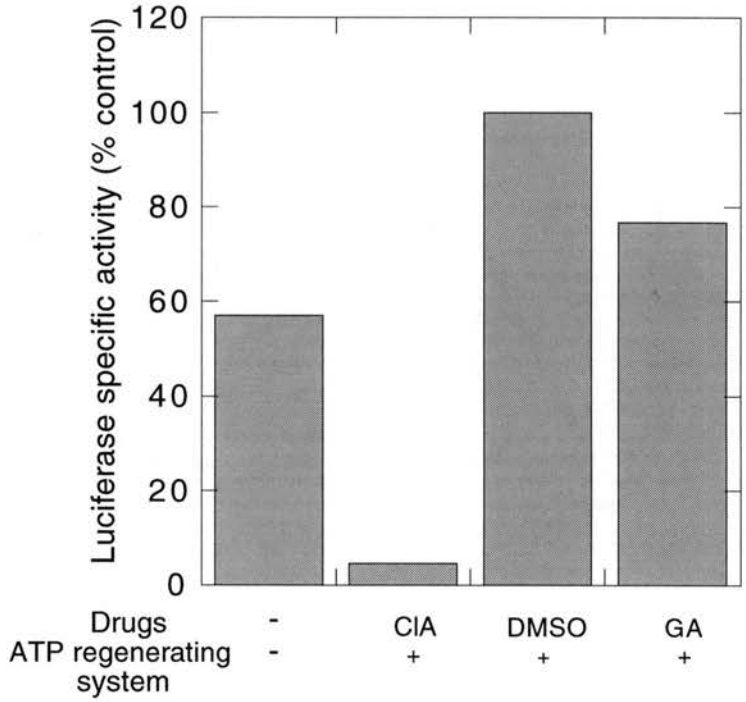
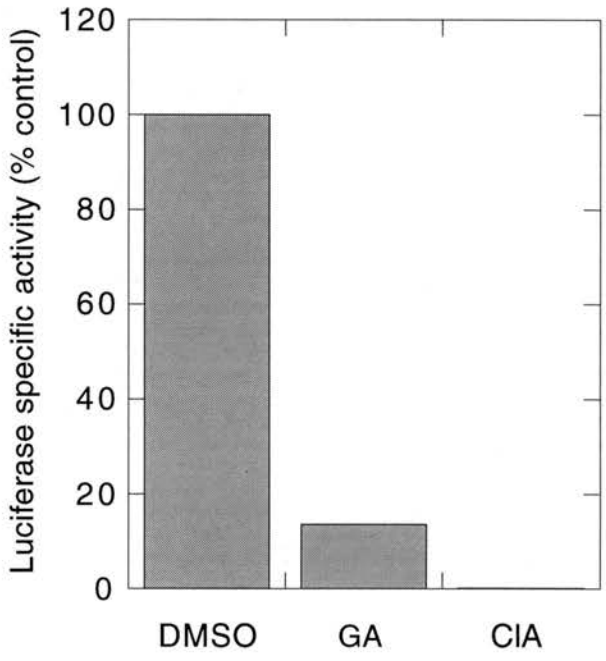

Luciferase specific activity (% control)
120
100
80
60
40
20
0
Drugs
ATP regenerating system
-
-
CIA
+
DMSO
+
GA
+

Figure 14. Fully folded luciferase requires ongoing chaperone support at 42 °C

Purified luciferase was added to rabbit reticulocyte lysate containing an ATP-regenerating system and DMSO (DMSO), geldanamycin (GA), or clofibric acid (CIA) as indicated. Reactions were incubated for 1 hr at 42 °C and luciferase activities assayed. Luciferase activity is expressed as a percentage of that recovered from control incubations in lysate containing an ATP-regenerating system but no drugs.


Luciferase specific activity (% control)
120
100
80
60
40
20
0
DMSO
GA
CIA

CHAPTER IV

HSC70 NEGATIVELY REGULATES HRI

Introduction

The heme-regulated translational inhibitor (HRI) is a protein kinase that specifically phosphorylates the 38 kDa α-subunit of eukaryotic initiation factor-2, eIF-2 (reviewed in (Courtneidge & Bishop, 1982, Oppermann et al., 1981, Tai et al., 1992)). The phosphorylation of eIF-2α in heme-deficient rabbit reticulocyte lysate (RRL) results in the inhibition of protein chain initiation upon sequestration of eIF-2B, the initiation factor responsible for catalyzing guanine nucleotide exchange which is required for the recycling of eIF-2 in the initiation of translation (Matts et al., 1983, Siekierka et al., 1984). The unavailability of eIF-2B results in the accumulation of eIF-2 in complexes with GDP and in the inhibition of the formation of the eIF-2•GTP•Met-tRNA$_i$ ternary complex. Since the amount of eIF-2B present in the lysate is much lower than the amount of eIF-2 present, phosphorylation of only 20-40% of the eIF-2α present is sufficient to render eIF-2B unavailable to catalyse GTP/GDP exchange (Matts & London, 1984).

In addition to heme-deficiency, activation of HRI in rabbit reticulocyte lysates (RRL) occurs in response to a number of agents that induce the heat shock or stess response in whole organisms or cultured cells. These agents include: heat shock, sulfhydryl reagents, oxidants, glucose deficiency, heavy metal ion treatment, and ethanol (Bonanou-Tzedaki et al., 1978, Ernst et al., 1978, Matts et al., 1991, Wu, 1981). As HRI activates it becomes progressively more phosphorylated. Activation of HRI may also

involve sulfhydryl oxidation or sulfhydryl/disulfide bond rearrangements (Ernst et al., 1979, Farrell et al., 1978, Gross & Rabinovitz, 1972, Kosower et al., 1972). The relationship between the molecular forms of HRI activated in response to heme-deficiency to forms of active HRI generated in hemin-supplemented lysate in response to heat or oxidative stress is not well understood.

HRI activation also appears to be regulated through its interaction with the heat shock proteins (hsps) and hsp cohorts, hsp90, hsc70, FKBP52 and p23 *in situ* in RRL (Matts & Hurst, 1989, Matts et al., 1992, Xu et al., In preparation) The sensitivity of HRI to activation in response to heat and oxidative stress in hemin-supplemented RRL correlates inversely to the quantities of hsc70 present in lysate preparations (Matts & Hurst, 1992). In addition, HRI in heme-deficient RRL containing low levels of hsp70 converts more rapidly to an activated state that is resistant to inhibition by hemin (Matts & Hurst, 1992). Recently, Gross and coworkers have identified the Supernatant Factor, a purified component of RRL that maintains protein synthesis when added to heme-deficient lysate, as a hsp70 family member (Gross et al., 1994). The ability of the Supernatant Factor to suppress the activation of partially purified HRI *in vitro* supports the hypothesis that hsc70 plays a negative role in the activation of HRI.

In this chapter, we have examined whether purified hsc70 has the ability to suppress the activation of HRI in hemin-supplemented RRL that occurs in response to heat shock or exposure to GSSG or Hg^{2+}. The data presented here indicate that: (i) biologically active pure hsc70 lowers the level of phosphorylated eIF-2α and maintains eIF-2B activity in heme-deficient RRL, and in hemin-supplemented RRL exposed to heat shock, RCM-BSA, GSSG or Hg^{2+}; (ii) hsc70 completely suppressed protein synthesis inhibition caused by Hg^{2+}, but only partly reversed inhibition caused by GSSG and heat shock; (iii) the inability of hsc70 to fully protect protein synthesis from inhibition due to heat shock and GSSG was due to its inability to protect eIF-4E from heat-induced dephosphorylation, and its inability to protect translational elongation from GSSG-induced inhibition,

respectively. These results are consistent with the notion that hsc70 negatively regulates the activation of HRI in RRL.

Experimental procedures

Materials.

Reticulocyte lysate was prepared from anemic rabbits as described (Hunt et al., 1972), using buffered saline containing 5 mM glucose to wash the reticulocytes prior to their lysis (Matts et al., 1991). Nitro blue tetrazolium (NBT), 5-bromo-4-chloro-3-indolyl phosphate (BCIP) salicylic acid, clofibric acid, indomethacin, ibuprofen, and 7-methyl GTP were obtained from Sigma. Alkaline phosphatase conjugated to goat anti-rabbit IgG and rabbit anti-mouse IgG was obtained from ICN. L-[^{14}C]Leucine(340 mCi/mmole) and [γ-^{32}P]ATP (1000-3000 Ci/mmol) were obtained from Du Pont-New England Nuclear. DEAE cellulose resin was from Toyohaas. eIF-2 was isolated from reticulocyte lysate obtained from Green Hectar as described previously (Hurst et al., 1987). 7-Methyl GTP-Sepharose-4B was obtained from Pharmacia Biotech. Rabbit 4322 anti-hsp90/hsp70 polyclonal antiserum (Erhart et al., 1988) was provided by Dr Stephen J. Ullrich (National Cancer Institute). YDJ-1 was generously provided by David Toft (Mayo Medical School, Rochester, MN).

Buffers

TB, 10 mM Tris-HCl (pH 7.5); TB/50, 10mM Tris-HCl (pH 7.5) containing 50 mM NaCl; TBS, 10 mM Tris-HCl (pH 7.5) containing 150 mM NaCl; HE, 20 mM HEPES (pH 7.4), 0.5 mM EDTA; HE/50, 20 mM HEPES (pH 7.4), 0.5 mM EDTA, 50 mM KCl, 50 mM NaF; HEM/50, 20 mM HEPES, 0.5 mM EDTA, 50 mM KCL, 50 mM NaF, 2 mM $MgCl_2$, 1 mM ATP, 0.2 mM GTP; GB, 25 mM Tris-HCl (pH 7.5) containing

100 mM KCl, 15 mM $Mg(OAc)_2$, and 0.2 mM EDTA; 1xSDS sample buffer, 62.5 mM Tris-HCl (pH 6.8), 5% SDS, 90 mM dithithreitol, 5% glycerol, and 0.005% Bromophenol blue: alkaline phosphatase reaction buffer, 100 mM TRIS-HCl (pH 9.5) containing 100 mM NaCl, 100 mM $Mg(OAc)_2$, 300 µg/ml NTB and 150 µg/ml BCIP.

Protein synthesis and eIF-2α phosphorylation in reticulocyte lysates

Protein synthesis was carried out at 30 °C in standard reticulocyte lysate reaction mixtures with or without the addition of [^{14}C]leucine as described (Hunt et al., 1972; Ernst et al., 1978). Hemin supplemented lysates contained 20 µM hemin-HCl. Protein synthesis was determined by measuring the incorporation of [^{14}C]leucine into the acid-precipitable protein at 30 °C in standard reticulocyte reaction mixtures (Hunt et al., 1972; Ernst et al., 1978). eIF-2α phosphorylations in 2 µl of protein synthesis mixes were analysed as previously described by western blotting of one-dimensional isoelectric focusing slab gels (Maurides et al., 1989).

Immunoadsorption of BSA , RCM-BSA, and HRI from rabbit reticulocyte lysates

Immunoadsorptions of lysate proteins with the the anti-HRI mAB F or with anti-BSA were carried out as described (Matts et al., 1993, Matts et al., 1992).

Analysis of eIF-4E phosphorylation

After preincubation for 18 min at 42 °C (heat shocked) or 35 °C (control), hemin-supplemented protein synthesis mixes were pulsed with [γ-^{32}P]ATP (0.4 mCi to 200 µl of protein synthesis mix) for 5 min, followed by the addition of ice cold NaF and EDTA to a final concentration of 50 mM and 10 mM, respectively. eIF-4E was adsorbed by the

addition of 30 µl of 7-methyl GTP-Sepharose to the samples (1:1 mix in HE buffer) on ice, followed by stirring in the cold room for 2 hr. The 7-methyl GTP-Sepharose was then washed sequentially with 1 ml each of HE, HE/100, HEM, and HE/100. The bound eIF-4E was then eluted with HE/100 buffer containing 50 µM 7-methyl GTP. Samples were analyzed by SDS-PAGE in 10% gels, followed by transfer of the protein onto the PVDF membrane and autoradiography.

Assay for eIF-2B guanine nucleotide exchange activity

RRLs were incubated under protein synthesizing conditions at 30 °C or 42 °C (heat shock) in the absence of [^{14}C]Leu and eIF-2B activity was measured as described previously (Matts 1984). Briefly, at the times indicated, 50 µl of protein synthesis mix was mixed with 130 µl of ice cold dilution buffer (40 mM Tris HCl, pH7.4, 100 mM KCl, 50 mM KF, 2 mM $Mg(OAc)_2$, 10% glycerol, 40 µM GDP), and 20 µl of preformed eIF-2•[^{3}H]GDP complex. Reaction mixes were then incubated at 30 °C for 2 min. Exchange assays were stopped by the addition of 1 ml ice cold wash buffer, followed by filtration of the reaction mixture through nitrocellulose filters (HAWP 02500, Millipore) which rapidly binds the remaining eIF-2•[^{3}H]GDP complex. Filters were then washed with an additional 15 ml of ice cold wash buffer to remove any unbound [^{3}H]GDP.

Polyribosome gradients

Hemin-supplemented protein synthesis mixes (100 µl) were incubated in the presence or absence of 1.3 mM GSSG for 20 min at 30°C. These reaction mixes were diluted with 150 µl of ice cold GB buffer layered over a 15-40% (w/v) sucrose gradient and centrifuged for 1 h at 45,000 rpm in a Sorvall AH-650 rotor. The polyribosomal profile was analyzed using an ISCO Model 640 density gradient fractionator as described

previously (Matts et al., 1991). Inhibition of elongation was assayed by the determining the ability of agents to inhibit run-off of polyribosomes after the addition of aurin tricarboxylic acid (ATA), an initiation inhibitor. Specifically, hemin-supplemented protein synthesis mixes were supplemented with 120 μM ATA and incubated in the presence or absence of 1.3 mM GSSG (1.3 mM) for 1.5 min at 30 °C prior to analysis as described above.

Purification of hsc70

Hsc70 was purified from the post-ribosomal supernatant of fresh rabbit reticulocyte lysate obtained from Green Hectar by a slight modification of the procedure described by Gross and co-workers for the purification of the Supernatant Factor (Gross, 1976, Gross et al., 1994). The inital purification of hsc70 from post-ribosomal supernatant consisted of acid precipitation at pH 5.4, reprecipitation at 40-80% saturated ammonium sulfate, HPLC chromatography on DEAE-Toyopearl from ToyoHaas, chromatography on phosphocellulose, followed by affinity chomatography on ATP-agarose. The hsc70 obtained was highly pure and biologically active in reversing protein synthesis inhibition due to heme deficiency. Hsc70 purified in a similar manner from rabbit liver or brain is as pure, but is not active in reversing the protein synthesis inhibition in heme-deficient RRL. In agreement with the report of Gross and coworkers (Gross et al., 1994) bovine brain hsp70 purchased from StressGen was inactive or inhibitory to protein synthesis.

