TRANSLATIONAL REGULATION BY HEAT SHOCK PROTEINS

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Submitted to the Faculty of the
Graduate College of the
Oklahoma State University
in partial fulfillment of
the requirements for
the Degree of
DOCTOR OF PHILOSOPHY
May, 1993

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HEAT SHOCK PROTEINS

Thesis Approved:

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PREFACE

The translational regulation by heat shock proteins (hsps) in rabbit reticulocyte lysates has been studied. First, using the techniques of immunoprecipitation and Western blot analysis, the interactions of the heme-regulated eIF-2α kinase (HRI) with the 90 kDa heat shock protein (hsp90), hsp70, and hsp56 or the EC1 antigen in rabbit reticulocyte lysates have been determined, which indicates that HRI exists in reticulocyte lysates as a multiprotein complex with heat shock proteins. Second, the function of heat shock proteins in regulating the activation and activity of HRI in normal or stressed conditions including the addition of denatured proteins, heat shock, and the addition of oxidants, such as N-ethylmaleimide or heavy metal ions (Hg^{++}), have been investigated. Third, to better understand the biochemical function of the associations of HRI with hsps, the mechanism for assembly of HRI-hsps complex, and perhaps also general mechanisms for the assembly of multiprotein complexes *in vivo*, a serious of experiments for reconstituting the HRI-hsps complex have been carried out. Finally, based on the observations described above, a general model for the translational regulation by heat shock proteins in rabbit reticulocyte lysates has been proposed. The research presented here greatly enriches our knowledge in translational control and the function of heat shock proteins and strongly supports the prevailing concept that heat shock proteins, working as molecular chaperones, play the fundermental roles in living cells.

As I am completing this dissertation, I wish to express my deepest appreciation to my research advisor Dr. Robert L. Matts for his intelligent guidance, constructive advice, invaluable assistance, and unforgetable friendship throughout the years. Many thanks go to other committee members, Dr. Richard Essenberg, Dr. Robert Gholson, Dr. Franklin

Leach, and Dr. John Sauer, who have played essential roles in my training and in the exectution of this project.

I wish to express my sincere gratitude to Robin Hurst for her thoughtful suggestion, kind assistance, and sincere friendship. I would also like to thank her for performing the experiments of denatured proteins inhibiting protein synthesis and their binding to hsp70 described in Chapter III. I am grateful to Dr. Ulrich Melcher for his careful reading and correcting my proposal for the oral exam. I appreaciate the suggestion, advisement, assistance, and friendship from Dr. Steven Hartson and Mr. Yu Lu. I also sincerely thank the faculty of the Department for their teaching, collaboration, and friendship. I appreciate the Department for financial support.

I wish to thank Dr. Jane-Jane Chen (Massachusetts Institute of Technology), Dr. Gary Perdew (Purdue University), Dr. David Toft (Mayo Clinic), Dr. Lee Faber (Medical College of Ohio), Dr. William Welch (University of California, San Francisco), and Dr. Stephen Ullrich (National Cancer Institute) for providing the antibodies used in this research.

Finally, I wish to express my greatest appreciation to my wife Chenghua for her invaluable assistance, understanding, encouragement, and love. My deepest appreciation is extended to my parents, Shinan and Shusheng, my brother Zhengye, and my sister Qiaoming, for their understanding, moral support, and love. My sincere gratitude is due to my brother Zhengye for his disinterested support that made it possible for me to came to the United States for the study. I wish this dissertation will be a piece of consolation to them.

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LIST OF ABBREVIATIONS

BCIP	5-bromo-4-chloro-3-indolyl-phosphate
BPB	bromophenyl blue
BSA	bovine serum albumin
cDNA	complementary DNA
DMSO	dimethylsulfoxide
DOC	deoxycholate
dsRNA	double-stranded RNA
DTT	dithiothreitol
eIF-2	eukaryotic initiation factor 2
eIF-2(αP)	eIF-2 phosphorylated on α-subunit
eIF-2α	38 kDa α-subunit of eIF-2
GAG	goat anti-mouse IgG
GAM	goat anti-mouse IgM
GEF	guanine nucleotide exchange factor
HRI	heme-regulated eIF-2α kinase
HRI	heme-regulated inhibitor
hsc70	constitutively expressed hsp70
HSE	heat shock element
HSF	heat shock factor
HSP	heat shock protein
hsp	heat shock protein
hsp70	members of 70 kDa heat shock proteins

hsp90	members of 90 kDa heat shock proteins
IFCS	inactivated fetal calf serum
IgG	immunoglubin G
IgM	immunoglubin M
kDa	kilodaltons
LA	bovine α-lactalbumin
LG	bovine β-lactogloblin A
mAb	monoclonal antibody
NBT	nitro blue tetrazolium
NFDM	non-fat dry milk
NEM	N-ethylmaleimide
PAGE	polyacrylamide gel electrophoresis
PPI	peptidylproline cis-trans isomerase
RCM	reduced carboxymethylated
RF	reversing factor
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis

CHAPTER I

INTRODUCTION

The rabbit reticulocyte lysate was the first in vitro eukaryotic translation system developed. It is still the most efficient mammalian cell-free system, synthesizing protein at the same rate as the intact cell for periods of 60 min or more (Lamfrom and Knopf, 1964; Adamson et al., 1969; Jackson et al., 1983). Studies of the control on protein synthesis in rabbit reticulocytes and their lysates, have provided us with much of our understanding of translational regulation. The finding that heme is necessary for the initiation of protein synthesis (Bruns and London, 1965; Zucker and Schulman, 1968; Adamson et al., 1969; Hunt et al., 1972) was a stepping stone to our current knowledge of the translational regulation at the initiation stage. The principal mechanism involved in this regulation has gradually been elucidated. In heme-deficiency, protein synthesis in reticulocyte lysates is inhibited due to activation of the heme-regulated inhibitor (HRI), which specifically phosphorylates the α-subunit of the eukaryotic initiation factor 2 (eIF-2α). Phosphorylation of eIF-2α results in the binding and sequestration of the reversing factor (RF) which is required for the recycling of eIF-2. The unavailability of RF results in the shut-off of polypeptide chain initiation (reviewed by Hunt,1979; Pain, 1986; London et al., 1987; Hershey, 1991; Jackson, 1991).

Inhibition of initiation via phosphorylation of eIF-2α in reticulocyte lysates is also observed upon the addition of low levels of double-stranded RNA (dsRNA) (Kosower et al., 1972). The low level of dsRNA activates a dsRNA-dependent eIF-2α kinase (dsI) (Farrell et al., 1977; Levin and London, 1978), which is different from HRI (Petryshyn et

al., 1979). The work presented here deals with HRI and heat shock proteins in translational control.

Initiation of Protein Synthesis in Eukaryotic Cells

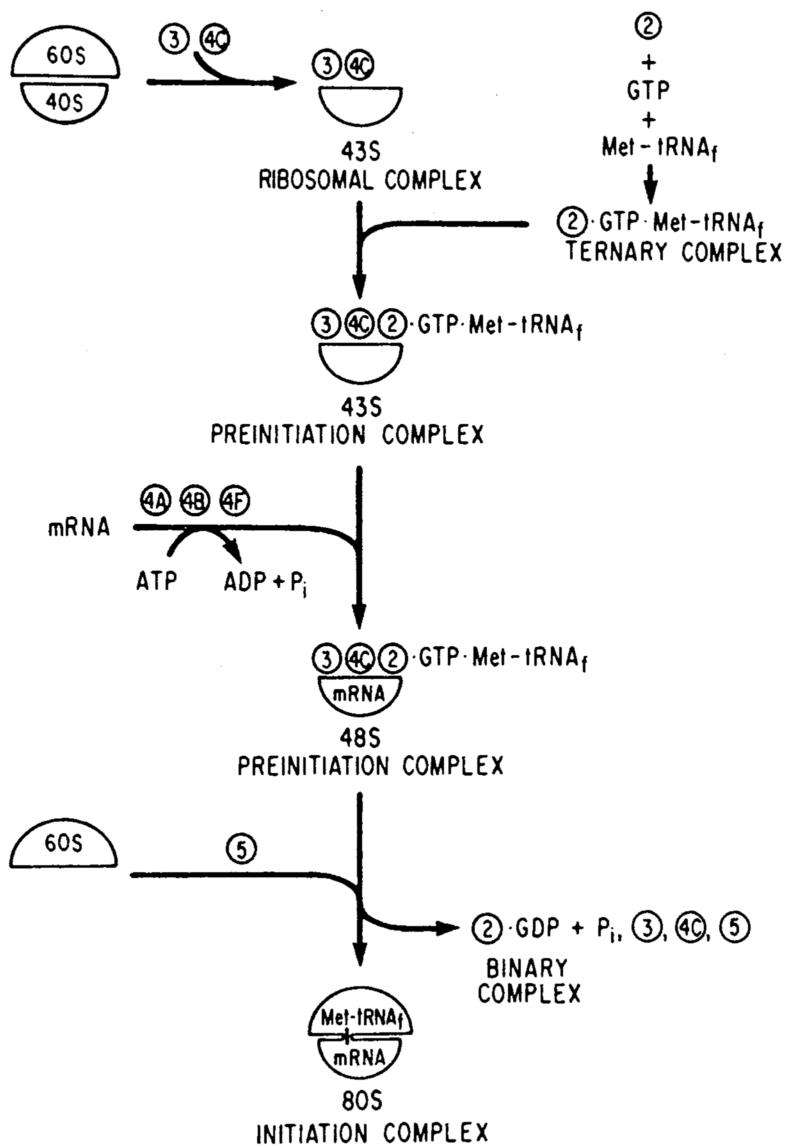
Briefly, there are four steps in the eukaryotic translational initiation pathway: (1) the formation of free ribosomal subunits; (2) the formation of the 43S preinitiation complex; (3) the formation of the 48S preinitiation complex; and (4) the formation of the 80S initiation complex (Fig. 1) (London et al., 1987).

First, 40S ribosomal subunits bind the initiation factors eIF-3 and eIF-4C to form a 43S ribosomal complex, which is unable to associate with 60S subunit. Next, the eIF-2·GTP·met-tRNA$_f$ ternary complex binds to the 43S ribosomal complex to form the 43S preinitiation complex. Then with the help of eIF-4A, eIF-4B, and eIF-4F, mRNA binds to the 43S preinitiation complex in an ATP-dependent reaction to form the 48S preinitiation complex. Finally, eIF-5 catalyzes the hydrolysis of the eIF-2 bound GTP, stimulating the release of eIF-3, eIF-4C, and eIF-2·GDP. The 48S preinitiation complex subsequently associates with the 60S subunit to form the active 80S initiation complex.

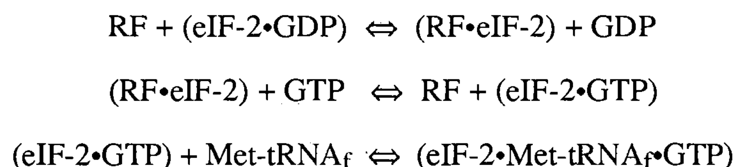
eIF-2 Recycling and the Reversing Factor

The recycling of eIF-2 after the last step in initiation requires the replacement of GDP by GTP, which is catalyzed by the reversing factor, RF (London et al., 1987). This factor has also been designated GEF, guanine nucleotide exchange factor (Panniers and Henshaw, 1983; Pain and Clemens, 1983) and eIF-2B (Konieczny and Safer, 1983). RF was originally purified from rabbit reticulocyte lysates by assaying its ability to stimulate protein synthesis in heme-deficient lysates (Amesz et al., 1979; Siekierka et al., 1981, 1982; Matts et al., 1983). It consists of five asymmetric subunits with approximate molecular weights of 82, 65, 55, 40, and 32 kDa, and forms a 1:1 complex with eIF-2 (Matts et al., 1983; Panniers and Henshaw, 1983; Konieczny and safer, 1983; Amesz et

Figure 1. Summary of Eukaryotic Initiation of Translation (London et al., 1987). Numbers in circles refer to eukaryotic initiation factors.


60S
40S
3
4C
43S
RIBOSOMAL COMPLEX
2
+
GTP
+
Met - tRNAf
2 · GTP · Met - tRNAf
TERNARY COMPLEX
3 4C 2 · GTP · Met - tRNAf
43S
PREINITIATION COMPLEX
4A 4B 4F
mRNA
ATP
ADP + Pi
3 4C 2 · GTP · Met - tRNAf
mRNA
48S
PREINITIATION COMPLEX
60S
5
2 · GDP + Pi, 3, 4C, 5
BINARY
COMPLEX
Met-tRNAf
mRNA
80S
INITIATION COMPLEX

al., 1979; Siekierka et al., 1981). RF catalyzes the recycling of eIF-2 by markedly stimulating the rate of dissociation of GDP from the eIF-2·GDP complex (Siekierka et al., 1982; Matts et al., 1983; Panniers and Henshaw, 1983; Mehta et al., 1983; Pain et al., 1983; Matts et al., 1988). The binding of RF to eIF-2 decreases the affinity of eIF-2 for GDP (Kd GDP=1.8×10^{-7} M) and increases the affinity of eIF-2 for GTP (Kd GTP=1.7×10^{-7} M) (Konieczny and Safer, 1983; Safer, 1983). Since GTP is present physiologically at concentrations 10- to 20-fold greater than GDP (Jagus and Safer, 1981; Safer et al., 1982), the net effect is that upon the binding of RF to eIF-2•GDP, GTP is exchanged for GDP. Thus, in the presence of Met-tRNA$_f$, the formation of the eIF-2•GTP•Met-RNA$_f$ ternary complex is promoted (London et al., 1987). RF catalyzed recycling of eIF-2 may be summarized as:



$$\text{RF} + (\text{eIF-2•GDP}) \Leftrightarrow (\text{RF•eIF-2}) + \text{GDP}$$

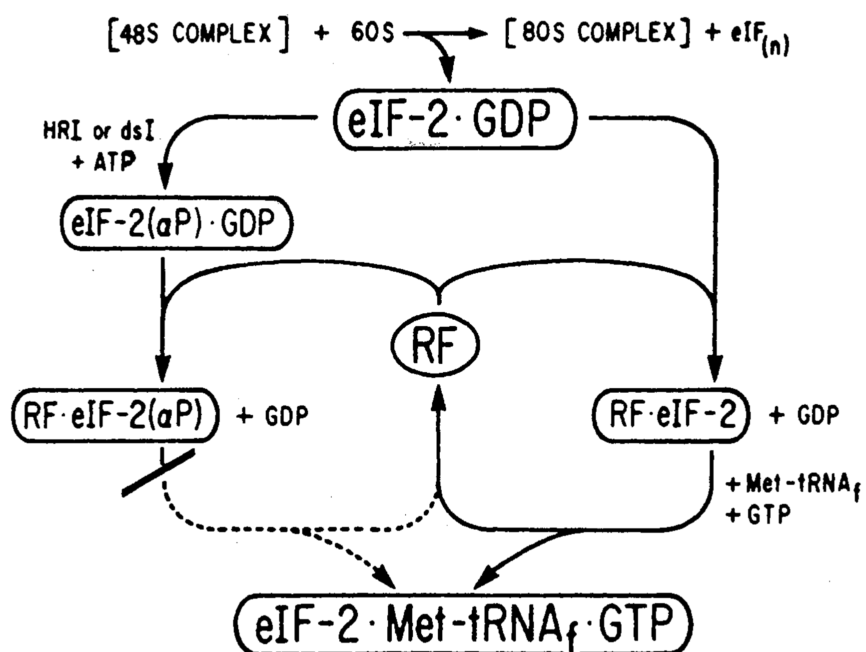
$$(\text{RF•eIF-2}) + \text{GTP} \Leftrightarrow \text{RF} + (\text{eIF-2•GTP})$$

$$(\text{eIF-2•GTP}) + \text{Met-tRNA}_f \Leftrightarrow (\text{eIF-2•Met-tRNA}_f\text{•GTP})$$

Inhibition of Initiation and HRI

The recycling of eIF-2 is disrupted by the heme-regulated eIF-2α kinase (HRI) and dsRNA-dependent eIF-2α kinase (dsI) (London et al., 1987). HRI is a cAMP-independent kinase, which specifically phosphorylates the 38 kDa α-subunit of eIF-2 (eIF-2α) (Levin et al., 1976; Kramer et al., 1976; Ranu and London, 1976; Farrell et al., 1977; Gross and Mendelewski, 1977; Levin and London, 1978; Ernst et al., 1978). The phosphorylated [eIF-2(αP)•GDP] binary complex interacts with RF to form a [RF•eIF-2(αP)] that is not readily dissociable and therefore non-functional (Fig. 2) (Matts et al., 1983; Hurst and Matts, 1987). Since RF is present in lysates at a limiting concentration relative to eIF-2 (Matts et al., 1983; Thomas et al., 1984), the phosphorylation of only 20-40% of lysate eIF-2 is sufficient to sequester all the lysate RF and to shut off initiation of protein synthesis (Matts and London, 1984).

Figure 2. Effect of Phosphorylation of eIF-2α on Availability of Reversing Factor for Recycling of eIF-2 (London et al., 1987).



[48S COMPLEX] + 60S → [80S COMPLEX] + eIF(n)
eIF-2·GDP
HRI or dsI
+ ATP
eIF-2(αP)·GDP
RF
RF·eIF-2(αP)
+ GDP
RF·eIF-2
+ GDP
+ Met-tRNAf
+ GTP
eIF-2·Met-tRNAf·GTP

Highly purified HRI has been characterized as a dimer (~150 kDa) of a single polypeptide (Trachsel et al., 1978; Hunt, 1979) with a sedimentation coefficient of ~6.6S in glycerol gradients (Levin et al., 1980). On sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), HRI migrates as an 80-92 kDa molecule, depending upon the percentage of the polyacrylamide gel (London et al., 1987; Chen et al., 1991a). Recently, the cDNA coding for HRI has been cloned and sequenced (Chen et al., 1991b). The HRI cDNA contained a G+C rich (80%) 5'-flanking region and a 1878 nucleotide long open reading frame that encodes a 626-amino acid polypeptide (Chen et al., 1991b). It contains all eleven catalytic domains of protein kinases and the consensus sequences of protein-serine/threonine kinases (Hanks et al., 1988; Chen et al., 1991a,b).

HRI is present in hemin-supplemented reticulocyte lysates in an inactive form (proinhibitor) (Gross and Rabinovitz, 1972; Hunt, 1979). It is activated in lysates by heme deficiency (Howard et al., 1970; Maxwell et al., 1971; Gross and Rabinovitz, 1972). During heme-deficiency HRI is thought to go through a multistage process of activation: progressing from an inactive proinhibitor form to a hemin-reversible form, then to an intermediate, and finally to a form that is irreversibly activated (Hunt, 1979). The formation of the irreversible inhibitor is prevented by the presence of hemin. HRI can also be activated in hemin-supplemented reticulocyte lysates by treatment with the sulfhydryl reagent N-ethylmaleimide (NEM) (Kosower et al., 1972; Ernst et al., 1978a,b), oxidized glutathione (GSSG) (Kosower et al., 1972; Ernst et al., 1978a,b), high partial pressure of O_2 (Almis-Kanigur et al., 1982), ethanol (Wu, 1981), heavy metal ions (Hurst et al., 1987; Matts et al., 1991), and heat shock (Bonanou-Tzedzki et al., 1978;Ernst et al., 1982). However, little is known about the mechanism for the activation of HRI under these conditions.

Heat Shock Response and Heat Shock Proteins

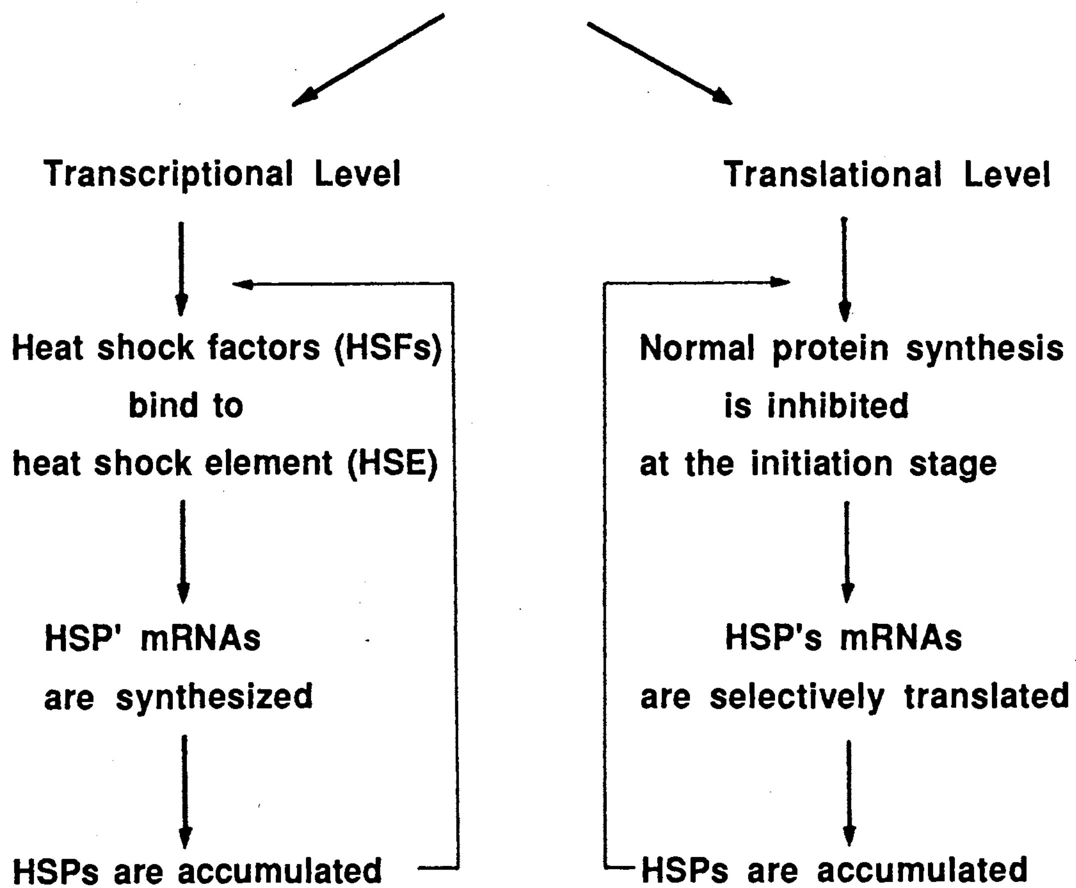
The exposure of cells from a wide variety of species to an increase in temperature results in the enhanced synthesis of a small number of highly conserved proteins, the heat shock proteins (hsps) (Ritossa, 1962; Schlesinger et al., 1982; Craig, 1985; Lindquist, 1986). This phenomenon has been called the heat shock response even though recovery from anoxia, ethanol, inhibitors of oxidative phosphorylation, and a number of other chemicals which induce the synthesis of the same proteins. From bacteria to human being, this response is universal. It is found in nearly every cell- and tissue-type of multicellular organisms, in explanted tissues, and in cultured cells.