Upon storage of aliquots of concentrated stocks of purified hsc70 at -70 °C, a significant amount of the protein is observed to be present in aggregates upon thawing. In addition, the hsc70 was observed to aggregate if the purified hsc70 was concentrated too extensively. These observations are consistent with reports that ATP bound to purified hsc70 hydrolyzes slowly, leading to the accumulation of hsc70•ADP. Most studies suggest that purified hsc70 occurs as a mixture of monomers, dimers and polymers in

ADP, while in ATP it occurs mostly as monomer molecules that readily form aggregates upon storage (Carlino et al., 1992, Palleros et al., 1993, Schlossman et al., 1984, Schmid et al., 1985, Toledo et al., 1993). Moreover polymerization of hsc70 is proportional to protein concentration (Gao et al., 1996). Polymerized hsc70 has either no effect or is inhibitory to protein synthesis in RRL (not shown). Aggregated hsc70 disaggregates when ATP is exchanged for bound ADP (Gao et al., 1996). To activate aggregated hsc70, hsc70 was preincubated in hemin supplemented lysate in the presence of a creatine phosphate/creatine phosphokinase ATP regenerating system 10 min prior to the initiation of protein synthesis. Moreover, addition of low levels of YDJ-1 (yeast DnaJ homolog) to RRL, enhanced the ability of hsc70 to inhibit HRI activation.

Results

Heat shock causes a loss in the functional pool of hsc70

Previously an inverse correlation between the degree of heat shock-induced protein synthesis inhibition and hsc70 levels in RRL has been observed. It was hypothesized that protein synthesis inhibition under heat stress occurs when heat shock-induced denatured proteins accumulate to a level that sequesters the available pool of functional hsc70. To test this hypothesis two different lysates were compared which had different resistance to heat stress (Fig. 15). One lysate was very sensitive to heat shock and shut off protein synthesis within 5 min of incubation at 42 °C (lysate 2), whereas the other lysate was more resistant and shut off protein synthesis after 15 min of incubation (lysate 1). These two lysates were compared for the availability of active hsc70 pool to bind to RCM-BSA, at 5 min and 15 min. RCM-BSA and BSA were added to the lysates and then immunoadsorbed with anti-BSA at the end of 5 min and 15 min. Western blot analysis showed that when the lysates were incubated with RCM-BSA at 30 °C the amount of hsc70 interacting with RCM-BSA did not vary with time, in both the sensitive and resistant lysates. But when the mixture

was incubated at 42 °C, there was a rapid loss of hsc70 that binds to RCM-BSA within 5 min in the sensitive lysate, whereas in the resistant lysate this occurred only at the end of 15 min, correlating very well with the time of protein synthesis shut off. This further indicates that heat shock reduces the active pool of hsc70 that can bind to substrates.

Heat shock blocks the association of hsc70 with HRI

It has been proposed previously that the mechanism by which denatured proteins activate HRI, is by binding and sequestering hsc70, and blocking the interaction of hsc70 with HRI. To confirm if a similar mechanism occurs under heat stress, we analyzed the level of hsc70 interacting with HRI during heat shock. HRI was adsorbed from control and heat shocked hemin-supplemented lysate with anti-HRI mAbF. Western blot analysis indicated that hsp70 was coadsorbed with HRI from the control lysate, but not from the heat shocked lysate, indicating that hsp70 dissociates from HRI during heat shock (Fig. 16). This is consistent with the observation that denatured protein has the ability to block the interaction of hsp70 with HRI, upon its activation in the presence of denatured proteins.

Hsc70 partially suppressed inhibition due to RCM-BSA.

It has been hypothesized that RCM-BSA inhibits protein synthesis by sequestering hsc70 from HRI, and thereby activating HRI. Consistent with this hypothesis, we observed that exogenously purified hsc70 when added to RCM-BSA supplemented lysate partially reversed inhibition in protein synthesis (Fig. 17A). In addition, exogenously added hsc70 also lowered eIF-2α phosphorylation and increased eIF-2B activity in RCM-BSA treated RRL (Table III). These results support the hypothesis that denatured proteins activate HRI by titrating the active pool of hsc70.

Effect of hsc70 on heat shock induced eIF-2α phosphorylation and eIF-4E dephosphorylation

The phosphorylation state of eIF-4E correlates positively with the rate of translation. Both the dephosphorylation of eIF-4E and the phosphorylation of eIF-2α have been implicated in playing a role in translational inhibition induced by heat shock in cultured cells (Hershey, 1991, Pain & Clemens, 1991). To examine the mechanism of translational arrest in heat shocked RRL, the phosphorylation status of eIF-4E and eIF-2α were examined in control and heat shocked hemin-supplemented RRL by pulse-labeling with [γ-^{32}P]ATP. Consistent with observations made *in vivo*, we observed that translational inhibition in heat shocked lysates was accompanied by dephosphorylation of eIF-4E (Fig. 18) and an increase in phosphorylation of eIF-2α.

To examine the role played by hsc70 in the regulation of translation in response to heat shock, the effect of purified hsc70 on protein synthesis was examined in control and heat shocked RRL. The addition of hsc70 to heat shocked lysate only partially restored protein synthesis (Fig. 17B). Examination of the phosphorylation status of eIF-2α and eIF-4E indicated that while hsc70 suppressed the phosphorylation of eIF2α caused by heat shock (Table III), hsc70 had no effect on the heat-induced dephosphorylation of eIF-4E (Fig. 18). Consistent with its effects on eIF-2α phosphorylation, hsc70 enhanced eIF-2B activity when added to heat shocked RRL (Table III). These observations suggest that hsc70 reverses inhibition of translation caused by the activation of HRI, but has no effect on the translational inhibition due to the dephosphorylation of eIF-4E.

Hsc70 lowers eIF-2α phosphorylation and increases eIF-2B activity in heme deficient RRL

Hsc70 has been shown to decrease the rate at which protein synthesis shuts off in heme deficient RRL. However, no evidence was presented that eIF-2α phosphorylation

was suppressed and eIF-2B activity was maintained by the addition of hsc70 to heme-deficient RRL. Therefore, we examined the effect of hsc70 on eIF-2α phosphorylation and eIF-2B activity the hallmarks of translational regulation via eIF-2α kinase activation. Addition of hsc70 to heme-deficient lysate suppressed the rate at which protein synthesis was inhibited (Fig. 19A), decreased the level of eIF-2α phosphorylation and increased eIF-2B activity (Table III). These observations are consistent with the notion that the interaction of hsc70 with HRI suppresses HRI activation.

YDJ-1 enhances the ability of hsc70 to maintain protein synthesis in heme deficient RRL.

It has been previously demonstrated that DnaJ depolymerizes hsc70. As the purified hsc70 was partially polymerized, we examined the effect of the yeast DnaJ homolog, YDJ-1 on the ability of hsc70 to suppress HRI activation in RRL. We observed that addition of both hsc70 and YDJ-1 together to heme deficient lysate further enhanced protein synthesis by 20% above the effect of hsc70 alone (Fig 20). YDJ-1 alone had small but significant effect on protein synthesis.

Hsc70 enhances protein synthesis in $HgCl_2$ and GSSG treated hemin-supplemented RRL

Protein synthesis also shuts off in hemin-supplemented RRL in response to eIF-2α phosphorylation caused by oxidative stress. Correlative data suggests that hsp70 protects translation from shut-off in response to oxidative stress generated by either treatment of RRL with GSSG, heavy metal ions (e.g., Hg^{2+}) or methylene blue (Ernst et al., 1978, Hurst et al., 1987, Kosower et al., 1972, Matts et al., 1991). To test this hypothesis, we examined the effect of hsc70 on protein synthesis in hemin-supplemented RRL treated with either GSSG and $HgCl_2$. Hsc70 decreased the level of eIF-2α phosphorylation, maintained the eIF-2B activity (Table III), and completely suppressed inhibition of protein

synthesis due to $HgCl_2$ (Fig. 19B). However, while hsc70 lowered the level of eIF-2α phosphorylation, and increased eIF-2B activity in GSSG-treated RRL (Table III), the addition of hsc70 only partially protected protein synthesis from inhibition (Fig. 21).

GSSG inhibits both the initiation and elongation phases of translation

Unlike Hg^{2+}, which inhibits protein synthesis in a biphasic manner that is typical of inhibitors that exclusively affect initiation, GSSG inhibited the translation rate from the beginning of the incubation, and as incubation time progressed the rate of translation slowly tapered off. The effect of GSSG on the initial rate of translation suggested that GSSG may be inhibiting translational elongation. To test this hypothesis we examined the polyribosomal profile of GSSG treated hemin-supplemented RRL. Relative to untreated RRL (Fig 22A), the polyribosomal profile of GSSG treated RRL showed an increase in 80S ribosomes characteristic of initiation inhibition (Fig, 22B). However, large polyribosomes were also present after 20 min of incubation. The maintenance of polyribosomes under conditions where eIF-2α phosphorylation is enhanced and eIF-2B activity is nearly completely inhibited suggested that GSSG was causing an inhibition of elongation. To examine the possibility that GSSG also inhibits elongation, the polyribosomal profile of control and GSSG-treated hemin-supplemented RRL was studied in the presence of ATA, an initiation inhibitor. In the presence of ATA, the polyribosomes present in untreated hemin-supplemented RRL ran-off and accumulated as inactive 80S monomers (Fig. 22C) within 1 min of incubation (not shown). However, in the presence of ATA, large polyribosomes were still present in GSSG-treated RRL after 3 min of incubation (Fig. 22D). The failure of the polyribosomes to run-off in the presence of an inhibitor of initiation indicates that GSSG, besides inhibiting protein synthesis at the initiation stage, is also inhibiting protein synthesis at the level of elongation. The ability of hsc70 to lower the phosphorylation of eIF-2α and increase the eIF-2B activity, but only

partially reverse protein synthesis inhibition due to GSSG, suggest that hsc70 is able to reverse the inhibition of initiation caused by GSSG-induced HRI activation, but that hsc70 can not reverse GSSG-induced inhibition of elongation.

Discussion

The data presented in this report indicate that besides suppressing the activation of HRI in response to heme-deficiency in RRL, hsc70 also suppresses the activation of HRI in hemin-supplemented RRL that occurs in response to heat shock, or the addition of RCM-BSA, GSSG, or Hg^{2+} to RRL. The ability of hsc70 to decrease the level of eIF-2α phosphorylation and enhance eIF-2B activity in heme-deficient RRL and in hemin-supplemented RRL exposed to heat shock, RCM-BSA, GSSG or Hg^{2+} clearly indicates that the ability of hsc70 to suppress HRI activation is not limited to HRI activation in response to heme-deficiency. While, it has been previously demonstrated that hsc70 reversed protein synthesis inhibition under heme deficiency (Gross, 1976, Gross et al., 1994), the ability of hsc70 to maintain protein synthesis was the only criteria used in that report to assess the effect of hsc70 on HRI activation. We show here that hsc70 also lowered the amount of phosphorylated eIF-2α and also suppressed the inhibition of eIF-2B GDP exchange activity, besides slowing the rate of protein synthesis inhibition during heme deficiency.

Heat shock inhibits protein synthesis by causing changes in the translational machinery at a number of levels. Heat shock induced inhibition of protein synthesis has been correlated with eIF-2α phosphorylation and eIF-4E dephosphorylation in cultured cells. Similar to the observations made *in vivo*, eIF-2α phosphorylation and eIF-4E dephosphorylation were observed in response to heat shock in RRL. Hsc70 partially reversed inhibition due to heat shock. This partial protection of translation can be explained by the observation that, while hsc70 reduced the level of eIF-2α phosphorylation, it had no

effect on eIF-4E dephosphorylation. Consistent with the notion that under heat shock there was a loss of hsc70 bound to HRI, inhibition of protein synthesis caused by HRI activation correlated with loss of hsc70 that was available to bind RCM-BSA indicating that heat shock leads to an accumulation of denatured proteins . The denatured proteins sequestered hsc70 from HRI, leading to the activation of HRI. Furthermore, addition of purified hsc70 lowered eIF-2α phosphorylation and suppressed inhibition of eIF-2B catalyzed GDP exchange. Moreover, with increased times of incubation hsc70 lost its ability to suppress inhibition of eIF-2B GDP exchange activity. The inability of hsc70 to maintain translation suggest that hsc70 becomes limiting as more denatured proteins are produced with time. This is consistent with the observation that with increasing time the amount of hsc70 available to bind to RCM-BSA under heat shock decreases.

Hsc70 completely reversed protein synthesis inhibition due to mercury treatment. Heat-shock protein 70 is a stress-induced protein and is suggested to play a protective role against heavy metal ion treatment. It has been previously demonstrated that lysates with higher level of hsc70 showed higher tolerance to heavy metal ion treatment. Consistent with this hypothesis we observed that addition of purified hsc70 completely reversed protein synthesis inhibition due to mercury. In addition, purified hsc70 lowered eIF-2α phosphorylation and suppressed inhibition of eIF-2B GDP exchange.

GSSG inhibited protein synthesis both at the initiation level and at the elongation level. Gross *et al* had previously concluded that GSSG could be inhibiting protein synthesis by a different mechanism than heme deficiency, since hsc70 slightly suppressed GSSG induced inhibition of protein synthesis. We observed that hsc70 partially suppressed GSSG induced inhibition of protein synthesis, due to its ability to suppress the activation of HRI. Furthermore, hsc70 was able to completely suppress the activation of HRI caused by Hg^{2+}, another sulfhydryl reactive compound that inhibits protein synthesis exclusively at the initiation level. Hsc70 suppressed phosphorylation of eIF-2α resulting from GSSG and Hg^{2+} treatment and also suppressed inhibition of eIF-2B GDP exchange

activity. The ability of hsc70 to partially revert GSSG induced inhibition of protein synthesis could be explained by hsc70 suppressing inhibition of initiation, but having no effect on inhibition at the elongation level. Consistent with this hypothesis it was shown previously that lysates with higher level of hsp70 were more resistant to protein synthesis inhibition due to oxidised glutathione and mercury.

DnaJ may also play a role in regulating HRI activation. We observed that purified YDJ-1 together with hsc70 increased the rate of protein synthesis in heme deficient lysate by 20 % more than when only hsc70 was added. The ability of YDJ-1 to suppress HRI activation may be due to (i) the ability of YDJ-1 to enhance the activity of added hsc70 in general; and/or (ii) the ability of YDJ-1 to bind to HRI and directly enhance the interaction of hsc70 with HRI. Our purified hsc70 preparation contained some insoluble material indicating that the hsc70 was partially polymerised. Previously it has been shown that DnaJ helps in the depolymerisation of hsc70. Since only monomeric hsc70 is functional, DnaJ could be enhancing the activity of hsc70 by depolymerising hsc70. The observation that DnaJ alone had a rather modest effect on protein synthesis in heme deficient RRL suggests that YDJ-1 is not a rate-limiting activity for maintaining HRI in an inactive state in RRL. However, we have observed that 5 % of HRI in RRL can be immunoprecipitated with anti-DnaJ (data not shown). This observation suggests that DnaJ might have a direct effect on HRI, by directing hsc70 to HRI. Also the amount of DnaJ may become limiting only on the addition of exogenous hsc70 particularly if the hsc70 is polymerized, since the polymerised hsc70 would bind endogenous DnaJ during its depolymerization. The relationship between DnaJ, hsc70 and HRI activation is currently under investigation.

Figure 15. Heat shock causes a loss in functional hsc70 pool (Courtesy of Robin Hurst).

A) Protein synthesis was measured in standard reaction mixtures in the lysate 1 (heat resistant) and lysate 2 (heat sensitive) at 30 °C (A)and 41 °C (F). The amount of [^{14}C]leucine incorporated into acid precipitable protein in a 5 µl aliquot was determined in the times indicated in the figure. B) Lysates 1 and 2 were incubated at either 30 °C or 42 °C. RCM-BSA or BSA were added at 0 min or 10 min. At the end of 5 min and 15 min, aliquots were taken and immunoabsorbed for BSA and RCM-BSA, as

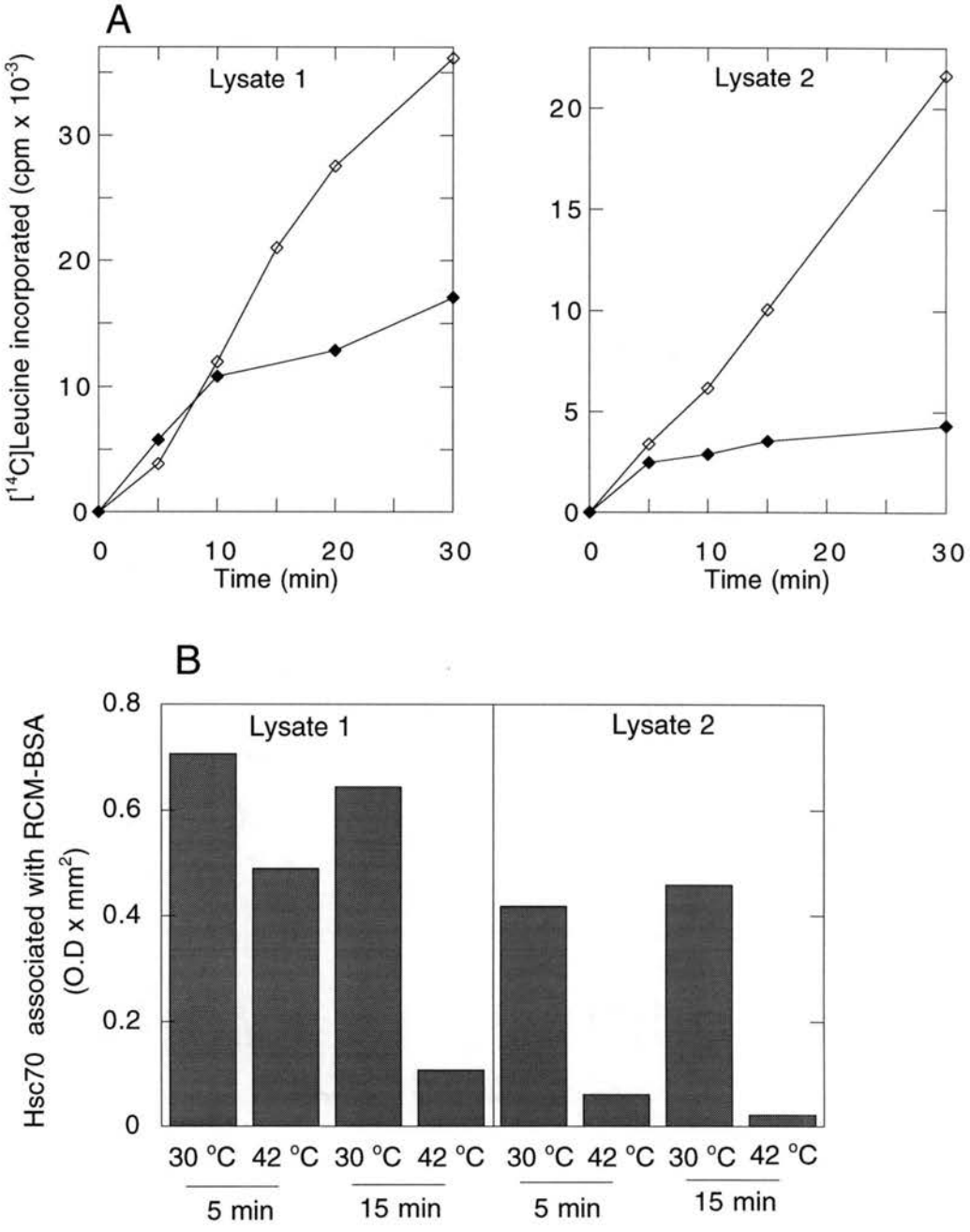

A
Lysate 1
Lysate 2
[14C]Leucine incorporated (cpm x 10-3)
Time (min)
Time (min)
B
Hsc70 associated with RCM-BSA (O.D x mm2)
Lysate 1
Lysate 2
30 °C 42 °C 30 °C 42 °C 30 °C 42 °C 30 °C 42 °C
5 min
15 min
5 min
15 min

Figure 16. Heat shock blocks the interaction of HRI with hsc70 (Courtesy of Zouyu Xu).

Hemin supplemented protein synthesis mixes were incubated at 30 °C or 42 °C for 20 min followed by apyrase treatment (one unit of apyrase per 10μl of protein synthesis mixes) on ice for 15 min. Proteins from 10 μl of the apyrase treated protein synthesis mixes were adsorbed with the non-immune control (C) or the anti-HRI (I) mAb F in the presence of TBS. The adsorbed fractions were western blotted with the 4322 anti-hsp90/hsp70 antibody to detect the presence of hsp70, HC, heavy chain of IgG.

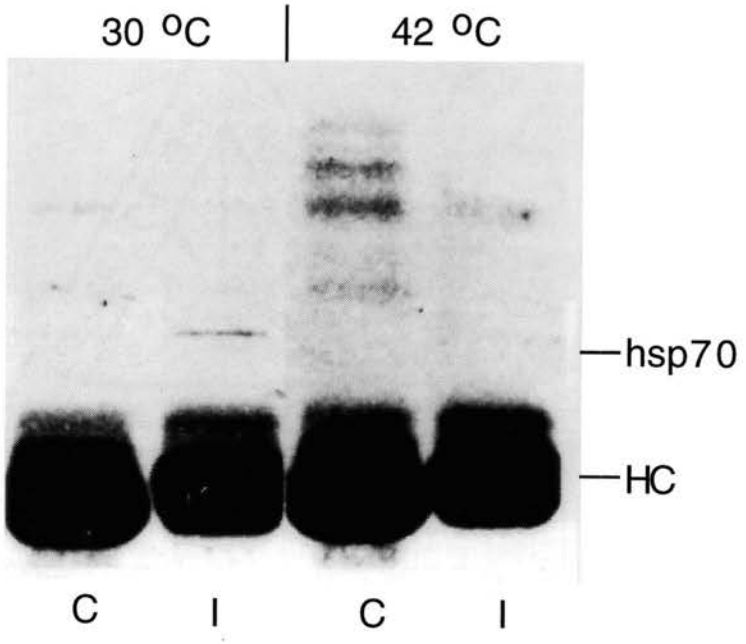

30 °C
42 °C
hsp70
HC
C
I
C
I

Figure 17. Hsc70 partially reversed inhibition due to RCM-BSA and heat shock.

Protein synthesis mix was preincubated with either hsc70 (0.1 mg/ml; O, ◆) or the buffer (◇) for 10 mins at 30 °C. The reaction mixes were transferred to iceand mixed with [^{14}C]leucine. Protein mixes were then either A) supplemented with BSA (O) or RCM-BSA (1mg/ml; ◆, ◇), and transferred to 30 °C; or B) transferred to 30 °C (O) or 42 °C (◆, ◇). The amount of [^{14}C]leucine incorporated into acid precipitable protein in a 5 µl aliquot was determined in the times indicated in the figure.

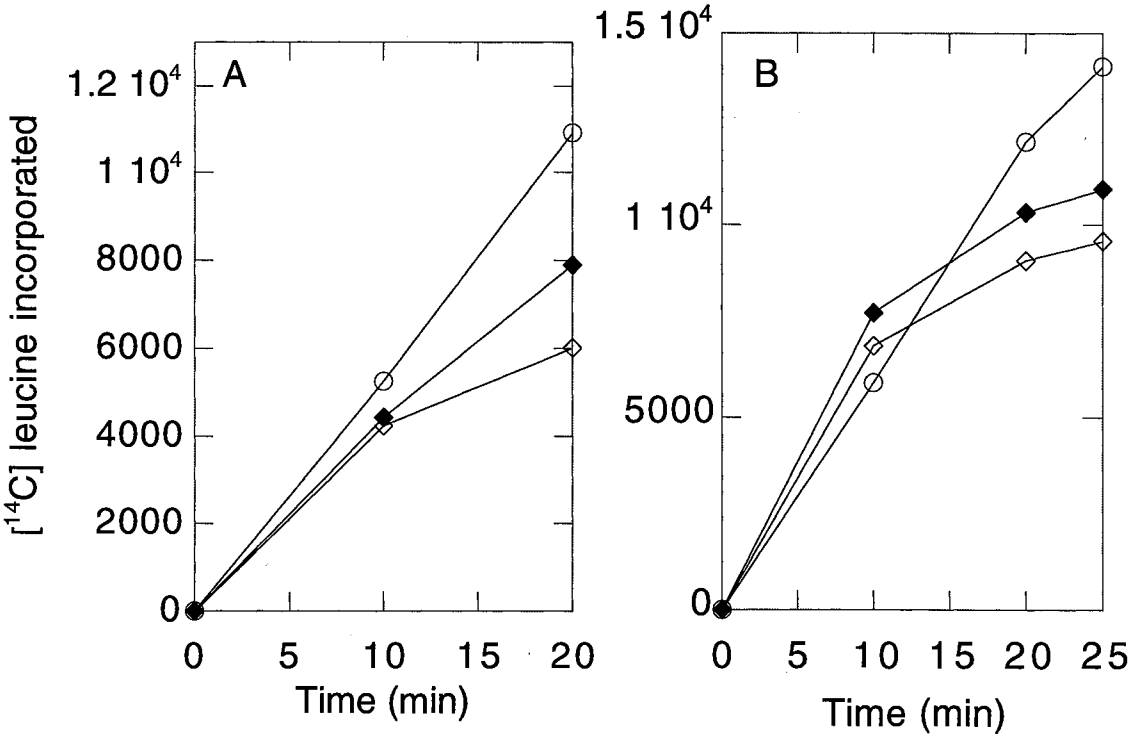

A
B
[^{14}C] leucine incorporated
1.2 10^4
1 10^4
8000
6000
4000
2000
0
1.5 10^4
1 10^4
5000
0
0 5 10 15 20
0 5 10 15 20 25
Time (min)
Time (min)

Figure 18. Heat shock causes eIF-4E dephosphorylation.

Protein synthesis mixes (200 µl) were incubated at 35 °C and 42 °C in the presence or absence of added hsc70 (75 µg/ml). At the end of 18 min the reaction mix was pulsed with 0.2 mCi of γ^{32}P ATP and transferred to ice at the end of 23 min. eIF-4E was adsorbed to m^7GTP-Sepharose for 2 hrs at 4 °C. Pellets were extensively washed and eIF-4E eluted with m^7GTP. The pellets and the m^7GTP eluent were then run on a 10% gel.

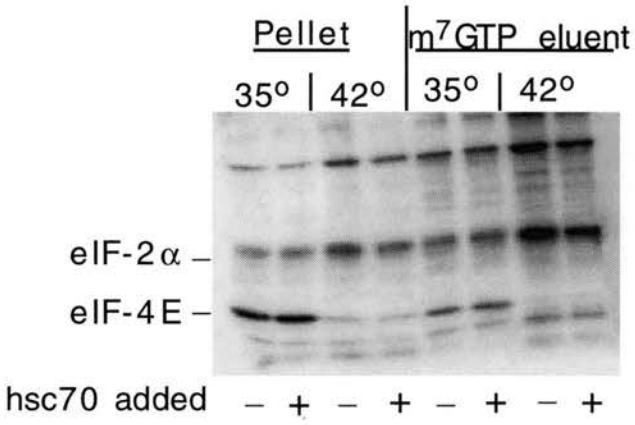

Pellet
m7GTP eluent
35° | 42° | 35° | 42°
eIF-2α
eIF-4E
hsc70 added – + – + – + – +

Figure 19. Hsc70 reverses inihibition due to heme deficiency and Hg^{++} treatment.

A) Protein synthesis was carried out in the presence (⊙) and absence (◇ ◆) of heme at 30 °C, with (⊙ ◆) or without (◇) the addition of purified hsc70 (0.05 mg/ml). B) Hemin-supplemented protein synthesis mixes were incubated at 30 °C in the presence (◇ ◆) or absence (O) of 20 μM $HgCl_2$ with (◆) or without (◇) the addition of purified hsc70 (0.05 mg/ml). Protein synthesis was measured by following incorporation of [^{14}C]leucine into acid-precipitable protein at the times indicated.

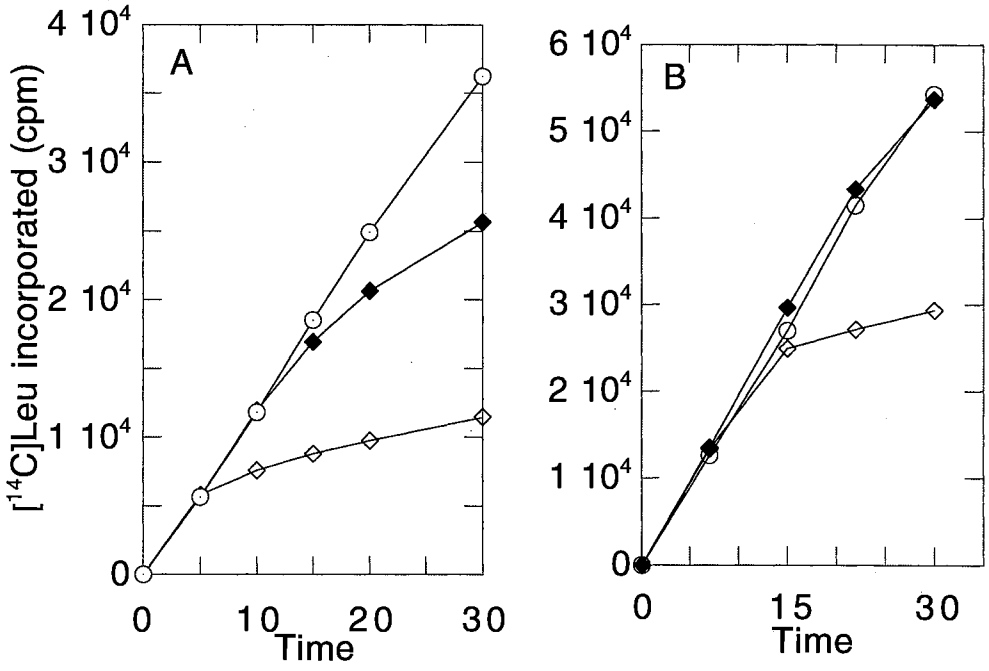

A
B
[14C]Leu incorporated (cpm)
4 10^4
3 10^4
2 10^4
1 10^4
0
6 10^4
5 10^4
0
10
20
30
15
Time

Figure 20. Effect of YDJ-1 and hsc70 on protein synthesis in heme deficient RRL.