The heat shock response is characterized by the transcriptional activation of a number of "heat shock" genes and is usually accompanied by a shutdown of normal protein synthesis at the level of initiation and the selective translation of the mRNAs encoding for heat shock proteins (Lindquist and Craig, 1988; Morimoto et al., 1992). Therefore, the heat shock response is regulated at both transcriptional and translational levels (Fig. 3).

In prokaryotes, as examplified by *Escherichia coli*, the major heat shock proteins are coded by single genes expressed constitutively at all temperatures. The heat shock response is positively regulated at the transcriptional level by the σ^{32} polypeptide, the product of the *rpo*H (*htpR*) gene and by the σ^{24} polypeptide (Neidhardt and VanBogelen, 1987; Gross et al., 1990; Georgopoulos et al., 1990). The gene coding for σ^{24} has not been identified.

In eukaryotes, the heat shock response is controlled mostly at the transcriptional level by a positively acting heat shock factor (HSF), which binds to specific regions of DNA called the heat shock element (HSE). HSEs contain repeats of the pentameric sequence nGAAn, located upstream of heat shock gene promoters (Sorger, 1991; Hightower, 1991). Recent reports have indicated the existence of multiple heat shock factors, some of which are themselves heat shock inducible (Scharf et al., 1990; Schuetz et al., 1991; Rabindran et al., 1991; Sarge et al., 1991). In the yeast *Saccharomyces cerevisiae*, HSF constitutively binds DNA and undergoes heat-induced phosphorylation

Figure 3. Heat Shock Response and Its Regulation.



Regulation of Heat Shock Response
Transcriptional Level
Translational Level
Heat shock factors (HSFs)
bind to
heat shock element (HSE)
Normal protein synthesis
is inhibited
at the initiation stage
HSP' mRNAs
are synthesized
HSP's mRNAs
are selectively translated
HSPs are accumulated
HSPs are accumulated

with an increase in the transcriptional activity of the adjacent gene (Sorger, 1990). In higher eukaryotes, HSF is maintained in a non-DNA binding state in unstressed cells. In response to heat shock, HSF oligomerizes and binds to the HSEs in the promoters of all heat shock-responsive genes (Jakobsen and Pelham, 1988, 1991; Westwood et al., 1991; Abravaya et al., 1991a, 1991b; Clos et al., 1992;Jurivich et al., 1992; Rabindran et al., 1992).

Heat shock proteins (hsps) are generally defined as those whose synthesis is sharply and dramatically induced at high temperatures. They show a remarkable conservation throughout evolution. The proteins are named according to their apparent molecular weights on SDS-PAGE and placed into different families based upon structural homologies. Very often in eukaryotes each family of hsps includes several subsets: HSCs (heat shock cognates) which are expressed constitutively; HSPs (heat shock proteins) which are largely expressed under conditions of stress; and sometimes a third class of proteins which are expressed constitutively, but whose rate of synthesis is significantly augmented following stress (Ang et al., 1991). The synthesis of some members of this last category is increased following glucose starvation, and they have been named GRPs (glucose regulated proteins).

Heat shock proteins function to protect cells during heat shock or related stresses, but they are also important components of normal cells. Some heat shock proteins are also molecular chaperones (Ellis and van-der Vies, 1991; Georgopoulos, 1992). Under normal cellular conditions, hsps appear to carry out a diversity of essential functions mediated primarily by their binding to other proteins. Through protein-protein interactions, hsps are thought to assist in such actions as intracellular protein trafficking and folding-unfolding of proteins. In some cases, these interactions directly or indirectly influence the activity of other proteins (Lindquist and Craig, 1988; Welch, 1990; Schlesinger, 1990; Gething and Sambrook, 1992; Edwards et al., 1992; Georgopoulos, 1992).

The 90 kDa heat shock protein (hsp90), the 70 kDa heat shock protein (hsp70), and the 56 kDa heat shock protein (hsp56) are abundant in cells. Hsp90 and hsp70 have been shown to associate specifically with a number of physiologically important viral and cellular proteins including: steroid hormone receptors; the oncogene product tyrosine kinase pp60$^{v\text{-}src}$; the antioncogene product p53, and HRI (Courtneidge and Bishop, 1982; Brugge et al., 1983; Evans, 1988; Matts and Hurst, 1989; and Hainaut and Milner, 1992). Recently, hsp56 has been found to be a binding protein for the immunosuppressant FK506 (Lebeau et al., 1992; Massol et al., 1992; Ruff et al., 1992; Tai et al., 1992; Yem et al., 1992). Therefore, research into the functions and mechanism of action of heat shock proteins in cells has become one of the hottest areas in biochemical and biomedical sciences.

CHAPTER II

HRI/HEAT SHOCK PROTEINS COMPLEX

Introduction

The rabbit reticulocyte lysate has been commonly used as a model system for studying the regulation of protein synthesis at the level of translation because of its lack of interfering transcriptional effects (Ochoa, 1983; Pain, 1986; Mattews, 1986; and London et al., 1987). The inhibition of initiation of protein synthesis in heme-deficient rabbit reticulocytes and their lysates is due to the activation of a heme-regulated inhibitor (HRI) which specifically phosphorylates the 38-kilodalton α-subunit of the eukaryotic initiation factor eIF-2 (eIF-2α) (Levin et al., 1976; Kramer et al., 1976; Ranu and London, 1976; and Farrell et al., 1977). The phosphorylation of eIF-2α results in the inhibition of protein chain initiation upon sequestration of the reversing factor (RF) responsible for eIF-2 recycling (Matts et al., 1983; Siekierka et al., 1983). Since the amount of RF 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 RF unavailable to catalyze GTP/GDP exchange (Matts and London, 1984; Matts et al., 1986).

Inhibition of protein synthesis in hemin-supplemented rabbit reticulocyte lysates occurs in response to a variety of conditions such as depletion of glucose-6-phosphate (Ernst et al., 1978; Jackson et al., 1983a, 1983b; Hunt et al., 1983; Michelson et al., 1984), addition of ethanol, sulfhydryl reactive agents, or oxidants (Wu, 1981; Kosower et al., 1972; Gross and Rabinovitz, 1972; Ernst et al., 1978; Palomo et al., 1985; Hurst et al., 1987), and heat shock (Bonanou-Tzedzki et al., 1978; Ernst et al., 1982). Many of these same agents or conditions usually induce the heat shock or stress response in

eukaryotic cells (Craig, 1985; Lindquist, 1986; Lanks, 1986, Bond and Schlesinger, 1988). The inhibition of protein synthesis under these conditions occurs at least in part due to the activation of HRI. Phosphorylation of eIF-2α in response to heat shock has been reported in HeLa and Ehrlich ascites cells (Duncan and Hershey, 1984; DeBenedetti and Baglioni, 1986; Scorsone et al., 1987), apparently due to the activation of an eIF-2α kinase with antigenic properties similar to HRI (DeBenedetti and Baglioni, 1986). However, the mechanism for the activation of HRI is not well understood. Studies have indicated that the capacity to reduce disulfide bonds present in proteins is required for maintaining HRI in an inactive form in hemin-supplemented reticulocyte lysates (Jackson et al., 1983; Matts et al., 1991). However, whether it is the redox status of sulfhydryl groups within HRI or some other cytosolic proteins that regulate the activity of HRI has not yet been determined.

Prokaryotic and eukaryotic cells respond to heat shock and certain other environmental abuses by synthesizing a small set of highly conserved proteins, heat shock proteins (hsps) (Ritossa, 1962; Schlesinger et al., 1982; Craig, 1985, Lindquist, 1986; Lindquist and Craig, 1988; Pelham, 1989; Schlesinger, 1990; Ang et al., 1991). The functions of hsps have long been a matter for speculation. It is widely assumed that these proteins protect cells from the effects of stress and act as molecular chaperones and foldases, mediating the transport and folding of nascent polypeptide chains and their assembly into or their disassembly from oligmeric structures (Pelham, 1984; Craig and Jacobsen, 1985; Pelham, 1986; Welch and Suhan, 1986; Lindquist and Craig, 1988; Beckman et al., 1990, Ellis and van der Vies, 1991; Wickner et al., 1991; Georgopoulos, 1992). The mechanisms of action of a number of hsps remain unclear.

Several major heat shock proteins (hsp90, hsp70, and hsp60) are known to be members of multi-gene families (Lindquist, 1986; Schlesinger, 1990; Hightower, 1991; Sorger, 1991). Some hsps are constitutively expressed in cells at normal temperatures and are abundant in the cytoplasm (Lindquist, 1986). The constitutively expressed heat shock proteins have been proposed to play a role in mediating the transport and folding of nascent

polypeptides while the stress induced ones have been proposed to play a role in protein renaturation (Pelham, 1986, 1989; Rothman, 1989; Hartle and Newpert, 1990; Beckman et al., 1990; Morimoto et al., 1990; Ang et al., 1991; Sherman and Goldberg, 1992; Beckman et al., 1992; Georgopoulas, 1992).