Protein synthesis mixes were carried out in the presence (O) or absence (◇, ◆, ×, Δ) of heme at 30 °C, with (O, ◆, Δ) or without (◇, ×) the addition of purified hsc70 (0.1 mg/ml) or YDJ-1 (6 μg/ml; O, ◆, ×). Protein synthesis was measured by following incorporation of [^{14}C]leucine into acid precipitable protein at the times indicated.

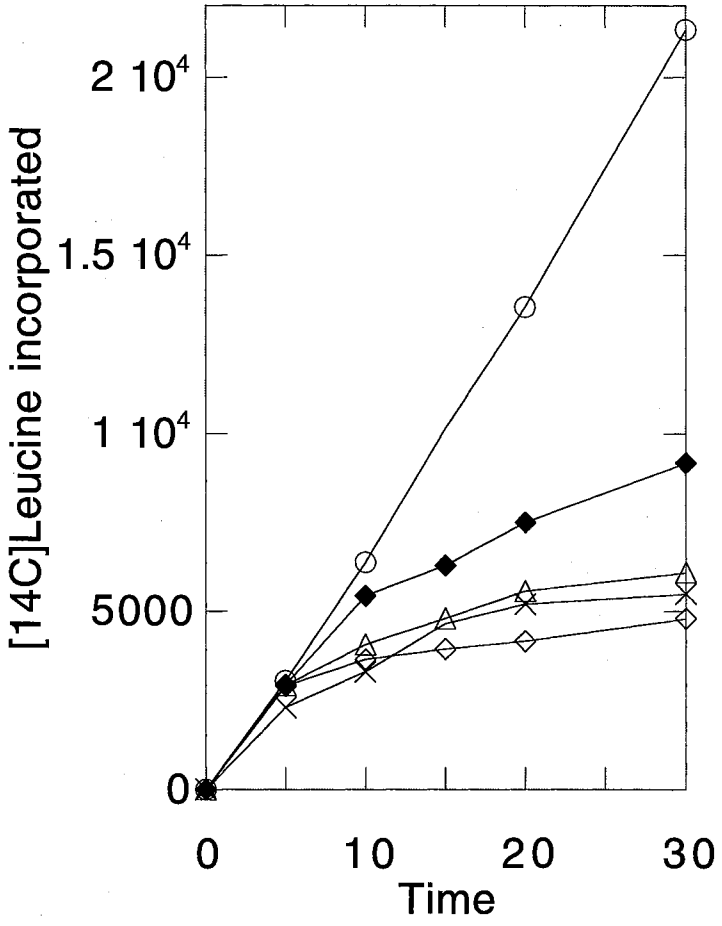

[14C]Leucine incorporated
2 10^4
1.5 10^4
1 10^4
5000
0
0
10
20
30
Time

Figure 21. Hsc70 partially reversed protein synthesis inhibition due to GSSG.

A) Hemin-supplemented protein synthesis mixes were incubated at 30 °C in the presence (◇, ◆) or absence (O) of 1.6 mM GSSG with (O, ◆) or without (◇) the addition of purified hsc70 (0.05 mg/ml). Protein synthesis was measured by following incorporation of [^{14}C]leucine into acid-precipitable protein at the times indicated.

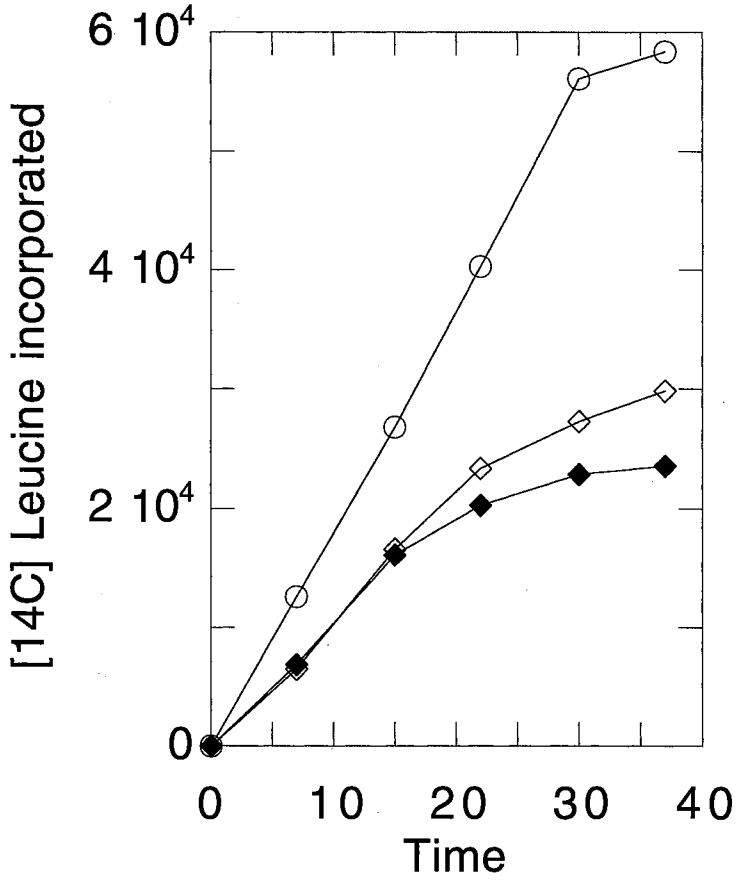

6 10^4
4 10^4
2 10^4
0
[14C] Leucine incorporated
0
10
20
30
40
Time

Figure 22. Ribosome profile of GSSG treated lysates (Courtesy of Yan Gu).

Protein synthesizing lysates were incubated for 20 min with no addition (A) or with 1.3 mM GSSG (B). Samples were diluted and centrifuged for 1 hr in sucrose gradients as described before.

Protein synthesis mix supplemented with 120 μM ATA were incubated in the presence (D) and absence (C) of 1.3 mM GSSG for 3 min at 30 °C. Samples were diluted with gradient buffer and supplemented with cycloheximide (0.4 mM). Samples were centrifuged for 1 hr in sucrose gradients.

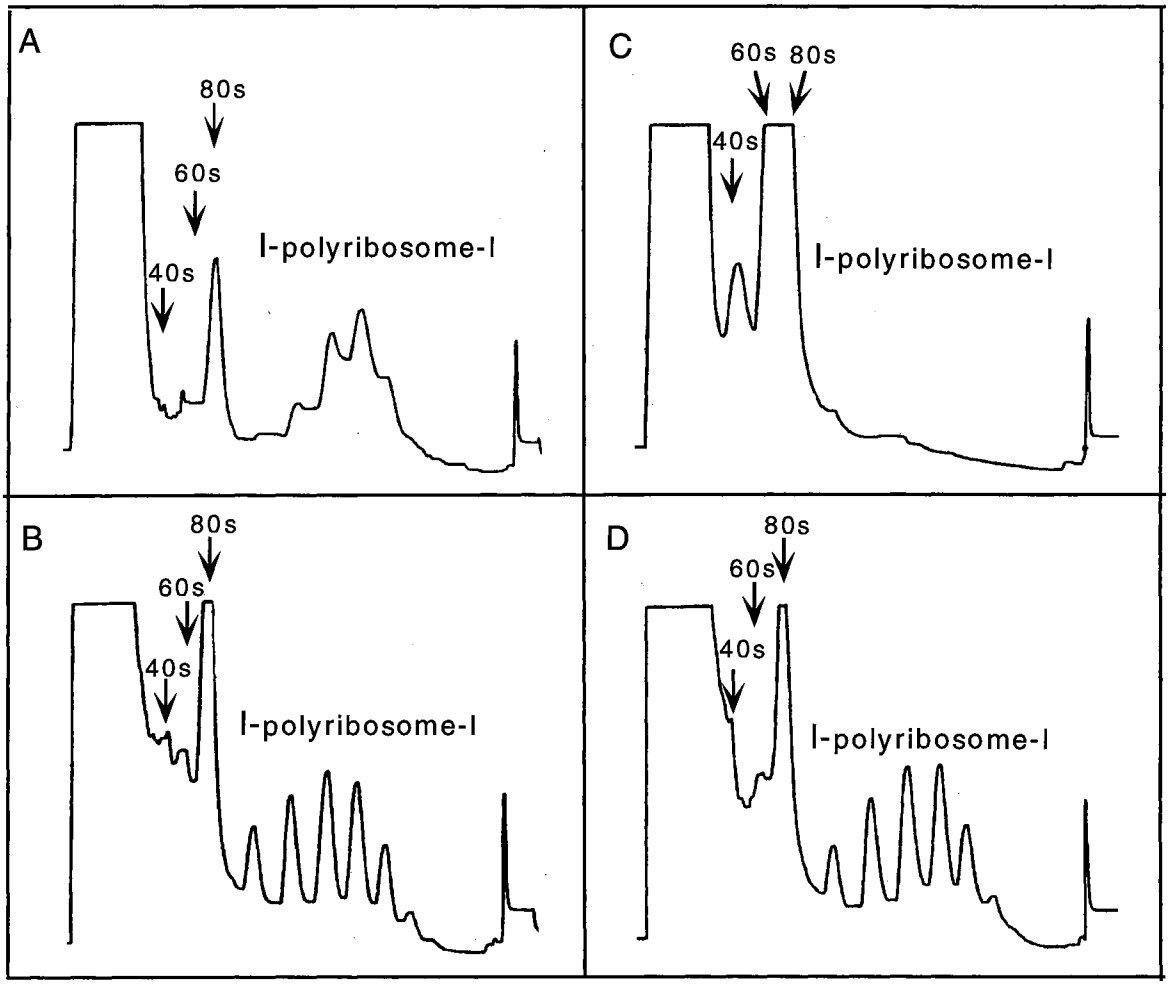

A
80s
60s
40s
I-polyribosome-I
C
60s 80s
40s
I-polyribosome-I
B
80s
60s
40s
I-polyribosome-I
D
80s
60s
40s
I-polyribosome-I

Table III. Effect of hsc70 on eIF-2α phosphorylation and eIF-2B exchange under different stress condition.

Heme-deficient protein synthesis mixes(experiment 1) were incubated at 30 °C in the presence or absence of hsc70 (0.1 mg/ml). After 5 min, an aliquot of the reaction mix was analysed for eIF-2B activity. At the end of 10 min, an aliquot of the reaction mix was taken for analysis of eIF-2α phosphorylation by IEF. With the exception of heme deficiency (experiment 1) in all the other experiments hemin supplemented protein synthesis mixes with added hsc70 (0.1 mg/ml) or the buffer were preincubated for 10 min at 30 °C to activate the hsc70, before exposure to different stress agents. After 5 mins of incubation in the presence of $HgCl_2$, GSSG, 42 °C, or RCM-BSA (experiment 2, 3, 4, and 5), aliquots were taken for analysis of eIF-2α phosphorylation and eIF-2B activity. Experiments were repeated twice with similar results.

Treatments	eIF-2α Phosphorylation O.D x mm^2	% inhibition of GDP exchange
	Experiment 1a	Experiment 1b
- heme	2.475	100
-heme + hsc70	1.588	29
	Experiment 2a	Experiment 2b
$HgCl_2$ (10 μM)	0.099	64
$HgCl_2$ + hsc70	0.005	3
	Experiment 3a	Experiment 3b
GSSG (1.6mM)	0.238	106
GSSG + hsc70	0.022	60
	Experiment 4a	Experiment 4b
42 °C	0.387	94
42 °C + hsc70	0.125	3
	Experiment 5a	Experiment 5b
RCM-BSA (1.5 mg/ml)	0.300	56
RCM-BSA + hsc70	0.101	1

CHAPTER V

EFFECT OF HSC70 BINDING AGENTS ON PROTEIN SYNTHESIS, FOLDING, AND RENATURATION

Introduction

The hsp70 family of molecular chaperones is ubiquitous and highly conserved with diverse biochemical roles both under normal growth conditions and under cellular stress (Craig et al., 1994, Hendrick & Hartl, 1993). In cooperation with other chaperones, Hsp70 facilitates assembly and disassembly of proteins, protein renaturation, preventing aggregation of denatured proteins, and chaperones the transport and folding of newly translated polypeptides in the cytosol as well as within organelles. The hsp70s in mitochondria and the endoplasmic reticulum play an additional role by providing a driving force for protein translocation (Hohfeld & Hartl, 1994, Stuart et al., 1994). A link between the function of the hsp70 class of chaperones and cellular proteolysis has also been demonstrated (Craig et al., 1994). Finally, hsp70s are involved in signal transduction pathways in cooperation with hsp90 (Bohen & Yamamoto, 1994, Rutherford & Zuker, 1994).

Hsp70 recognizes short peptides (of at least seven amino acid residues) enriched in hydrophobic residues (Blond-Elguindi et al., 1993, Flynn et al., 1991, Takenaka et al., 1995). Peptide binding is modulated by ATP. The ATP-bound form of hsp70 binds and releases peptide rapidly, whereas the ADP-bound form binds and releases peptide slowly. Cycling of the hsp70 homolog DnaK between these nucleotide states depends on its interaction with regulatory proteins such as DnaJ and GrpE of *E.coli*. DnaJ activates the

ATPase of the bacterial hsp70 homolog DnaK. Unlike hsp70, DnaJ has more affinity for protein substrates exhibiting secondary and tertiary structure, but exhibits very low affinity for polypeptides in extended conformations. The ability of DnaJ to stimulate DnaK's substrate binding properties correlates with DnaJ's affinity for the various protein substrates. Specifically, DnaJ will activate DnaK to bind to a substrate for which DnaJ has a high affinity, but will effectively stimulate release of DnaK from substrates for which DnaJ has low affinity (Cyr et al., 1994).

GrpE functions as a nucleotide exchange factor allowing rebinding of ATP that in turn promotes substrate dissociation. Recently, p60 has been identified as a potential eukaryotic homolog of GrpE. DnaJ homologs have been identified in all compartments of eukaryotic cells that contain hsp70. Recently, a novel hsc70-interacting protein Hip (p48) has been identified in the mammalian cytosol. Hip stabilizes the ADP state of hsc70 that has a high affinity for protein substrates. Hip functions both as regulator of hsc70 and as a molecular chaperone.

In this study we have investigated the relationship between the regulation of protein synthesis and the ability to fold nascent polypeptides and renature denatured proteins. Rabbit reticulocyte lysate (RRL) was used as the model system because of its unique capabilities for *in vitro* protein synthesis. In addition RRL contains high amounts of molecular chaperones that are involved in protein folding. Luciferase was used as the model protein to study the role of hsc70 in chaperoning protein folding and renaturation, because of its rapid and sensitive luminescence assay. In RRL, protein synthesis is regulated at the initiation level by the heme regulated inhibitor (HRI). HRI, on activation phosphorylates the α subunit of eIF-2 and effectively stops protein synthesis at the initiation level (Levin et al., 1976, Ranu & London, 1976). Previous results indicate that hsc70 negatively regulates HRI. Thus, drugs which inhibit hsp70 function would be predicted to inhibit protein synthesis. Similarly, a requirement for hsc70 in protein folding in the cytosol has been demonstrated (Frydman et al., 1994). Hsc70 appears to act at the

earlier stages of protein folding when the polypeptide backbone is still in an extended conformation (Beckman et al., 1990, Frydman et al., 1994). In this report different hsc70 binding agents were used to determine the importance of hsc70 in the folding and renaturation of luciferase. The effects of different hsc70 binding agents on protein synthesis, luciferase folding and renaturation were compared. We observed that all agents which inhibit the folding of nascent luciferase activate HRI and inhibit protein synthesis. In contrast, peptide FYQLALT and geldanamycin which inhibited luciferase renaturation, did not inhibit either folding of nascent luciferase or protein synthesis. These findings suggest that the rate of protein synthesis is coordinated with the ability to fold newly synthesized protein.

Materials and methods

Materials

Aspirin, indomethacin, ibuprofen, clofibric acid, creatine phosphokinase (Type I), bovine serum albumin (acetylated), luciferin, and luciferase were obtained from Sigma. PepN (NIVRKK), pepF (FYQLALT), and pepSH3 (SPPTPKPRPPRPLPVAPGS) were obtained from Sarkeys Biotechnology Research Laboratory (OSU). L-[^{14}C]Leucine and L-[^{35}S]Methionine were purchased from Du Pont-NEN. TnT coupled reticulocyte lysate and affinity pure anti-luciferase antibody were obtained from Promega. Geldanamycin (GA) was provided by the Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute. The F5 anti -p60 and the JJ3 anti-p23 monoclonal antibodies (mAbs) were provided by David Smith (U. of Nebraska, Omaha) and David Toft (Mayo Medical School, Rochester, MN), respectively. The 4322 anti-hsp70/90 and anti-hsp90 (84/86) antiserum was provided by Stephen Ullrich, NCI. Anti-luciferase affinity purified polyclonal antibodies was purchased from

Promega. N27 anti-hsp70 mAb was purchased from StressGen. Anti-DnaJ were provided by William Welch (University of California, San Francisco).

Buffers

The buffers used were as follows: Assay buffer (AB) consisting of 25 mM Tricine-HCl (pH 7.8), 8 mM $MgSO_4$, 0.1 mM EDTA, 33 mM DTT, 470 μM D-luciferin, 240 μM coenzyme A, and 0.5 mM ATP; stability buffer (SB) consisting of 25 mM Tricine-HCl (pH 7.8), 8 mM $MgSO_4$, 0.1 mM EDTA, 10 mg/ml bovine serum albumin, 10% glycerol, and 1% Triton X-100; Tris buffer (TB) consisting of 10 mM Tris-HCl (pH 7.4); Tris buffered saline (TBS) consisting of TB and 150 mM NaCl; TB/500 consisting TB and 500 mM NaCl; and TB/50 consisting of TB and 50 mM KCl; TBS detergent buffer (TBSD) consisting of TBS and 1% deoxycholate, 1% Triton X-100. and 0.1% SDS.

Luciferase renaturation assay

Firefly luciferase (0.5 mg/ml) in SB (unless otherwise specified) was denatured by incubation at 41^0 C for 10 min (Schumacher et al., 1994). After being cooled on ice, aliquots were diluted 20-fold into heme-deficient protein synthesis mixes (Matts et al., 1991) containing 10 mM creatine phosphate, 20 U/ml creatine phosphokinase. Samples were incubated at 28°C for 10 min, luciferase activity present in 1.5 μl aliquots was determined by dilution into 50 μl AB. Light production was measured for 10 sec in a Lumac (3M) Bioluminometer.

De novo folding of luciferase

Luciferase was synthesized in TNT lysate at 30°C. After 10 min, translational reactions were synchronised by the addition of aurintricarboxylic acid to inhibit re-initiation. Protein synthesis reactions were then incubated for 6 min to allow polyribosomes to run off. Following run off, the different hsp70 binding agents were added to the lysates. Protein synthesis was monitored by incorporation of [^{35}S]met into TCA-insoluble material. At various time points the luciferase activity present in 1.5µl aliqouts was determined by dilution into 50 µl AB. Light productions was measured for 10 sec in a Lumac (3M) Bioluminometer.

Immunoadsorptions of proteins

Affinity pure anti-luciferase antibody (1 µl) was adsorbed to 20 µl of anti-rabbit IgG-agarose in the presence or absence (control) of luciferase (10 µg) for 2 hr on ice as described previously. Heme-deficient protein synthesis mix (30 µl), which had been preincubated for 10 min at 30 °C with different drugs, were added to the agarose pellet. The mixtures were incubated for 10 min at 30 °C with periodic mixing to prevent settling of the agarose. The unadsorbed proteins were separated from adsorbed proteins (pellets) by centrifugation. Pellets were washed with 750 µl of TB/50, three times with TBSD, and once with TB/50.

eIF-2α phosphorylation

RRL's were incubated under protein synthesis condition at 30 °C for 10 min in the presence of the various drugs. Two µl of these mixes were then separated on an isoelectric focussing gel, western blotted and analysed for phosphorylation of eIF-2α, as described previously (Maurides et al., 1989).

eIF-2α GTP/GDP exchange

RRL's were incubated under protein synthesizing conditions at 30 °C in the absence of [^{14}C]Leucine as described (Hunt 1972, Ernst 1978). The rate at which [^{3}H]GDP was exchanged from pre-formed eIF-2•[^{3}H]GDP complexes were measured as described previously (Matts & London, 1984). At the times indicated, 50 µl of PS mix was mixed with 130 µl of ice cold dilution buffer (40 mM Tris HCl, pH7.4, 100 mM KCl, 50 mM KF, 2 mM $Mg(OAc)_2$, 10% glycerol, 40 µM GDP), and 20 µl of preformed eIF2•[^{3}H]GDP complex. Reactions mixes were then incubated at 30 °C for another 2 min. Exchange assays were stopped by the addition of 1 ml ice cold wash buffer, followed by filtration of the reaction mixture through nitrocellulose filters (HAWP 02500, Millipore) which rapidly binds the remaining eIF-2•[^{3}H]GDP complex. Filters were washed with 15 ml of ice cold wash buffer to remove any unbound [^{3}H]GDP.

Polyribosomal gradients

Protein synthesis mixes (100 µl) were pretreated with different drugs for 20 min at 30 °C. These reaction mixes were diluted with 150 µl of ice cold gradient buffer and layered over a 15-40% (w/v) sucrose gradient and centrifuged for 1 h at 45,000 rpm in a Sorvall AH-650 rotor. Polyribosomal profile in the gradient was analyzed as described previously (Matts et al., 1991). Since the drugs showed accumulation of polyribosomes, polyribosomal profiles were also studied in the presence of ATA, an initiation inhibitor, to determine if the large polyribosomes that accumulated in the presence of some of the drugs ran off with time. Failure to run off indicates an inhibition of the elongation phase of translation. Protein synthesis mixes, supplemented with various drugs and 120 µM ATA, were incubated at 30 °C for 1.5 min.

Western blot analysis

Samples were prepared for SDS-PAGE, separated in 10% gels, and transferred to PVDF membrane as previously described (Matts & Hurst, 1992, Matts et al., 1992). Hsp70 and DnaJ was detected with N-27 and anti-DnaJ respectively.

Results

Effect of pharmacological agents on the folding and renaturation of luciferase.

To determine whether hsc70 could be pharmacologically inhibited in RRL, we examined the effect of a number of agents on two processes that are known to require hsc70: folding of newly synthesized protein and protein renaturation. Agents were selected because they had been demonstrated to interact with hsc70 *in vitro*, or we predicted that they might do so. The effect of the agents on both the folding of newly synthesized protein and protein renaturation was examined because previous results with the hsp90 binding drug geldanamycin (Chapter III) indicated that pharmacological agents exist that differentially inhibited the two processes.

Clofibric acid (ClA) an hsc70 binding agent, inhibits luciferase folding and renaturation - ClA, a pharmacological agent that causes peroxisomal proliferation, has been demonstrated to be a specific hsc70 binding drug. Therefore, we examined whether the binding of ClA to hsc70 inhibited its function. ClA was observed to be a strong inhibitor of both the folding of newly synthesized luciferase and renaturation thermally denatured luciferase (Figure 23A and 24A).

Nonsteroidal anti-inflammatory drugs inhibit luciferase folding and renaturation - Aspirin, ibuprofen, and indomethacin are nonsteroidal anti-inflammatory drugs (NSAD) used to treat inflammation and other chronic diseases (Wiessman 1991). NSAD's are inhibitors of cyclooxygenase, with the order of potency of inhibition as indomethacin > ibuprofen > aspirin. It has been shown previously that salicylate and indomethacin treatment induces DNA binding activity of heat shock factor 1 (HSF1) (Jurivich 1992, Lee 1995). These drugs potentiate the effect of heat shock, by maintaing the HSF1 in the activated DNA-binding state for a period twice as long as the control, resulting in enhanced and prolonged hsp70 mRNA transcription. Hsp70 has been suggested to negatively regulate HSF1 DNA binding activity, and it has been proposed that one mechanism through which heat shock causes the activation of HSF1, is by titrating functional hsp70 with denatured protein (Abravaya et al., 1992). This hypothesis is further supported by the observation that constitutive overexpression of the major inducible heat shock protein, hsp70, in transfected human cells reduces the extent of HSF1 activation after a heat stress (Mosser et al., 1993). Thus, one mechanism by which NSAD agents might potentiate the DNA binding activity of HSF-1 is through their ability to interfere with hsc70 function. To test the hypothesis that NSAD agents are hsp70 inhibitors, we examined the effects of salicylic acid, ibuprofen and indomethacin on luciferase folding and renaturation, which are hsc70 dependent processes. Luciferase folding (Fig 23A) and renaturation (Fig 24A) processes were observed to be strongly inhibited by NSAD agents. Similar to the order of potency for NSAD-induced inhibition of cyclooxygenase and activation of HSF-DNA binding activity (Jurivich et al., 1992, Lee et al., 1995, Weissman, 1991), indomethacin showed a stronger inhibition than ibuprofen, which was a stronger inhibitor than salicylate in luciferase folding and renaturation.

Effects of peptides and unfolded protein on luciferase folding and renaturation -

Through the use of phage display peptide libraries, the binding specificities of hsp70 family members have been defined (Blond-Elguindi et al., 1993, Gragerov et al., 1994). Hsp70 family members bind to peptides of seven amino acids in length or longer. The best defined specificity is that of alternating large hydrophobic amino acids. The peptide FYQLALT (pepF) is optimal for binding to hsc70. Recently, it has been shown that basic amino acids C-terminal to several hydrophobic residues, such as NIVRKK (pepN), also have a very high affinity for hsc70.

Peptides- pepSH3 and pepN strongly inhibit both luciferase folding and renaturation- Since hsc70 has a very high affinity for both pepN and pepF, we examined whether these peptides could interfere with the function of hsc70. Consistent with this hypothesis, we find that pepN strongly inhibits both luciferase folding (Fig 23A) and renaturation (Fig 24A), processes known to involve active hsc70 (Frydman et al., 1994, Schumacher et al., 1994). Another peptide, SPPTPKPRPPRPLPVAPGS (pepSH3) which like pepN, contains both hydrophobic and basic amino acids also inhibited luciferase folding and renaturation. PepSH3 was observed to be a slightly stronger inhibitor than pepN in renaturation (Table IV). However, unlike the basic peptides pepN and pepSH3 which inhibit both luciferase folding and renaturation, the high affinity hydrophobic pepF had no effect on luciferase folding, but strongly inhibited luciferase renaturation. The IC50 for luciferase renaturation by pepF was 0.3mM, which is 10 times lower than the IC50 for pepN (Table IV).

RCM-BSA slowed the folding of luciferase and had selective effect on luciferase renaturation - The addition of RCM-BSA has been shown to bind hsc70, and block the interaction of hsc70 with the heme-regulated eIF-2α kinase, and inhibit protein synthesis. To confirm the role of hsc70 in chaperoning the folding of nascent polypeptide chains and protein renaturation, we examined the effect of RCM-BSA on the folding of newly

synthesized luciferase and renaturation of thermally denatured luciferase. Sequestration of hsc70 by the addition of RCM-BSA in reticulocyte lysate significantly delayed the time between completion of luciferase synthesis and the acquisition of enzymatic activity (Fig 23B). However, RCM-BSA needed to be added at much earlier times than pepN for RCM-BSA to have an effect on luciferase folding. In contrast to pepN, RCM-BSA added after the completion of luciferase synthesis did not delay the folding of luciferase. Similarly, RCM-BSA had no effect on the initial rate of renaturation of luciferase that had been denatured in the presence of TritonX-100 and glycerol, unless lysate was pre-incubated with RCM-BSA for 20 min prior to the renaturation assay. Little or no pre-incubation of RRL with RCM-BSA was required for RCM-BSA to inhibit the renaturation of luciferase that had been denatured in the absence of TritonX-100 and glycerol (Fig 24B). Analysis of luciferase sedimentation velocity in glycerol gradients indicated that luciferase that was denatured in the absence of TritonX-100 and glycerol was present in much larger aggregates than luciferase denatured in the presence of these agents.

NSAD, peptides and clofibric acid inhibit the interaction of hsp70 with the denatured luciferase.

While it has been previously demonstrated that peptides and RCM-BSA inhibit the interaction of hsc70 with substrates, it is not known whether ClA or NSAD agents inhibit the interaction of hsc70 with substrates. Therefore, we examined the interaction of hsc70 with denatured luciferase in the presence of these drugs. Hsc70 was coimmunoprecipitated with denatured luciferase in the presence of inhibiting concentration of different drugs in RRL (Fig 25). ClA and NSAD agents clearly inhibited the interaction of hsc70 with denatured luciferase, in a manner similar to that observed for the peptides and unfolded proteins.

Effect of the pharmacological agents and peptides on protein synthesis.