The constitutively expressed form of the hsp90 family is abundant in the cytoplasm of normal eukaryotic cells, and has been found to form noncovalent complexes with a variety of cellular proteins (Schlesinger, 1990). Most steroid hormone receptors appear to be associated with the constitutively expressed form of hsp90 prior to the activation of the receptor upon steroid binding (Dougherty et al., 1984; Joab et al., 1984; Sullivan et al., 1985; Sanchez et al., 1985; Schuh et al., 1985; Catelli et al., 1985; Mendel et al., 1986; Redeuilh et al., 1987; Sanchez et al., 1987; Howard and Distelhorst, 1988; Denis et al., 1988; Simth et al., 1990). Another protein, the EC1 antigen (also termed p59) which reacts with the KN382/EC1 monoclonal antibody, has also been shown to be a common component of several untransformed rabbit steroid hormone receptors (Tai et al., 1986; Sanchez et al., 1990). The EC1 antigen or p59 has been identified as a heat shock protein called hsp56 (Sanchez, 1990) and a binding protein for the immunosuppressant FK506 (Yem et al., 1992; Tai et al., 1992). Studies have demonstrated that the constitutive form of hsp70 associates with the progesterone and glucocorticoid receptors (Estes et al., 1987; Kost et al., 1989; Sanchez et al., 1990; Smith et al., 1990). Similarly, HRI has been demonstrated to associate with hsp90 in hemin-supplemented reticulocyte lysates *in situ* (Matts and Hurst, 1989), and hsp90 has been reported to affect the activation or activity of partially purified HRI *in vitro* (Rose et al., 1989; Szyszka et al., 1989). To understand the mechanism of the regulation of HRI activation or activity, we first characterized the HRI-hsp90 complex. The data reported here indicated that, like steroid hormone receptors, HRI interacts with hsp90, hsp70, and hsp56 (the EC1 antigen) to form a complex in hemin-supplemented reticulocyte lysates. The association of HRI with hsp90 and the EC1 antigen is hemin dependent, while the interaction of HRI with hsp70 is not hemin dependent. The

data suggest that the heat shock proteins may play a role in regulation of HRI activation and activity.

Materials and Methods

Materials

Reticulocyte lysates were 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). Goat anti-mouse IgM and IgG were obtained from Jackson Immunoresearch Laboratories. Apyrase (grade VIII), nitro blue tetrazolium (NBT), 5-bromo-4-chloro-3-indolyl phosphate (BCIP), p-nitrophenyl-agarose, mouse IgM (TEPC 183) and mouse IgG (MOPC 21) were obtained from Sigma. Alkaline phosphatase-conjugated to rabbit anti-mouse IgG and goat anti-rabbit IgG were obtained from ICN. L-[^{14}C]Leucine (340 mCi/mmol) and [γ-^{32}P]ATP (1000-3000 Ci/mmol) were obtained from Du Pont-New England Nuclear. eIF-2 was isolated from reticulocyte lysate as described (Hurst et al., 1987). Anti-hsp90 IgM monoclonal antibody (8D3) (Perdew, 1988; Perdew and Whitelaw, 1991) was provided by Dr. Gary H. Perdew (Purdue University), anti-hsp90 IgG monoclonal antibody (AC88) (Riehl et al., 1985) was provided by Dr. David Toft (Mayo Clinic, Rochester, MN), KN382/EC1 anti-p59 IgG monoclonal antibody (EC1) (Tai et al., 1986) was provided by Dr. Lee E. Faber (Medical College of Ohio, Toledo, OH), the N27F3-4 (N27) (Kost et al., 1989) and C92F3A5 (C92) (Welch and Suhan, 1986) anti-hsp70 IgG monoclonal antibodies were provided by Dr. William J. Welch (UC San Francisco), and the rabbit anti-hsp90/hsp70 polyclonal antiserum (4322) (Erhart et al., 1988) was provided by Dr. Stephen J. Ullrich (NCI, NIH). The anti-HRI IgG monoclonal antibody (Pal et al., 1991) was provided by Dr. Jane-Jane Chen (MIT). Purified p59 (the EC1 antigen) from rabbit uterus cytosol (Tai et al., 1986) was provided by Dr. Ping-Kaung Tai (Medical College of Ohio, Toledo, OH).

General Procedures and Buffers

Protein synthesis was carried out at 30 °C in standard reticulocyte lysate reaction mixtures 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. Samples were denatured in SDS sample buffer followed by separation in 10% SDS-PAGE gels (9x14x0.15 cm; acrylamide:bis = 37.5:1) for analysis (unless noted otherwise). Proteins separated by the gels were transferred to Immobilon-P (Millipore) at a current density of 2.5 mA/cm^2 for 40 min for Western blot analysis as previously described (Matts and Hurst, 1989). Buffers: 10 mM Tris-HCl (pH 7.5), TB; 10 mM Tris-HCl (pH 7.5) containing 50 mM NaCl, TB/50; 10 mM Tris-HCl (pH 7.5) containing 150 mM NaCl, TBS; 10 mM Tris-HCl (pH 7.5) containing 500 mM NaCl, TB/500; 1xSDS sample buffer, 62.5 mM Tris-HCl (pH 6.8), 5% SDS, 90 mM dithiothreitol, 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.

Immunoprecipitations

p-Nitrophenyl-agarose was rinsed free of isopropanol, and was coupled to goat anti-mouse IgG (GAG) or goat anti-mouse IgM (GAM) as previously described (Livingston, 1975; Matts and Hurst, 1989). The procedure is identical to that used for coupling antibody to cyanogen bromide activated-agarose. However, the bond formed during the coupling does not generate a positive charge, which significantly reduces the non-specific binding of hsp90, HRI and other lysate proteins to the matrix during the immunoadsorptions (Matts and Hurst, 1989). The preparations contained approximately 1.6 mg of antibody coupled per ml of packed agarose. GAG-agarose and GAM-agarose were used as a 1:1 slurry in TBS. The following general procedure describes the quantities of antibodies that were determined to quantitatively adsorb hsp90, EC1 antigen or HRI

from 10 μl of reticulocyte lysate protein synthesis mix: for the adsorption of hsp90, 8D3 anti-hsp90 monoclonal antibody (mAb) from 62.5 μl of ascites fluid was bound to 87.5 μl of GAM-agarose; for the adsorption of the EC1 antigen, 62.5 μl of EC1 mAb (2.8 mg/ml) was bound to 100 μl of GAG-agarose; or for the adsorption of HRI, anti-HRI mAb F from 8 μl of ascites fluid was bound to 20 μl of GAG-agarose. Non-immune control incubations contained equivalent volumes of TEPC 183 mouse IgM or MOPC 21 mouse IgG ascites, with a concentration of approximately 5 mg of antibody per ml.

Antibodies were bound to the GAM or GAG-agarose for 2 hours on ice, with stirring every 15 min. The GAM- or GAG-agarose was then washed sequentially with 0.5 ml each of : TBS; TB/500; TBS; and protein adsorption buffer (either TB or TBS, with or without 20 μM hemin-Cl as indicated in the legend of the figures). The GAM- or GAG-agarose was pelleted by a 10 sec centrifugation (full speed burst) in a Brinkman model 5415 microfuge. The adsorption buffer contained 20 μM hemin-Cl for the immunoadsorption of proteins from hemin-supplemented protein synthesis mixes. Rabbit reticulocyte lysates were incubated under standard conditions for protein synthesis (Hunt et al., 1972; Ernst et al., 1978) in the presence (hemin-supplemented lysates) or absence (heme-deficient lysates) of 20 μM hemin for 20 min at 30°C. After the removal of excess adsorption buffer from the GAM- or GAG-agarose pellets containing the bound antibodies, 10 μl of protein synthesis mixes were added directly for the adsorption of hsp90, the EC1 antigen, or HRI, and their associated proteins. Samples were incubated at 4°C for 90 min with continuous mixing. The samples were then diluted ten fold with 90 μl of adsorption buffer, and the nonadsorbed proteins (supernatants) were separated from the proteins adsorbed to the agarose (pellets) by centrifugation. Supernatants were removed and stored at -70°C for further analysis. For the analysis of hsp70 associated with hsp90, the EC1 antigen, or HRI, we found it is necessary to dilute the protein synthesis mixes ten fold with adsorption buffer (90 μl) prior to the 90 min adsorption of the proteins, to reduce the

nonspecific binding of hsp70. This resulted in a slightly less efficient adsorption of HRI in association with the hsp90 or the EC1 antigen.

The pellets were then washed five times with 0.5 ml of the buffers described below to remove nonadsorbed proteins: For the adsorption of hsp90 or the EC1 antigen, and their associated proteins with the 8D3 anti-hsp90 or the EC1 mAbs, the pellets were washed sequentially: once with TB, twice with TB/50, and twice with TB. For the adsorption of HRI and its associated proteins with the anti-HRI mAb F, the pellets were washed 5 times with TBS (for the analysis of associated hsp90), or the pellets were washed sequentially: once with TBS; three times with TBS containing 1% sodium deoxycholate (DOC), 1% Triton X-100, and 0.1% SDS; and once with TBS (for the analysis of associated hsp70). Pellets were diluted with an equal volume of TB, and stored at -70°C prior to analysis. Samples were prepared for SDS-PAGE by addition of one volume of 2xSDS sample buffer followed by heating in a boiling water bath for 2.5 min.

Two proteins, present in ascites fluid, with molecular weights and immunological properties similar to heat shock protein cognate 73 and hsp72 (Welch and Suhan, 1986; Kost et al., 1989), were non-specifically bound to GAM- and GAG-agarose. These proteins were not removed from the pellet by washing with high salt (0.5 M NaCl) or upon incubation of the pellet with MgATP. The affinity of the anti-HRI mAb F was high enough that in experiments examining the association of hsp70 with HRI, non-specifically bound proteins were removed from the pellets by washing with TBS containing detergents as described above. In experiments examining the interaction of hsp70 with hsp90, proteins specifically adsorbed with hsp90 by the 8D3 anti-hsp90 mAb were eluted from the pellets by washing with high salt. Excess buffer was removed from the pellet, and adsorbed proteins were eluted by washing twice with 3 volumes of TB/500 (30 µl of buffer per 10 µl of packed agarose), followed by wash with TB/50; the washes were combined; and samples were prepared for SDS-PAGE after the addition of 1/4 volume (30 µl) of 4xSDS sample buffer as described above. The high salt elution also facilitates the detection of

hsp70 specifically associated with hsp90, since most of the IgM, whose heavy chain migrates with a molecular weight of approximately 70 kDa, remains bound to the GAM-agarose.

Western Blot Analysis

Samples were separated by SDS-PAGE, and proteins were transferred from gels to Immobilon-P membrane for Western blot analysis as described above. Blots were blocked with TBS containing 2.5 mg/ml bovine serum albumin (BSA) or TBS containing 5% (w/v) non-fat dry milk (skim milk, Difco Laboratories) for one hour at room temperature. The primary antibody was then reacted with the blots at 4°C overnight. After reacting with the primary antibodies, the blots were washed twice with TBS containing 0.5% Tween-20, once with TBS, and then blocked with TBS containing 10% heat inactivated fetal calf serum (IFCS) or 5% (w/v) skim milk prior to reaction with alkaline phosphatase-conjugated rabbit anti-mouse IgG or goat anti-rabbit IgG diluted 1:1000 in TBS containing 1% IFCS or 1% skim milk for two hours at room temperature. Blots were washed twice with TBS containing 0.5% Tween-20, and twice with TBS. Proteins were then detected by incubating blots in alkaline phosphatase reaction buffer at 35°C for 1 to 5 min.

For the detection of HRI, ascites fluid containing the anti-HRI mAb F was diluted 1:250 in TBS containing 500 μg/ml BSA (TBS/BSA). AC88 mAb (20 μg/ml) in TBS/BSA, or 4322 anti-hsp90/hsp70 antiserum diluted 1:50 in TBS containing 1% (w/v) skim milk, were used for the detection of hsp90 associated with the EC1 antigen or HRI, respectively. EC1 mAb (20 μg/ml) in TBS/BSA was used for the detection of the EC1 antigen. A mixture of N27 and C92 mAbs each diluted 1:2000 in TBS/BSA was used for the detection of hsp70.