In the last chapter, it was shown that hsc70 has a positive effect on translation, stimulating protein synthesis under a variety of stress conditions by negatively regulating HRI. Therefore, it would be predicted that pharmacological agents and peptides which inhibit hsp70 function may inhibit protein synthesis. Hence, we examined the effect of pharmacological agents and peptides on protein synthesis.

All the drugs used, except pepF, inhibited protein synthesis- ClA, indomethacin, ibuprofen, and salicylate all inhibited protein synthesis. The potency of the NSAD as inhibitors of translation correlated with their potency in inhibiting protein folding and renaturation. Indomethacin was the strongest inhibitor of protein synthesis among NSAD agents, followed by ibuprofen, with salicylic acid being the weakest inhibitor (Table IV). Similar to RCM-BSA, pepN (Fig 26A) and pepSH3 inhibited protein synthesis. However, the hydrophobic peptide, pepF was observed to have no effect on protein synthesis (Fig 26B).

Polysomal profiles indicated that most of these drugs inhibited protein synthesis both at the initiation and elongation level - Unlike RCM-BSA which inhibited protein synthesis with biphasic kinetics, a characteristic of inhibition at the initiation level, all the other drugs examined showed more linear inhibition. This observation suggested that elongation rate was also being inhibited. Therefore, to examine the mechanism of inhibition induced by these agents, polysomal profiles were analysed. Polysomal profiles indicated accumulation of 80S ribosomes in the presence of all the agents except for pepF (Fig 27A), indicating inhibition at the initiation level. In addition, NSAD, ClA and pepN (Fig 27B) and pepSH3 also showed accumulation of the larger polyribosomes. Polyribosome run off experiments were performed to determine whether inhibition at the

elongation step was also occuring. The polyribosomes present in ibuprofen and pepF (Fig 27C) treated lysates ran off at the end of 1.5 min, with all the polyribosomes accumulating the 80S ribosome complex, indicating that the polysomes observed in the absence of ATA were due to an incomplete inhibition of initiation. Whereas in the presence of pepSH3, pepN (Fig 27D), ClA, salicylic acid, and indomethacin the polyribosomes did not run off. A significant amount of larger polyribosomes were observed, indicating an inhibition of elongation.

Effect of the pharmacological agents and peptides on eIF-2α phosphorylation and the guanine nucleotide exchange activity of eIF-2B- To confirm that protein synthesis is inhibited at the initiation level due to the phosphorylation of eIF-2α, isoelectric focussing was performed to examine the phosphorylation state of eIF-2α. With the exception of pepF which was not inhibitory to protein synthesis, all of the other agents increased the phosphorylation level of eIF-2α (Table V).

NSAD, ClA, RCM-BSA and peptides, except for pepF, inhibited the eIF-2B GDP/GTP exchange - Exchange of bound GDP for GTP in the recycling of eIF-2 is catalyzed by eIF-2B. Phosphorylation of eIF-2α inhibits exchange GTP for GDP, because phosphorylated eIF-2 binds and sequesters eIF-2B in a poorly dissociable complex. To further confirm that these agents were causing eIF-2α to be phosphorylated to a level that was sufficient to inhibit eIF-2B activity and consequently the initiation of protein synthesis, the ability of RRL treated with these agents to catalyze guanine nucleotide exchange was determined. ClA, NSAD, pepN, pepSH3, and RCM-BSA inhibited eIF-2B catalyzed exchange of GDP from eIF-2 (Table VI), confirming that the phosphorylation of eIF-2 induced by these agents was sufficient to inhibit initiation of translation. Consistent with the lack of effect on translation and eIF-2α phosphorylation levels, pepF had no effect on eIF-2B activity.

Discussion

We have examined the importance of hsc70 in protein synthesis, folding and renaturation, by using different agents that interact with hsc70. The binding of hsc70 to RCM-BSA, pepN, pepF, and clofibric acid has been documented previously (Alvares et al., 1990, Huang et al., 1994, Matts et al., 1993, Takenaka et al., 1995). Clofibric acid and non steroidal anti-inflammatory drugs aspirin, ibuprofen and indomethacin, completely inhibited the binding of hsc70 to denatured luciferase (Fig 25). In addition to inhibiting the interaction of hsc70 with denatured luciferase, these drugs also lowered the nonspecific interaction of hsc70 with control antibodies. This observation suggests that these drugs inhibit the binding of hsc70 to any substrate. The ability of these drugs to inhibit the binding of hsc70 to substrate might be through the ability of these agents to bind to the substrate binding site of hsc70, or through their binding to a different site (eg. the ATP binding site) which allosterically affects the hsc70 substrate binding site.

The ability of the peptides to inhibit luciferase renaturation correlated with their hydrophobicity; the order of inhibitory potency of the peptides were pepF > pepSH3 > PepN. In contrast, pepF did not inhibit either *de novo* folding of luciferase or protein synthesis. Hsc70 complexed to pepF moves faster than uncomplexed hsc70, and uncomplexed hsc70 moves faster than hsc70 complexed to pepN in a non-denaturing gel (Takenaka et al., 1995). These observations indicate that these peptides are bound to hsc70 molecules that are in different conformations, and hence may affect the function of hsc70 differently. Moreover pepN is a weaker stimulator of the hsc70 ATPase activity compared to pepF (Takenaka et al., 1995). In addition, pepN has sequence similarity with HDJ-2, a DnaJ homolog from humans (Takenaka et al., 1995). Similarity of pepN with HDJ-2 suggests that pepN could be binding to the DnaJ binding site on hsc70, thus preventing DnaJ from binding to hsc70. Interaction between DnaJ and hsp70 is important because

hsp70 does not bind compact protein-folding intermediates with high affinity. DnaJ recognizes structural features of compact protein folding intermediates and stabilizes them in a conformation in which binding sites for hsp70 are exposed, and thus facilitates DnaK binding to substrate. Therefore, it is likely that inhibition of hsc70 will have different consequences depending on the conformation of the inhibited hsc70.

RCM-BSA inhibited the folding of luciferase only when added during the synthesis of luciferase. RCM-BSA added in the later stages of folding did not inhibit luciferase folding. This observation suggests that nascent proteins have higher affinity for hsc70 than RCM-BSA, and that RCM-BSA needs to be added earlier to successfully compete for and sequester the active pool of hsc70. This is consistent with the observation that RCM-BSA inhibits luciferase renaturation only when the lysate is preincubated with RCM-BSA prior to the addition of denatured luciferase. This observation again suggests that denatured luciferase is a better substrate for hsc70 than RCM-BSA. Less preincubation was required for RCM-BSA to inhibit luciferase renaturation when luciferase is denatured in the absence of triton and glycerol, suggesting that the luciferase that is more highly aggregated may titrate hsc70 faster and have an additive effect to RCM-BSA and inhibit renaturation of soluble denatured luciferase further.

Affinity of hsc70 for luciferase versus RCM-BSA may reflect the affinity of DnaJ for the two proteins, because DnaJ stabilizes complexes between hsp70 and the substrate proteins for which DnaJ has high affinity. In contrast, DnaJ stimulates dissociation of complexes between hsp70 and the substrate polypetides for which DnaJ has low affinity. DnaJ binds protein substrates exhibiting secondary and tertiary structures, like denatured luciferase and folding intermediates of nascent luciferase, but exhibits very low affinity for polypeptides in extended conformation like CMLA (Cyr et al., 1994, Wawrzynow et al., 1995). Since hsp70 can bind to polypeptides with extended conformation preincubation may be required because of the slow binding of hsc70 to RCM-BSA in the absence of the interaction of DnaJ.

Non steroidal anti-inflammatory drugs potentiate the heat shock response by inducing HSF1 DNA binding activity. One potential mechanism of action for NSAD may be through their ability to inhibit the chaperone function, since hsc70 has been suggested to be the negative regulator of HSF-1. Consistent with this possibility NSAD: (i) inhibited the interaction of hsc70 with denaturing luciferase; (ii) inhibited folding and renaturation of luciferase; and (iii) inhibited protein synthesis - processes which are known to require hsc70. Moreover, in the presence of higher doses of NSAD and clofibric acid, we observed that the background level of active folded luciferase is also reduced during de novo folding of luciferase. This is consistent with the observation that luciferase is not stable above 28 °C. At temperatures above 28 °C, hsc70 is required to maintain luciferase in an active folded conformation. In the presence of these agents the activity of existing luciferase is lost as it denatures and no hsc70 is available to renature the enzyme.

The data presented in this report indicate that chaperones which are involved in nascent protein folding are also intimately involved in the regulation of protein synthesis (Table VII). All agents tested that inhibited *de novo* folding of nascent luciferase also activated HRI and inhibited protein synthesis. However, not all agents that inhibited renaturation of thermally denatured luciferase inhibit *de novo* folding of luciferase or protein synthesis (Table VII). Geldanamycin (GA) an hsp90 binding drug, inhibits luciferase renaturation, but has no effect on protein synthesis or *de novo* luciferase folding (Thulasiraman et al., Submitted). Similarly, pepF did not inhibit the folding of nascent luciferase or protein synthesis. These observations are consistent with the finding that protein renaturation and *de novo* protein folding do not follow identical folding pathways (Frydman & Hartl, 1996, Thulasiraman et al., Submitted). This suggests that protein synthesis is coordinated with the ability to fold newly synthesized polypeptides. Thus protein synthesis becomes inhibited when the cell cannot fold the newly synthesized proteins. Agents that inhibit protein renaturation may also cause protein synthesis to become inhibited, when the amounts of denatured protein accumulates to a level that

sequesters sufficient amount of chaperones that then interfere with nascent protein folding. The ability of the protein synthesis inhibitor cycloheximide to protect cells from the toxic effects of heat shock (Lee et al., 1994) suggests that misfolded newly synthesized proteins are cytotoxic to cells. The ability of a cell to shut down protein synthesis may then be a critical mechanism for maintaining cell viability under adverse environmental conditions.

Figure 23. Effect of drugs on luciferase folding.

After 10 min of luciferase synthesis in TnT lysate at 30 °C, protein synthesis was inhibited at the initiation level by the addition of ATA. Polyribosomes were allowed to complete the synthesis and run off of luciferase for another 6 min. Water, 15 mM clofibric acid, 20 mM salicylic acid, 3.5 mM ibuprofen, 1.5 mM indomethacin, 10 mM pepSH3, 5 mM pepN, 0.5 mM pepF, or 5 μM geldanamycin were then added and aliquots were taken at 16 min and 23 min for determining protein synthesis and luciferase activity. Amount of protein synthesis under different conditions were similar. To determine the effect of BSA and RCM-BSA in luciferase folding, luciferase was allowed to synthesize for 6 min, followed by the addition of ATA, edeine and BSA/RCM-BSA (44 μM). Aliquots were taken at 18 min and 22 min for determining protein synthesis and luciferase activity. Protein synthesis under these two conditions were similar.

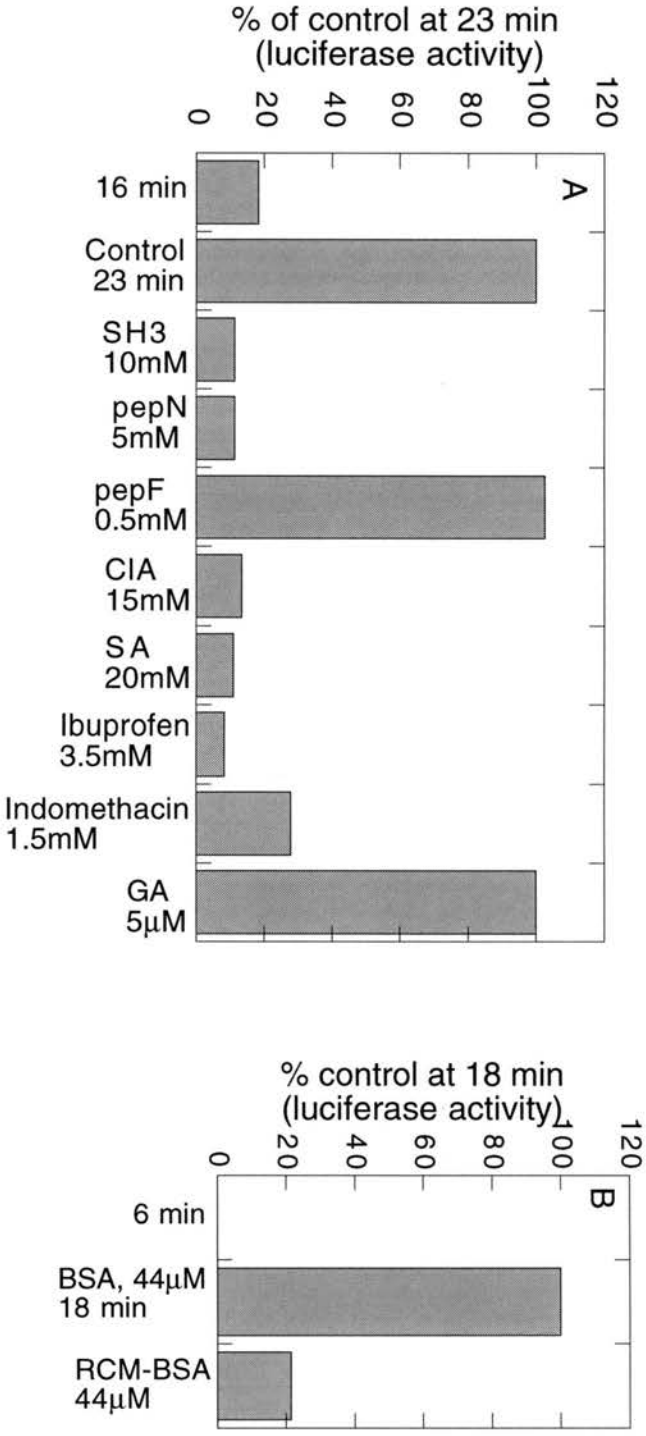

% of control at 23 min
(luciferase activity)
0
20
40
60
80
100
120
A
16 min
Control
23 min
SH3
10mM
pepN
5mM
pepF
0.5mM
CIA
15mM
SA
20mM
Ibuprofen
3.5mM
Indomethacin
1.5mM
GA
5μM
% control at 18 min
(luciferase activity)
0
20
40
60
80
100
120
B
6 min
BSA, 44μM
18 min
RCM-BSA
44μM

Figure 24. Effect of drugs on luciferase renaturation

A) Thermally denatured luciferase (1µl of 1mg/ml) was added to 20 µl of heme-deficient protein synthesis mixes containing either no additions or, 4 mM pepSH3, 6 mM pepN, 0.4 mM pepF, 17 mM clofibric acid, 20 mM salicylic acid, 3.3 mM ibuprofen, 1.3 mM indomethacin, or 0.7 µM geldanamycin. After 10 min the amount of luciferase renatured was measured. B) Luciferase which was denatured in the presence or absence of triton and glycerol was added to heme deficient protein synthesis mixes preincubated with RCM-BSA or BSA for 20 min or 0 min at 30 °C. The amount of luciferase renatured at the end of 10 min was measured.