Results

Interaction of HRI with hsp90 is hemin dependent

The 8D3 anti-hsp90 monoclonal antibody is one of two mAbs that are currently available which binds to steroid hormone receptor-hsp90 complexes in mammals (Perdew, 1988; Perdew and Whitelaw, 1991). The 8D3 mAb quantitatively removes hsp90 from both hemin-supplemented (Fig. 4) and heme-deficient rabbit reticulocyte lysates (data not shown, see Chapter IV). To verify the association of hsp90 with HRI, the 8D3 anti-hsp90 mAb GAM-agarose pellets were separated by SDS-PAGE followed by Western blot analysis using the anti-HRI mAb F (Pal et al., 1991). The anti-HRI mAb F detected HRI with an estimated molecular weight from 88 to 92 kDa, which migrates slightly in front of hsp90 under the SDS-PAGE conditions described above (Fig. 5). HRI was clearly co-immunoadsorbed with hsp90 by 8D3 anti-hsp90 mAb from hemin-supplemented reticulocyte lysates (Fig. 5). Significantly less HRI was present in the 8D3 anti-hsp90 immune pellets from heme-deficient lysates. The association of HRI with hsp90 appeared to be stabilized by the presence of hemin in the adsorption buffer, as the amount of HRI co-immunoprecipitated with hsp90 decreased significantly at hemin concentrations below 5 μM (Fig. 5). We observed that in heme-deficient lysates, HRI could reassociate with hsp90 over a 90 min time period of the immunoadsorption, if 20 μM was added to the adsorption buffer. The reassociation of HRI with hsp90 due to the addition of hemin to heme-deficient lysates was time and temperature dependent (data not shown, see Chapter IV).

Hsp90 interacts with hsp70 and the EC1 antigen in rabbit reticulocyte lysates.

The EC1 antigen, also termed p59, has been identified as a heat shock protein hsp56 (Sanchez, 1990) and a binding protein for the immunosuppressant FK506 (Yem et al., 1992; Lebeau et al., 1992; Tai et al., 1992). The EC1 antigen and hsp70 are common

components of several steroid hormone receptor-hsp90 complexes (Tai et al., 1986; Kost et al., 1989;Smith et al., 1990; Renoir et al., 1990; Sanchez et al., 1990a,b). To investigate whether the EC1 antigen and hsp70 interact with the HRI-hsp90 complex, the 8D3 anti-hsp90 immune pellets were analyzed by Western blotting with the anti-EC1 mAb and the N27/C92 anti-hsp70 mAbs, respectively. Probing with the EC1 mAb, the EC1 antigen was detected as a 56 kDa band which comigrated with immunoaffinity purified p59 from rabbit uterus cytosol (Fig. 6A). The 8D3 anti-hsp90 mAb co-adsrobed the EC1 antigen from both hemin-supplemented and heme-deficient lysates (Fig. 6A). Another protein band with a molecular mass of approximately 52 kDa, which reacted with the EC1 mAb, was also observed to be present in both the 8D3 pellets and the rabbit uterine EC1 antigen preparation (Fig. 6A). We have not yet determined whether this 52 kDa protein is a proteolytic fragment of the EC1 antigen, or a unique, but antigenically similar protein, which is also present in the complex with hsp90 and the EC1 antigen.

Using a mixture of N27 and C92 mAbs, hsp70 was detected to be specifically co-adsorbed with hsp90 by the 8D3 mAb from rabbit reticulocyte lysates (Fig. 6B). The association of hsp90 with hsp70 is not hemin dependent, since hsp70 could be co-immunoprecipitated by the 8D3 anti-hsp90 mAb from both hemin-supplemented and heme-deficient lysates (Fig. 6B).

<u>The EC1 Antigen interacts with HRI, hsp90, and hsp70.</u>

Though the data described above suggest that the EC1 antigen interacts with hsp90 in both hemin-supplemented and heme-deficient lysates, there is no direct evidence for the interaction of EC1 antigen with HRI or the HRI-hsp90 complex in rabbit reticulocyte lysates. To verify the interaction of the EC1 antigen with HRI or the HRI-hsp90 complex, the proteins co-adsorbed with the EC1 antigen were analyzed by Western blotting. The EC1 mAb quantitatively removed the EC1 antigen from both hemin-supplemented and heme-deficient reticulocyte lysates (Fig. 7). Western blot analysis using the anti-HRI mAb

F as a probe indicated that approximately 80% of the HRI was co-adsorbed together with the EC1 antigen from hemin-supplemented lysate, while little HRI was co-adsorbed from heme-deficient lysates (Fig. 8). When the AC88 anti-hsp90 mAb was used for probing the Western blot, we observed that hsp90 was co-adsorbed with the EC1 antigen from both hemin-supplemented and heme-deficient lysates (Fig. 9A). The EC1 mAb only coadsorbed a fraction of the hsp90 from the lysates, indicating that not all of the EC1 antigen associates with hsp90 in lysate and that a pool of hsp90, which is not associated with the EC1 antigen, is present (data not shown). The interaction of the EC1 antigen with hsp70 in reticulocyte lysates was verified by blotting the EC1 mAb immune pellets with a mixture of the N27 and C92 anti-hsp70 mAbs (Fig. 9B). Like hsp90, the interaction of the EC1 antigen with hsp70 was not hemin-dependent (Fig. 9B). Consistently less hsp70 was adsorbed together with the EC1 antigen from hemin-supplemented than from heme-deficient lysates. A similar observation was also observed in the fractions immunoadsorbed by the 8D3 anti-hsp90 mAb from hemin-supplemented and heme-deficient lysates (Fig. 6B).

<u>HRI interacts with hsp90 and hsp70.</u>

The anti-HRI mAb F can quantitatively remove HRI from both hemin-supplemented and heme-deficient reticulocyte lysates (Fig. 10). When the immune pellets of anti-HRI mAb F were washed 5 times with TBS followed by blotting with 4322 anti-hsp90/hsp70 polyclonal antibody, hsp90 was found to be co-adsorbed with HRI (Fig. 11A). A reduction in the amount of hsp90 associated with HRI was observed in anti-HRI F immunoprecipitates prepared from heme-deficient lysates as compared to those prepared from hemin-supplemented lysates (Fig. 11A). Although the amount of hsp90 associated with HRI in heme-deficiency from different lysates varies somewhat, reduction in the association of HRI with hsp90 is always observed in heme-deficient lysates (data not shown). To verify the association of HRI with hsp70, the immune pellets prepared using

the anti-HRI mAb F must be washed with TBS containing 0.1% SDS, 1% DOC, and 1% Triton X-100 to remove the non-specifically bound of hsp70 (data not shown). Western blot analysis of the detergent washed anti-HRI pellets with a mixture of the N27 and C92 anti-hsp70 mAbs, showed that hsp70 was co-adsorbed specifically with HRI (Fig. 11B). More hsp70 appeared to be associated with HRI adsorbed from heme-deficient lysates than from hemin-supplemented lysates (Fig. 11B). We were unable to determine whether the EC1 antigen was co-adsorbed with HRI from hemin-supplemented lysates by the anti-HRI mAb F. Since HRI is present in lysates in low abundance, the inability to detect the co-adsorbed EC1 antigen may be because the amount of this protein was under the detection limits of Western blotting with the EC1 mAb. For example, using the 4322 anti-hsp90/hsp70 polyclonal antiserum, the association of hsp90 with HRI in the immune pellet of the anti-HRI mAb F from hemin-supplemented reticulocyte lysates was readily detected. However, it was not detectable if the AC88 anti-hsp90 mAb was used.

Discussion

The interactions of the heme-regulated eIF-2α kinase with hsp90, hsp70, and the EC1 antigen in rabbit reticulocyte lysates have been examined. The results indicate that HRI exists in the lysates as a multiprotein complex with heat shock proteins. The HRI-HSPs complex consists of at least four components, HRI, hsp90, hsp70, and the EC1 antigen. The formation or stability of this complex is dependent upon the presence of hemin at a concentration of at least 5 μM. A hemin concentration of 5 μM is the minimum concentration sufficient to saturate the heme binding sites on partially purified HRI and inhibit its activation (Fagard and London, 1981). The interaction of HRI with hsp90 and the EC1 antigen is regulated by hemin, while the interaction of HRI with hsp70 is not hemin-dependent, suggesting that hsp90 and hsp70 may bind to HRI at different sites. The anti-HRI mAb F co-adsorbed more hsp70 with HRI from heme-deficient than from hemin-supplemented lysates. However, it is not certain at this time whether this apparent increase

in the binding of hsp70 to HRI in heme-deficient lysates is specific, since an increase in the association of hsp70 with non-immune control pellets was observed in heme-deficient lysates. The observation that hsp90 could bind back to HRI in heme-deficient lysates, if hemin was added to the adsorption buffer, suggests that there is a dynamic equilibrium in the association of HRI with hsp90. This equilibrium favors the formation of HRI-hsp90 complex, if hemin is present.

The Western blot analysis of the immune pellets prepared using the 8D3 anti-hsp90 mAb indicated that the EC1 antigen and hsp70 could be co-adsorbed with hsp90 from both heme-deficient and hemin-supplemented lysates (Fig. 5A and 5B). The observations that the interaction of the EC1 antigen with HRI is dependent upon the presence of hemin, but the association of the EC1 antigen with hsp90 is not hemin dependent, suggests that the interaction of the EC1 antigen with HRI may be mediated through its association with hsp90. The interaction of the EC1 antigen with steroid hormone receptors via hsp90 has been proposed (Renoir et al., 1990). However, the actual mechanism through which the EC1 antigen interacts with HRI remains to be characterized. The observation that only about 50 to 70% of the EC1 antigen in reticulocyte lysate could be co-adsorbed with hsp90 by the 8D3 anti-hsp90 mAb from either heme-deficient or hemin-supplemented lysates (data not shown), suggests that not all EC1 antigen associates with hsp90 in rabbit reticulocyte lysates. Also some dissociation of the EC1 antigen from hsp90 may occur over the time course of the experiment, if there are less stable complexes of the hsp90-EC1 antigen in lysates. Western blot analysis of the immune pellets prepared using the EC1 mAb showed that hsp70 associates with the EC1 antigen whether hemin is present in reticulocyte lysates or not. These observations suggest that a complex containing hsp90, hsp70, and the EC1 antigen is present in reticulocyte lysate, and that the formation of this complex is not dependent on the presence of hemin.

The fact that the 8D3 anti-hsp90 and the EC1 mAbs are able to adsorb HRI nearly quantitatively from hemin-supplemented reticulocyte lysates suggests that these proteins are

present in a complex with a minimum stoichiometry of 1:1:1 (HRI:hsp90:EC1 antigen). It is difficult to show the stoichiometry of hsp70 in the HRI-HSPs complex at this time, since we currently do not have an anti-hsp70 mAb available which is capable of quantitatively adsorbing hsp70 from reticulocyte lysates.

Two other proteins with molecular weights of approximately 100 and 60 kDa were also reported to be associated with hsp90 in rabbit reticulocyte lysates (Matts et al., 1992). This is consistent with the observation that hsp90 exists in the cytosol of Hepa cell extracts in heteromeric complexes with proteins that have molecular weights similar to those described here (Perdew and Whitelaw, 1991). However, the functions of these proteins within the hsp90 heteromeric complex have not yet been characterized. A recent report has shown that the reconstitution of purified avian progesterone receptor with hsp90 in rabbit reticulocyte lysate occurs in conjunction with the reassociation of the receptor with several proteins, including hsp70, the EC1 antigen, and a 60 kDa protein (p60) (Smith et al., 1990). Since no one has been successful in reconstituting the binding of purified steroid hormone receptors to purified hsp90 *in vitro*, it has been suggested that one or more of these hsp90-associated proteins may be involved in the assembly of steroid hormone receptor-hsp90 complexes (Smith et al., 1990; Scherrer et al., 1990). Because of the low abundance of HRI in reticulocyte lysates, we are unable to determine directly whether HRI interacts with the 60 kDa protein described above, although we have observed that about 5% of the HRI from hemin-supplemented reticulocyte lysate was co-adsorbed with the 60 kDa protein by an anti-p60 mAb (data not shown).

The major heat shock proteins have been proposed to act as molecular chaperones (Schlesinger, 1990; Ellis and van der Vies, 1991; Gething and Sambrook, 1992). The functions of the various heat shock proteins is currently being studied intensely. The interaction of hsp90 with a number of protein kinases has been reported to affect their activities (Matts and Hurst, 1989; Rose et al., 1989; Szyszka et al., 1989; Nygard et al., 1991; Miyata and Yahara, 1992). Several oncogene products with tyrosine kinase activity are reported to associate with hsp90 upon their biosynthesis (Brugge et al., 1981;

Ziemiecki et al., 1986). Activation of the tyrosine kinase activity of pp60$^{v\text{-}src}$ requires its dissociation from hsp90 and insertion into the plasma membrane (Brugge et al., 1981; Brugge, 1986). The glucocorticoid receptor (GR) is recovered from hormone-free cells as a heteromeric complex containing hsp90 (Pratt, 1990). When GR is bound to hsp90, it is maintained in a high-affinity steroid binding conformation, which does not bind DNA. Binding of hormone results in the dissociation of hsp90 and associated proteins and the aquisition of high affinity DNA binding activity (Sanchez et al., 1987; Bresnick et al., 1989; Dalman et al., 1989; Scherrer et al., 1990). Hsp90 has been found to bind and protect casein kinase II from self-aggregation and enhance its kinase activity (Miyata and Yahara, 1992). Hsp90 apparently associates with HRI, keeping HRI in an inactive form in the presence of hemin. Recently, Matts and Hurst found that the ability of hemin to restore protein synthesis in heme-deficient lysates correlated with the levels of hsp90 present (Matts and Hurst, 1992). In all these cases, hsp90 appears to regulate the function of target proteins by forming complexes with the proteins.

The EC1 antigen, which participates in the heterooligomeric form of steroid receptors in association with hsp90 (Tai et al., 1986; Renoir et al., 1990), has been recently identified as a novel heat shock protein, hsp56 (Sanchez, 1990). The cloning and sequencing of the gene suggest that the EC1 antigen may bind ATP and calmodulin, and that it may also belong to the peptidylproline cis-trans isomerase (PPI) protein family (Lebeau et al., 1992). Consistent with this proposal, the EC1 antigen has been demonstrated to bind the immunosuppressant FK506 (Callebaut et al., 1992; Yem et al., 1992; Tai et al., 1992) and calmodulin (Massol et al., 1992). The association of HRI with the EC1 antigen implies that the polypeptide structure and function of HRI may be modulated by the PPI activity of the EC1 antigen.

The 70 kDa heat shock proteins interact with other proteins and maintain or alter their conformational states (Morimoto et al., 1990; Pelham, 1990; Gething and Sambrook, 1992). They have been shown to participate in protein folding, translocation of proteins

across membranes, and assembly or disassembly of oligomeric protein complexes (Pelham, 1990; Wickner et al., 1991). Among proteins that are known to interact with hsp70 are a number of cellular and viral proteins such as SV40 large T antigen, polyoma virus middle T antigen, mutant forms of the cellular antioncogene protein p53, steroid hormone receptors, and heat shock factors (HSF) (Pinhasi-Kimsi et al., 1986; Hinds et al.,1987; Walter et al., 1987; Clarke et al., 1988; Kost et al., 1989; Pallas et al., 1989; Sawai and Butel, 1989; Smith et al., 1990; Hainut and Milner, 1992; Abravaya et al., 1992). The data presented here demonstrate that hsp70 associates with HRI and this interaction, unlike hsp90, is not hemin dependent. The functional significance of these associations of HRI with hsps, in addition to its interactions with hsp90 and the EC1 antigen, remains to be established.

Figure 4. The Ability of the 8D3 Anti-hsp90 mAb to Remove Hsp90 from Rabbit Reticulocyte Lysates.

Proteins from 2.5 μl of hemin-supplemented protein synthesis mixes were adsorbed with non-immune control (*C*) or 8D3 anti-hsp90 (*I*) mAb bound to GAM-agarose as described under "Experimental Procedures". Non-adsorbed (*Sup*) and adsorbed (*Pel*) fractions were immunoblotted with the 4322 anti-hsp90/hsp70 antibody to detect the presence of hsp90. *L*, 2.5 μl of unincubated whole protein synthesis mix.

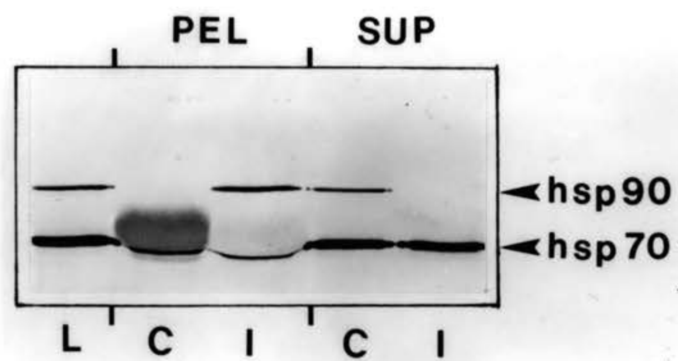
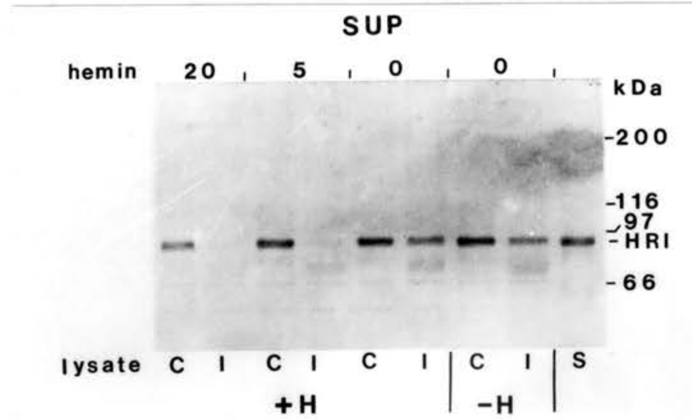

PEL
SUP
hsp90
hsp70
L
C
I
C
I

Figure 5. Effect of Hemin Concentration on the Coadsorption of HRI and Hsp90.

Proteins from 2.5 μl of hemin-supplemented (*+H*) and heme-deficient (*-H*) protein synthesis mixes were adsorbed with the non-immune control (*C*) or 8D3 anti-hsp90 (*I*) mAb bound to GAM-agarose in the presence of TB containing 20, 5, or 0 μM hemin as described under "Experimental Procedures". Non-adsorbed (*Sup*) and adsorbed (*Pel*) fractions were immunoblotted for the presence of HRI. *S*, purified HRI standard; *HC*, heavy chain of IgM.


SUP
hemin 20 5 0 0
kDa
-200
-116
97
- HRI
- 66
lysate C I C I C I C I S
+H
-H

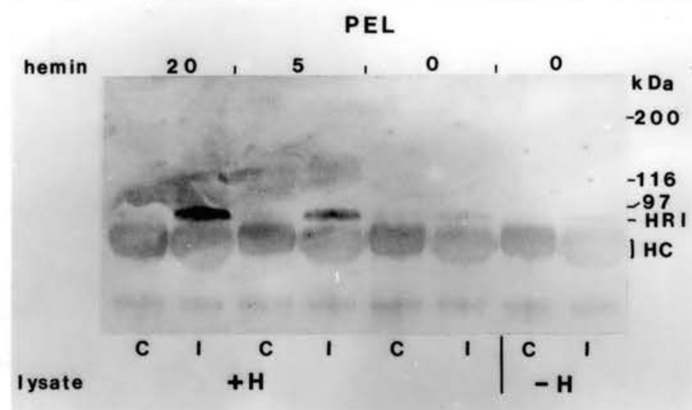
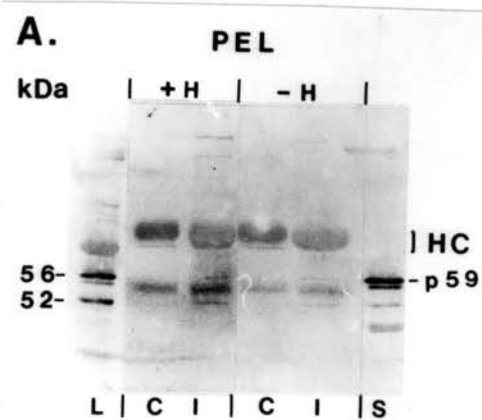

PEL
hemin 20 5 0 0
kDa
-200
-116
97
- HRI
] HC
C I C I C I C I
lysate
+H
-H

Figure 6. Coadsorption of the EC1 Antigen and Hsp70 with Hsp90 by the 8D3 Anti-hsp90 mAb.

Proteins from 2.5 μl of hemin-supplemented (+*H*) and heme-deficient (-*H*) protein synthesis mixes were adsorbed by control non-immune (*C*) or 8D3 anti-hsp90 (*I*) mAb in the presence of TB, as described under "Experimental Procedures". Proteins in the immunoprecipitates were extracted directly with SDS sample buffer for Western blotting with the EC1 mAb (*Panel A*); or proteins were extracted with TB/500, as described under "Experimental Procedures", followed by denaturation with SDS sample buffer for Western blotting with the N27 and C92 anti-hsp70 mAbs (*Panel B*). *S*, immunoaffinity purified EC1 antigen (p59) from rabbit uterine cytosol; L, 1.25 μl of unincubated whole reticulocyte lysate; *56* and *52 kDa*, the estimated molecular masses of the EC1 antigen and a lower molecular mass protein that reacts with the EC1 mAb; *HC*, heavy chain of IgM.