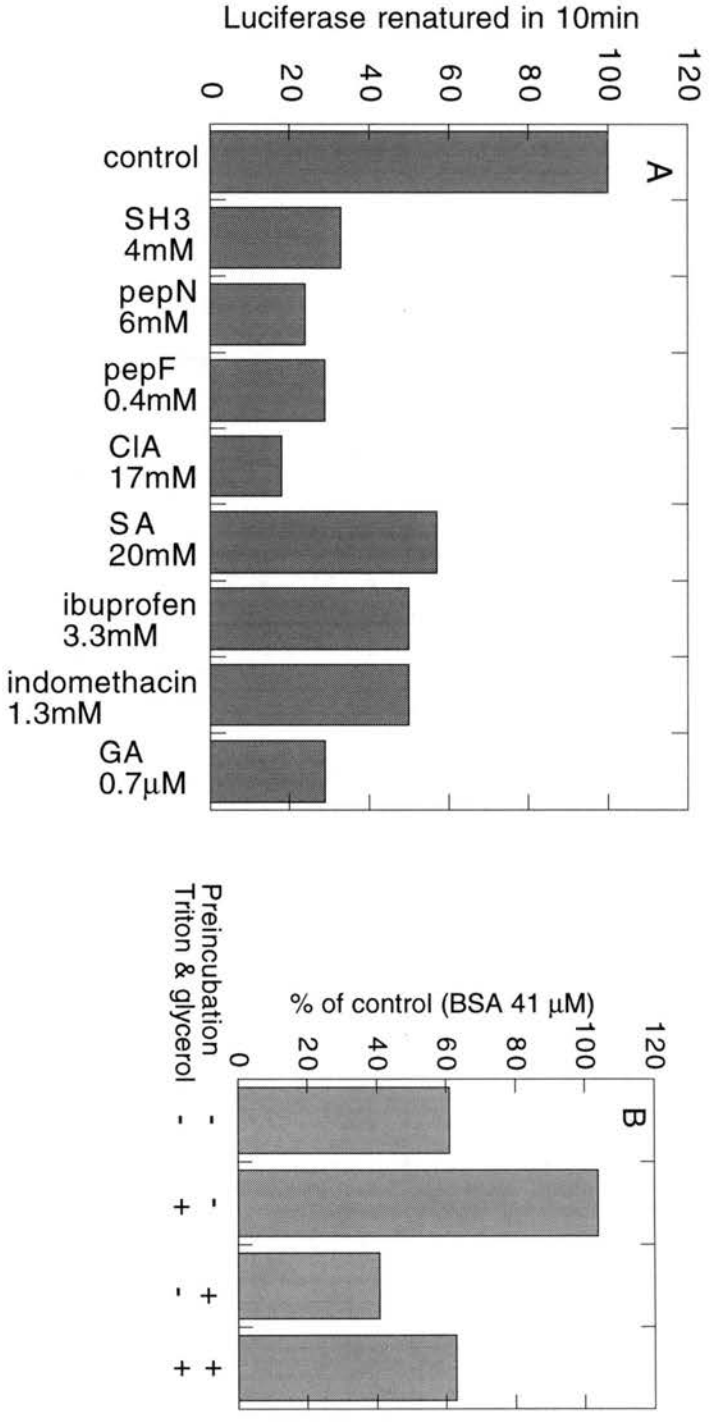

Luciferase renatured in 10min
0
20
40
60
80
100
120
control
SH3
4mM
pepN
6mM
pepF
0.4mM
ClA
17mM
SA
20mM
ibuprofen
3.3mM
indomethacin
1.3mM
GA
0.7μM
A
Preincubation
Triton & glycerol
% of control (BSA 41 μM)
B

Figure 25. Effect of the drugs on the interaction of hsc70 with luciferase.

Luciferase was adsorbed to affinity-purified anti-luciferase antibody bound to GaR-agarose as described previously. The immobilized luciferase was incubated for 10 min at 30 °C in heme-deficient protein synthesis mixes that were preincubated for 10 min at 30 °C in the presence either DMSO (lane 1), 1 mM pepF (lane 2), 69 μM BSA (lane 3), 69 μM RCM-BSA (lane 4), no addition (lane 5), 10 mM pepN (lane 6), 20 mM pepSH3 (lane 7), 60mM salicylic acid (lane 8), 10 mM ibuprofen (lane 9), 6.5 mM indomethacin (lane 10), or 60 mM clofibric acid (lane 11). The agarose pellets were washed, and analyzed by SDS-PAGE and western blotting as described under "Materials and Methods". The experiment was repeated two times with similar results.

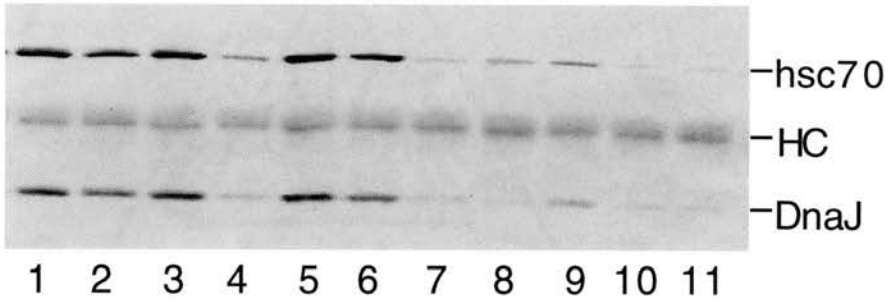
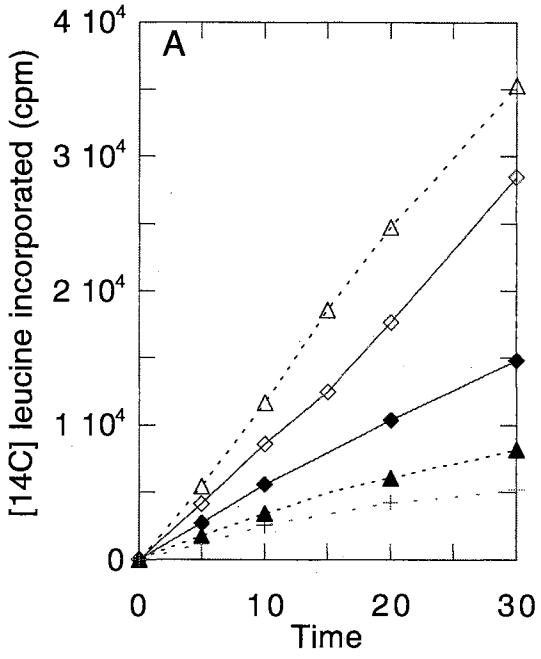

hsc70
HC
DnaJ
1 2 3 4 5 6 7 8 9 10 11

Figure 26. Effect of drugs on protein synthesis (Courtesy of Yan Gu).

A) Protein synthesis mixes supplemented with 0 mM (Δ), 1.25 mM (◇), 2.5 mM (◆), 3.75 mM (▲), and 5mM (+) concentration of pepN , were incubated at 30 °C. Protein synthesis was measured by following incorporation of [^{14}C]leucine into acid-precipitable protein at the times indicated. B) Protein synthesis mixes supplemented with no addition (◇) or 0.33mM pepF (◆) were incubated at 30 °C. Protein synthesis was measured by following incorporation of [^{35}S]methionine into acid-precipitable protein at the times indicated.


A
[14C] leucine incorporated (cpm)
4 10^4
3 10^4
2 10^4
1 10^4
0
0
10
20
30
Time

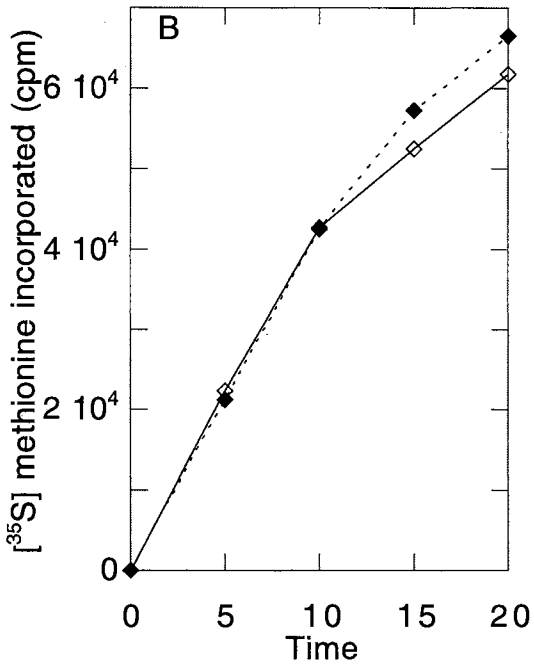

B
[35S] methionine incorporated (cpm)
6 10^4
4 10^4
2 10^4
0
0
5
10
15
20
Time

Figure 27. Ribosome profile of ribosomes in sucrose gradients (Courtesy of Yan Gu).

Protein synthesizing mixes supplemented with either 0.25 mM pepF (A) or 5 mM pepN (B) were incubated at 30 °C for 20 min. Samples were diluted and centrifuged for 1hr in sucrose gradients as described previously.

Protein synthesis mixes supplemented with 120 μM ATA and with either 0.25 mM pepF (C) or with 5 mM pepN (D) were incubated at 30 °C for 1.5 min. Samples were diluted with gradient buffer and supplemented with cycloheximide (0.4 mM). Samples were centrifuged for 1 hr in sucrose gradients.

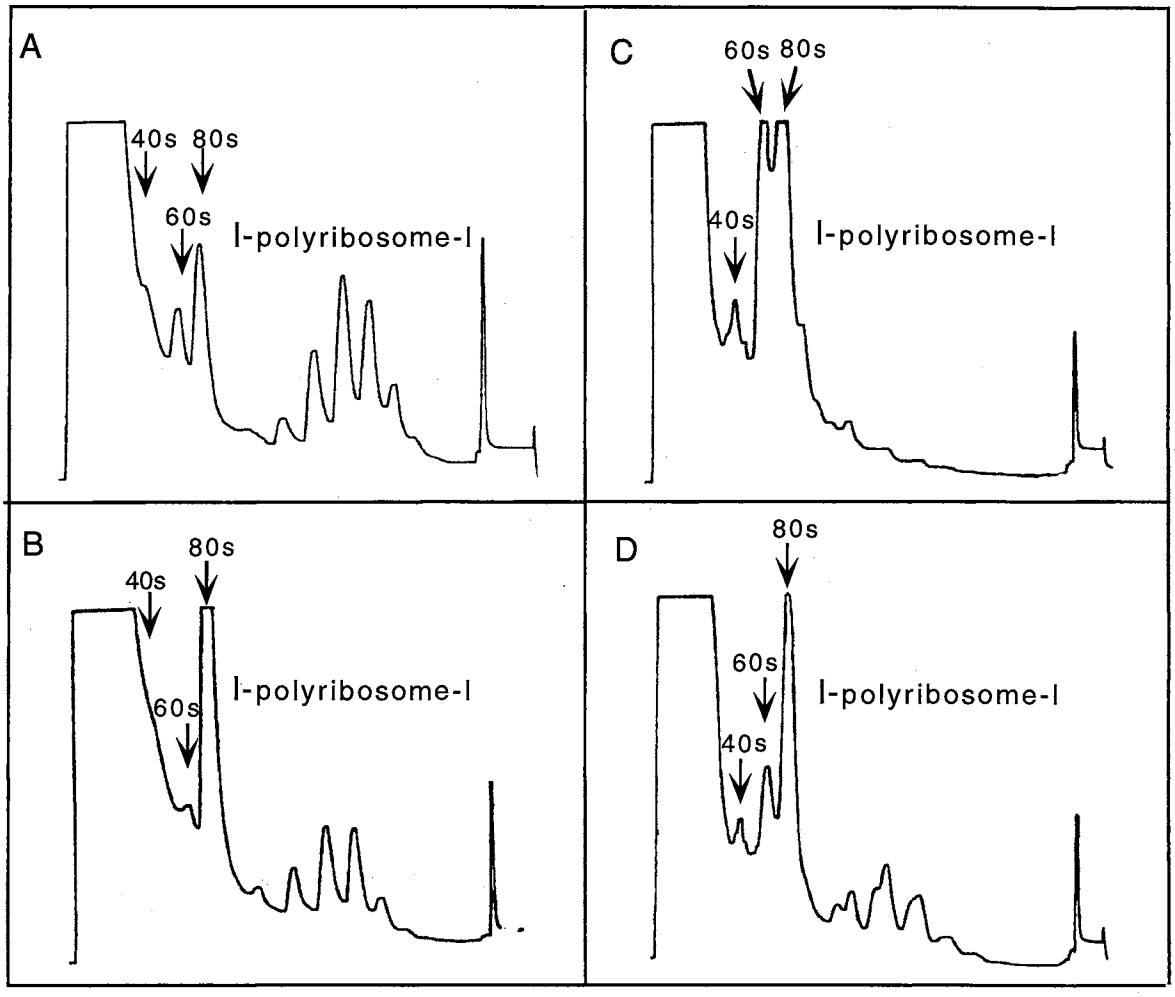

A
40s
80s
60s
I-polyribosome-I
C
60s
80s
40s
I-polyribosome-I
B
40s
80s
60s
I-polyribosome-I
D
80s
60s
40s
I-polyribosome-I

Table IV. IC50 for protein synthesis inhibition and luciferase renaturation for the drugs:

Protein synthesis was carried out in the presence of varying concentrations of different drugs, as described for pepN in fig 26. The concentration of drug required to inhibit protein synthesis by 50% relative to the control (IC50) was estimated from plots of drug concentration vs % inhibition. Similarly renaturation of thermally denatured luciferase was carried out in the presence of varying concentrations of each drug and their IC50 was determined.

Drugs	IC50 for protein synthesis (mM)	IC50 for luciferase renaturation (mM)
pepN	4.2	3
pepF	NA	0.3
SH3	6	2
ClA	8	11
aspirin	17	23
ibuprofen	5	3.33
indomethacin	2.8	1.3
Geldanamycin	NA	0.0002

Table V. Effect of the drugs on the phosphorylation of eIF-2α.

Protein synthesis mixes were supplemented with no addition (none), clofibric acid, NSAD's, pepN, pepF, SH3, RCM-BSA, and incubated at 30 C for 10 min. Protein synthesis mix (2μl) was mixed with IEF buffer and run on IEF gels and transferred onto a membrane and western blotted for eIF-2α. The values in the table indicate the densitometric reading of the phosphorylated band of eIF-2α.

DRUGS	Densitometric reading of phosphorylated eIF-2α (O.D x mm^2)
None	0.097
10 mM clofibric acid	1.149
20 mM salicylic acid	0.705
1.7 mM ibuprofen	0.678
0.9 mM indomethacin	1.009
pepF	0.087
3.3 mMpepN	0.477
6.6 mMSH3	0.686
22 μM RCM-BSA	0.300

Table VI. Inhibition of eIF-2B catalyzed GDP exchange by the drugs.