A.
PEL
kDa
+H
-H
56-
52-
HC
-p59
L
C
I
C
I
S

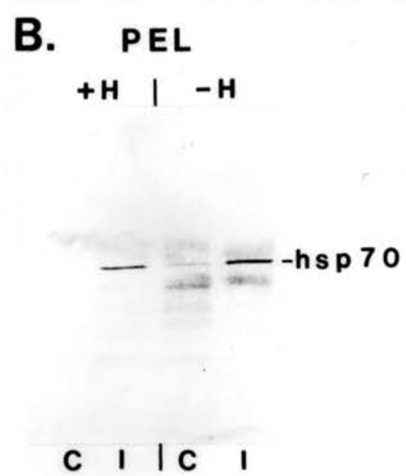

B.
PEL
+H
-H
-hsp70
C
I
C
I

Figure 7. The Ability of the EC1 mAb to Remove the EC1 antigen from Rabbit Reticulocyte Lysates.

Proteins from 2.5 μl of hemin-supplemented protein synthesis mixes were adsorbed with the non-immune control (*C*) or the EC1 mAb (*I*) bound to GAG-agarsoe in the presence of TB containing 20 μM hemin as described under "Experimental Procedures". Non-adsorbed (*Sup*) and adsorbed (*Pel*) fractions were immunobloted with the EC1 mAb for the presence of the EC1 antigen. *L*, 1.25 μl of unincubated whole reticulocyte lysate; *56 kDa*, the estimated molecular mass of the EC1 antigen; *EC1*, the EC1 antigen; *HC*, heavy chain of IgG.

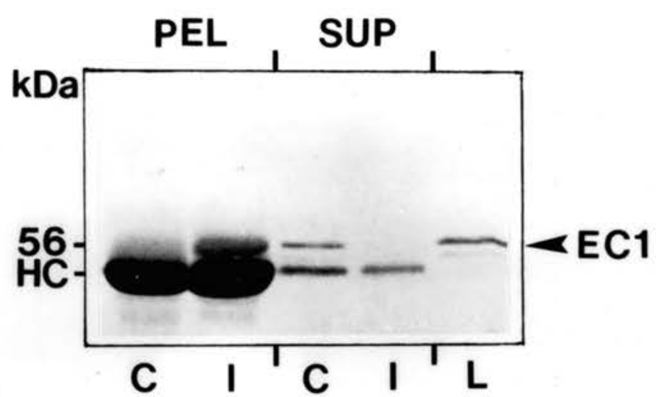
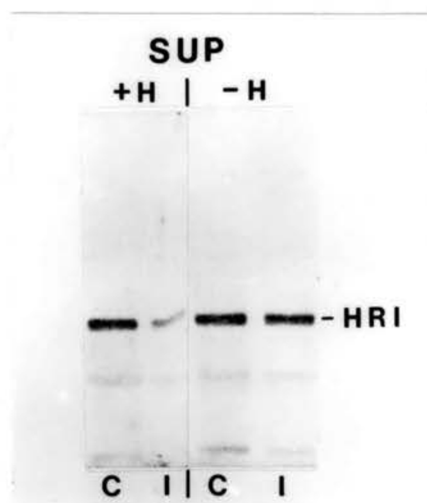

PEL
SUP
kDa
56
HC
EC1
C
I
C
I
L

Figure 8. The Ability of the EC1 mAb to Immunoadsorb HRI from Reticulocyte Lysates.

Proteins from 2.5 μl of hemin-supplemented (*+H*) and heme-deficient (*-H*) protein synthesis mixes were adsorbed with non-immune control (*C*) or EC1 (*I*) antibody bound to GAG-agarose in the presence of TB as described under "Experimental Procedures". Nonadsorbed proteins (*Sup*) and adsorbed proteins (*Pel*) were Western blotted for the presence of HRI. *HC*, heavy chain of IgG.


SUP
+H
-H
-HRI
C
I
C
I

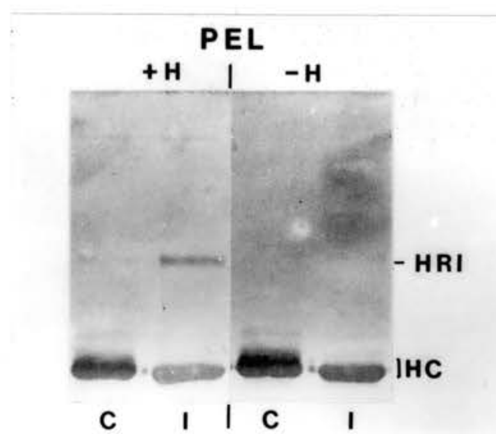
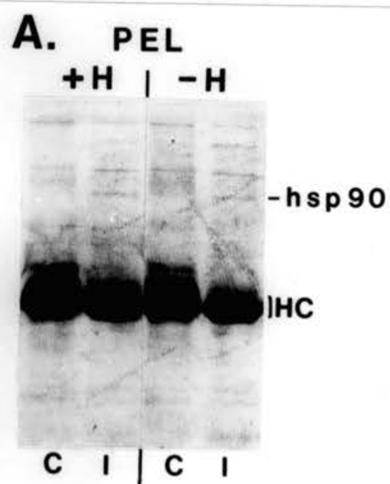

PEL
+H
-H
-HRI
HC
C
I
C
I

Figure 9. Coadsorption of Hsp90 and Hsp70 with the EC1 Antigen by the EC1 mAb.

Proteins from 2.5 μl of hemin-supplemented (*+H*) and heme-deficient (*-H*) protein synthesis mixes were adsorbed with non-immune control (*C*) or EC1 (*I*) antibody bound to GAG-agarose in the presence of TB, as described under "Experimental Procedures". Adsorbed proteins (*Pel*) were Western blotted with the AC88 anti-hsp90 mAb to detect the presence of hsp90 (*Panel A*) or a combination of N27 and C92 antibodies to detect the presence of hsp70 (*Panel B*). *HC*, heavy chain of IgG.


A.
PEL
+H
-H
-hsp90
HC
C
I
C
I

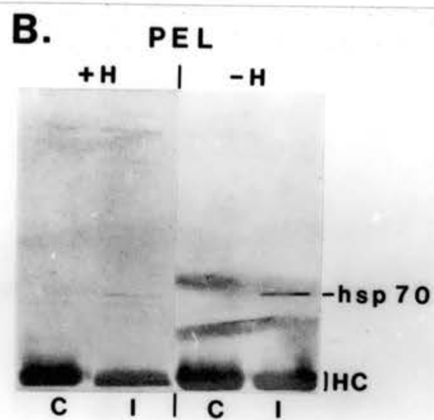

B.
PEL
+H
-H
-hsp 70
HC
C
I
C
I

Figure 10. The Ability of the Anti-HRI mAb F to Remove HRI from Rabbit Reticulocyte Lysates.

Proteins from 10 µl of hemin-supplemented (*+H*) and heme-deficient (*-H*) protein synthesis mixes were adsorbed with non-immune control (*C*) or the anti-HRI (*I*) antibody bound to GAG-agarose in the presence of TBS, as described under "Experimental Procedures". Nonadsorbed proteins (*S*) and adsorbed proteins (*P*) were Western blotted with the anti-HRI mAb F for the presence of HRI. *HC*, heavy chain of IgG.

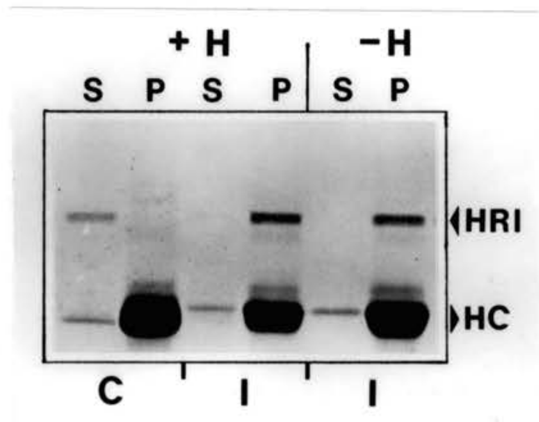
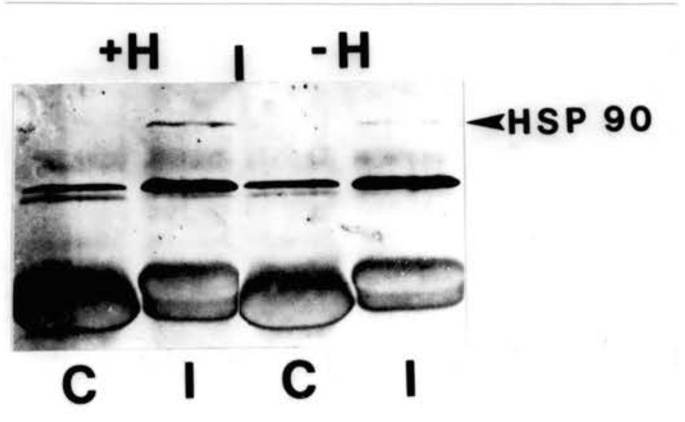
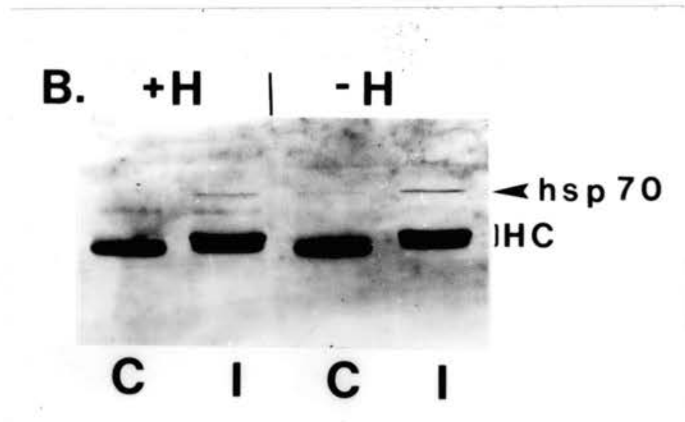

+ H
− H
S P S P S P
HRI
HC
C
I
I

Figure 11. Coadsorption of Hsp90 and Hsp70 with HRI by the Anti-HRI mAb F.

Proteins from 10 μl of hemin-supplemented (*+H*) and heme-deficient (*-H*) protein synthesis mixes were adsorbed with non-immune control (C) or the anti-HRI (*I*) antibody bound to GAG-agarose in the presence of TBS, as described under "Experimental Procedures". The adsorbed proteins were Western blotted with the 4322 anti-hsp90/hsp70 (*Panel A*) or with the N27 and C92 anti-hsp70 (*Panel B*) antibodies to detect the presence of hsp90 and hsp70, respectively. *HC*, heavy chain of IgG.