Lysates were preincubated with no addition, or clofibric acid, NSAD's, pepN, pepF, or SH3 at concentrations indicated in the table, for 20 min at 30 °C. At the end of 20 min, 50 µl of the mix was diluted with 150 µl of dilution buffer followed by the immediate addition of eIF-2•[^{3}H]GDP complex (Matts & London 1984). The amount of [^{3}H]GDP dissociated from eIF-2 in 2 min was determined as previously described.

DRUGS	Inhibit. of eIF-2B exchange (% of heme deficiency)
hemin deficiency	100
10 mM clofibric acid	92.8
25 mM salicylic acid	74
7.5 mM ibuprofen	101
6 mM indomethacin	104
pepF	no inhibition
pepN	65
SH3	10
14 μM RCM-BSA	66

Table VII. Overview of the effect of different drugs on protein synthesis, luciferase folding and renaturation: (+) indicates inhibition and (-) indicates no effect.

Drugs added	Protein synthesis inhibition	Luciferase renaturation	*de novo* folding of luciferase	Polysomal profile Initiation inhibiton	elongation inhibition	eIF-2α phosphory-lation	eIF-2α GTP/GDP exchange
hsp90 binding drug geldanamycin	-	+	-	-	-	-	-
hsp70 binding drug clofibric acid	+	+	+	+	+	+	+
NSAD salicylic acid	+	+	+	+	+	+	+
ibuprofen	+	+	+	+	-	+	+
indomethacin	+	+	+	+	+	+	+
Peptides & unfolded proteins pepN	+	+	+	+	+	+	+
PepF	-	+	-	-	-	-	+
SH3	+	+	+	+	+	+	+
RCM-BSA	+	-	+	+	-	+	+

CHAPTER VI

SUMMARY

Concluding Remarks

Evidence that hsp70 regulates stress response at the transcriptional level.

The heat shock response is characterised by the transcriptional activation of a number of heat shock genes and is accompanied by a shut down of normal protein synthesis at the level of initiation. There is selective translation of the mRNAs encoding for heat shock proteins. Accumulation of denatured polypeptides may be the signal that initiates the induction of the heat shock response (Craig, 1991, Morimoto et al., 1992, Sorger, 1991). Transcriptional activation of the heat shock genes occurs through the activation of heat shock transcription factor (HSF). Hsp70 has been proposed to bind HSF, maintaining it in an inactive conformation (Craig, 1991, Morimoto et al., 1992, Mosser et al., 1993, Sorger, 1991). Production of excess substrates for hsp70 (e.g., denatured proteins) would disrupt the hsp70/HSF binding equilibrium, inducing the heat shock response. Inactivation of the HSF would occur upon the reassociation of hsp70 with HSF when sufficient hsp70 has been synthesized (Mosser et al., 1993). The hypothesis that hsp70 is a negative regulator of HSF is supported by the observation that constitutive overexpression of hsp70 in transfected human cells reduces the extent of HSF activation in response to heat stress and heavy metal ion treatment (Mosser et al., 1993). Moreover treatment with cycloheximide, an inhibitor of protein synthesis, before and during heating facilitated the dissociation of HSF-HSE complex and terminated

transcription activity earlier (Lee et al., 1994). It is proposed that the free pool of hsp70 is increased by inhibiting protein synthesis. An increase in the level of free hsp70 will more effectively protect or repair thermolabile targets and consequently affect the regulation of heat shock response.

Evidence that hsc70 regulates stress response at the translational level.

Like the heat induced transcriptional effects, recovery of protein synthesis in heat stressed cells and the acquisition of resistance to stress-induced translational inhibition also correlate with the synthesis and accumulation of hsp70 within a variety of cells (DiDomenico et al., 1982, Laszlo, 1988, Mizzen & Welch, 1988). Moreover, HRI, which regulates protein synthesis at the initiation level, is associated with hsc70. Addition of denatured proteins and heat shock leads to the activation of HRI and dissociation of hsc70 from HRI. This observation led to the hypothesis that hsc70 negatively regulates HRI. This report shows that hsp70 negatively regulates HRI, since purified hsc70 prevented protein synthesis inhibition in RRL exposed to heat shock, heavy metal ions, and oxidising agents. Heat shock causes a decrease in the functional pool of hsc70, probably by causing an accumulation of denatured proteins that titrates the available hsc70 and thus leading to the activation of HRI and HSF. The effect of hsc70 on HRI activation is similar to the effect of hsp70 on HSF, suggesting that hsp70 regulates stress response both at the transcriptional and at the translational level.

Effect of inhibition of translation in response to stress - Protein synthesis inhibition in response to stress may be very useful in protecting cells from adverse physiological conditions. Protein synthesis inhibitors and agents that stabilize protein structure, protect cells from cytotoxicity of stress (Edington et al., 1989, Lee et al., 1990a, Lee & Dewey, 1986, Lee et al., 1987, Lee et al., 1990b). It has been proposed that denatured newly

synthesized nascent polypeptides are extremely cytotoxic to stressed cells (Beckman et al., 1990, Lee et al., 1990b), and that inhibition of protein synthesis is a necessary event required to maintain the viability of stressed cells. Consistent with this hypothesis we observe that all agents which inhibit nascent protein folding inhibited protein synthesis (Chapter V).

Role of hsc70 in inhibiting HRI under heme deficiency - HRI is also activated under heme deficiency. While HRI is known to be directly regulated by binding of hemin, accumulation of globin chains in the absence of sufficient heme to allow assembly of hemoglobin might sequester hsc70 and contribute to the degree to which HRI is activated during heme deficiency. This notion is consistent with the observation that addition of cycloheximide to hemin deficient lysate prior to polyribosome run off leads to increased HRI activity (Ramaiah et al., 1992). Moreover, addition of purified hsc70 reverts protein synthesis inhibition due to heme deficiency (Chapter IV).

Proposed mechanism of action of hsc70 in negatively regulating HRI - Hsc70 may affect HRI activation or activity by a number of mechanisms. Excess hsp70 might inhibit the folding of HRI to an active conformation. This speculation is supported by the results of computer simulation studies on chaperone-assisted folding (Sfatos et al., 1996). This model studies suggests that if the rate at which chaperones bind to folding substrate is faster than the rate at which the peptide folds, chaperones will retard the rate of protein folding. Since rate of chaperone binding is proportional to the rate constant for chaperone binding and the concentration of chaperone, changes in hsc70 concentration could either stimulate or inhibit protein folding depending on the protein target. This notion that hsc70 can inhibit protein folding is consistent with previous reports that luciferase renaturation is inhibited in the presence of excess hsc70 *in vitro* (Schumacher et al., 1994).

Alternatively hsc70 may be involved in maintaining the ability of the hemin binding domain in a conformation competent to bind hemin. HRI is negatively regulated by reversible binding by hemin. In the absence of hsc70, the heme binding domain of HRI may denature, causing the activation of HRI as its capacity to be regulated by the binding of hemin is lost. This is consistent with HRI in lysates containing low levels of hsc70 being converted more rapidly to a heme irreversible form (Matts & Hurst, 1992).

Inability to fold.

Evidence is now accumulating in the literature that the etiology of a number of human diseases involve altered protein folding (Thomas et al., 1995). Since protein folding in the cell is so complex, there is a significant likelihood of defects arising in the process (Fig 28). Potentially, thermodynamic destabilization of the native or an intermediate state, alteration of the folding kinetics, prolonged or inappropriate associations with molecular chaperones or folding enzymes, preferential formation of off-pathway or toxic conformations, or folding in an improper compartment, could all lead to loss of functional proteins and thus, a defective phenotype. Inability of an essential protein to form its native structure under physiological conditions may be the basis of a variety of human diseases. For example, (i) development of fatal disease cystic fibrosis is caused by mutations of the gene encoding cystic fibrosis transmembrane conductance regulator (CFTR) (Denning, 1992); (ii) mutation of fibrillin leads to marfan syndrome (Wu et al., 1995), (iii) mutation of α-ketoacid dehydrogenase complex leads to maple syrup urine disease (Chuang, 1994). Besides genetic deficiency, nutritional factors also lead to misfolding of proteins, for example scurvy is caused due to lack of vitamin C (Eyre, 1980). In addition, in some cases a stable fold is formed that itself is toxic to the cell, for example, prion proteins in scrapie (Gasset et al., 1993), and amyloid plaques in

Alzheimer's disease (Soto et al., 1995). Finally, sickle cell anemia and β-thalassemia are caused by conformers of hemoglobin, that while active, are prone to polymerization.

Involvement of hsps in disease states.

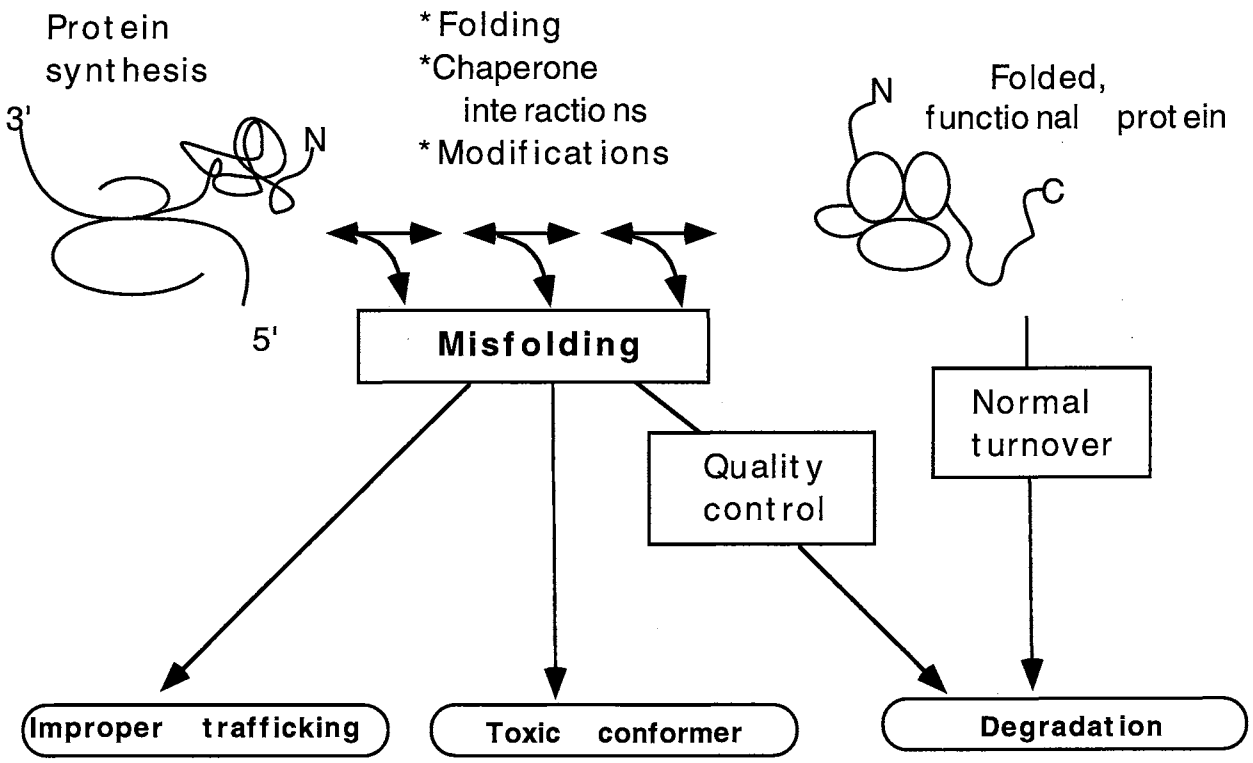
The elevated expression of genes encoding hsps has been detected in numerous disease states, including ischemia, reperfusion damage, oxidant injury, cardiac hypertrophy, fever, inflammation, bacterial and parasitic infection, metabolic diseases, neoplasia, and in sites of cell and tissue damage (Morimoto et al., 1994). The hsps activities are likely to exert themselves at multiple levels, by serving to detect the onset of physiological stress, to prevent subsequent damage resulting from the synthesis and accumulation of nonnative proteins, and as a key component of cellular repair processes following injury (Morimoto et al., 1994). Hsps, whether induced by prior exposure to nontoxic stress conditions or by overexpression of genes encoding hsps, have been shown to protect cells against a broad range of toxic conditions, including oxidative stress, tumor necrosis factor α, extreme temperatures, ethanol, heavy metals, and cellular damage following ischemia or sepsis induced injury (Angelidis et al., 1991, Jaattela et al., 1992, Li et al., 1992, Sanchez, 1992).

Altered interaction of mutant proteins with molecular chaperones has been observed in a variety of human diseases. For example, CFTR, misfolding of which causes cystic fibrosis, interacts with hsp70 and altered hsp70/CFTR interaction has been observed in cystic fibrosis (Pind et al., 1994). Besides CFTR, p53 also interacts with hsp70. The mutant oncogene product p53Val135 has a prolonged interaction with hsp70 relative to wild type p53 (Yehiely & Oren, 1992). Moreover, the ability of oncogenic p53 and Ras to induce neoplastic transformation was reduced by overproducing hsp70. Altered interaction with other chaperones besides hsp70 has also been observed in other human disease. For

example, in hereditary medium chain acyl-CoA dehydrogenase (MCAD) deficiency, the K304E variant of the tetrameric enzyme has altered interaction with hsp60. Expression of the bacterial hsp60, GroEL and its cohort, GroES, partially suppresses the inability of the K304E variant to fold productively in a heterologous expression system (Bross, 1993). Thus methods for altering chaperone levels deserve serious consideration as a means of combating human diseases.

Non steroidal anti-inflammatory drugs have been shown to delay Alzheimers disease and cystic fibrosis (Schehr, 1994). The primary action of these drugs is by inhibiting inflammation (Siegel et al., 1979). Another mechanism could be through poising cells to respond to stress by activating HSF to bind DNA and enhancing the production of hsp70, by maintaining trancriptional activity of HSF in an active state for an extended period of time (Lee et al., 1995). Consistent with this hypothesis exposure of HeLa cells to NSADs and mild heat shock, led to a stronger cytoprotection towards subsequent exposure to very high temperatures, when compared to exposure to mild heat stress alone (Lee et al., 1995). The ability of NSADs to potentiate stress response may have direct implications for inflammation and other diseases, as the heat shock response can now be modulated by drugs of clinical importance under physiological relevant conditions. Examining the effect of pharmacological agents on protein synthesis or protein folding in the RRL may represent a rapid method to screen for compounds that may either be toxic or be beneficial pharmacologically.

Figure 28. Diversity of protein misfolding in disease. (Thomas *et al.* 1995)


Protein
synthesis
3'
N
5'
* Folding
*Chaperone
interactions
* Modifications
N
Folded,
functional protein
C
Misfolding
Normal
turnover
Quality
control
Improper trafficking
Toxic conformer
Degradation

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