+H
-H
HSP 90
C
I
C
I


B.
+H
-H
hsp 70
HC
C
I
C
I

CHAPTER III

HEAT SHOCK PROTEINS REGULATE HRI ACTIVITY

Introduction

Protein synthesis in reticulocytes and their lysates is dependent on the presence of hemin (Bruns and London, 1965; Zucker and Schulman, 1968; Hunt et al., 1972; Adamson et al., 1969). In heme-deficiency, protein synthesis is inhibited at the level of initiation due to the activation of a heme-regulated inhibitor (HRI) (Howard et al., 1970; Maxwell et al., 1971; Pain, 1986; London et al., 1987). HRI is a cAMP-independent protein kinase, which specifically phosphorylates the 38 kDa α-subunit of the eukaryotic initiation factor eIF-2 (eIF-2α) (Levin et al., 1976; Farrell et al., 1977). Phosphorylation of eIF-2α in reticulocyte lysates results in the binding and sequestration of the reversing factor (RF) in an inactive RF-eIF-2α(P) complex (Amesz et al., 1979; Siekierka et al., 1982; Matts et al., 1983). The unavailability of RF, which is required for the exchange of GTP for GDP bound to eIF-2 in the recycling of eIF-2, results in the shut-off of protein synthesis (Matts et al., 1983; Panniers and Henshaw, 1983; Matts and London, 1984; Siekierka et al., 1984; Thomas et al., 1984; Matts et al., 1986).

Inhibition of protein synthesis also occurs in hemin-supplemented reticulocyte lysates in response to a variety of stress conditions, such as addition of oxidants and heat shock (Hunt, 1979; London et al., 1987; Jackson, 1991). These stress conditions commonly induce the heat shock or stress response in cells, which is characterized by transcriptional activation of heat shock genes, translational inhibition of normal protein synthesis, and selective translation of the mRNAs coding for heat shock proteins (Lindquist and Craig, 1988; Schlesinger, 1990; Morimoto et al., 1990; Ang et al., 1991;

Sorger, 1991; Hightower, 1991; Morimoto et al., 1992). The inhibition of normal protein synthesis in response to heat shock in HeLa and Ehrlich ascites cells correlates with the activation of an eIF-2α kinase with antigenic properties similar to those of HRI (Duncan and Hershey, 1984; DeBenedetti and Baglioni, 1986; Scorsone et al., 1987). However, the mechanism for the activation of HRI in response to these stress conditions is unclear.

It has been widely accepted that heat shock proteins play important roles in living organisms. The synthesis of hsps is induced in cells in response to a variety of environmental and pathophysiological stresses (Craig, 1985; Lindquist, 1986). Most agents or conditions that induce the heat shock response cause the accumulation of denatured and oxidized proteins (Lindquist and Craig, 1988; Craig, 1991). The level of hsp70 produced correlates quantitatively with the degree of stress, and the accumulation of specific quantities of hsp70 must occur before the transcription of hsp mRNAs is repressed (DiDomenico et al., 1982; Mizzen and Welch, 1988; Morimoto et al., 1992). Similarly, the accumulation of specific amounts of hsp70 must also occur before normal protein synthesis recovers in heat shocked cells (DiDomenico et al., 1982; Mizzen and Welch, 1988; Laszlo, 1988). The development of resistance to stress-induced translational inhibition in stressed cells also correlates with the levels of hsp70 present in the cells (Mizzen and Welch, 1988; Laszlo, 1988). These observations suggest that hsp70 may function as a cellular thermometer and regulate the heat shock response in cells. However, the molecular events that lead to the arrest and recovery of normal protein synthesis in response to heat shock and other stresses are still a mystery.

Recently, we have demonstrated that HRI interacts with hsp90, hsp70, and the EC1 antigen (hsp56) in rabbit reticulocyte lysates (Matts and Hurst, 1989; Matts et al., 1992). The association of HRI with hsp90 and the EC1 antigen is dependent upon the presence of hemin in lysates, while the interaction of HRI with hsp70 is not hemin-dependent (Matts et al., 1992). Furthermore, the levels of hsp90 present in the lysates were found to correlate with the ability of hemin to restore protein synthesis in heme-deficient lysates, while the

sensitivity of HRI to activation in response to heat or oxidative stress varied inversely with the levels of hsp70 present in lysates (Matts and Hurst, 1992). These findings prompted us to propose that the interaction of hsp70 with HRI maintains HRI in an inactive conformation and heat shock causes to the accumulation of denatured proteins which bind to hsp70, resulting in the activation of HRI. This model predicts that: addition of denatured proteins should activate HRI and inhibit protein synthesis in hemin-supplemented lysates; denatured proteins should bind to hsp70 and block the binding of hsp70 to HRI; and heat shock should block the interaction of hsp70 with HRI.

The data presented here indicate that: (1) it is the heat shock cognate hsc70 which binds to HRI; (2) release of hsc70 from HRI is dependent on ATP hydrolysis; (3) activation of HRI in heme-deficient lysates correlates with the alteration of the levels of hsp70 bound to HRI; (4) addition of denatured proteins, not native ones, inhibits protein synthesis in hemin-supplemented lysates; (5) the denatured proteins bind and sequester hsp70, prevent the association of hsp70 with HRI, and activate HRI; (6) heat shock blocks hsp70 interaction with HRI and activates HRI; and (7) addition of oxidants, such as N-ethylmaleimide (NEM) and heavy metal ions (Hg^{++}), activates HRI through a pathway differing from those of denatured proteins and heat shock.

Experimental Procedures

Materials

Goat anti-mouse IgM and IgG were obtained from Jackson Immunoresearch Laboratories. Bovine serum albumin (fraction V) (BSA), bovine β-lactoglobulin A (LG), bovine α-lactabumin (LA), rabbit anti-bovine serum albumin, apyrase (grade VIII), N-ethylmaleimide (NEM), nitro blue tetrazolium, 5-bromo-4-chloro-3-indolyl phosphate, p-nitrophenyl-agarose, mouse IgM (TEPC 183), and mouse IgG (MOPC 21) were obtained from Sigma. Alkaline phosphatase conjugated to rabbit anti-mouse IgG or to goat anti-

rabbit IgG was obtained from ICN. L-[^{14}C]Leucine (340 mCi/mmol) and [γ-^{32}P]ATP (1000-3000 Ci/mmol) were obtained from Du Pont-New England Nuclear. The 8D3 anti-hsp90 IgM monoclonal antibody (mAb) (Perdew, 1988; Perdew and Whitelaw, 1991) was provided by Dr. Gary Perdew (Purdue University); the AC88 anti-hsp90 IgG mAb (Riehl et al., 1985) was provided by Dr. David Toft (Mayo Clinic, Rochester, MN); the KN382/EC1 IgG mAb (EC1) (Tai et al., 1986) was provided by Dr. Lee Faber (Medical College of Ohio, OH); the N27F3-4 (N27) and C92F3A5 (C92) anti-hsp70 IgG mAbs (Kost et al., 1989; Welch and Suhan, 1986) were provided by Dr. William Welch (University of California, San Francisco); the anti-HRI F IgG mAb (Pal et al., 1991) was provided by Dr. J.J. Chen (Massachusetts Institute of Technology); and the rabbit 4322 anti-hsp90/hsp70 polyclonal antiserum (Erhart et al., 1988) was provided by Dr. Stephen J. Ullrich (National Cancer Institute).

Buffers

TB, 10 mM Tris-HCl (pH7.5); TB/50, 10 mM Tris-HCl (pH 7.5) containing 50 mM NaCl; TBS, 10 mM Tris-HCl (pH 7.5) containing 150 mM NaCl; TB/500, 10 mM Tris-HCl (pH 7.5) containing 500 mM NaCl; TBS/5% NFDM, TBS containing 5% (w/v) non-fat dry milk; 4X Sample Buffer, 250 mM Tris-HCl (pH 6.8) containing 16% (v/v) glycerol, 5% (w/v) SDS , 0.01% (w/v) bromophenyl blue (BPB), and 0.055% (w/v) dithiothreitol (DTT) (fresh DTT was added just before use).

Protein Synthesis and eIF-2α Phosphorylation in Reticulocyte Lysates

Reticulocyte lysates were prepared from anemic rabbits as described previously (Hunt et al., 1972), using buffered saline containing 5 mM glucose to wash the reticulocytes prior to their lysis (Matts et al., 1991). Protein synthesis was measured by the incorporation of [^{14}C]leucine into acid precipitable protein at 30 °C in standard reticulocyte reaction mixtures (Hunt et al., 1972; Ernst et al., 1978). Hemin-supplemented

control lysates contained 20 µM hemin-HCl. Protein synthesizing lysates were pulsed with [γ-^{32}P]ATP (0.5 mCi/µl protein synthesis mix final concentration) for 4 min at the times indicated in the figures, and 2.5 µl aliquots were analyzed by 8% SDS-PAGE as described (Matts et al., 1991).

Preparation of Denatured Proteins

Bovine serum albumin (BSA) was carboxymethylated with iodoacetic acid and bovine β-lactoglobulin A (LG) was carboxyamidomethylated with iodoacetamide as described previously (Ananthan et al., 1986). The reduced carboxymethylated BSA (RCM-BSA) and the reduced carboxyamidomethylated-LG (RCAM-LG) were then extensively dialyzed over a 72 hours period against six 2 liter volumes of 10 mM Tris-HCl (pH 7.6) containing 150 mM NaCl (TBS) and stored in aliquots at -70 °C. Solutions of the native proteins (~10 mg/ml) were prepared fresh in TBS prior to use.

Immunoprecipitation

p-Nitrophenyl-agarose was rinsed free of isopropanol, and was coupled to goat anti-mouse IgG (GAMG), or goat anti-mouse IgM (GAMM), or goat anti-rabbit IgG (GARG) as described previously (Matts et al., 1992). The preparations contained approximately 1.6 mg of antibody coupled per ml of packed agarose. GAMG-agarose, GAMM-agarose, and GARG-agarose were used as a 1:1 slurry in TBS. The following general procedure describes the quantities of antibodies that were determined quantitatively to adsorb hsp90, the EC1 antigen, HRI, BSA or RCM-BSA from 10 µl of reticulocyte lysate protein synthesis mix: for the adsorption of hsp90, 8D3 anti-hsp90 mAb from 62.5 µl of ascites fluid was bound to 87.5 µl of GAMM-agarose; for the adsorption of the EC1 antigen, 62.5 µl of EC1 mAb (2.8 mg/ml) was bound to 100 µl of GAMG-agarose; for the adsorption of HRI, anti-HRI mAb F from 15 µl of anti-HRI mAb F ascites fluid was bound to 25 µl of GAMG-agarose; for the adsorption of BSA or RCM-BSA, 75 µl of

rabbit anti-BSA antiserum was bound to 75 μl of GARG-agarose. Non-immune control incubations contained equivalent volumes of TEPC 183 mouse IgM or MOPC 21 mouse IgG ascites with a concentration of approximately 5 mg of antibody per ml, or a rabbit pre-immune antiserum.

Antibodies were bound to the GAMG-, or GAMM-, or GARG-agarose for two hours on ice. The GAMG-, or GAMM-, or GARG-agarose was then washed sequentially with 500 μl each of: TBS, TB/500; TBS; and adsorption buffer (either TBS or TB as indicated in the legend of the figures). The GAMG-, or GAMM-, or GARG-agarose was pelleted by a 10 sec centrifugation (full speed burst) in a Brinkman model 5415 Microfuge. The adsorption buffer contained 20 μM hemin-HCl for the immunoadsorption of proteins from hemin-supplemented protein synthesis mixes. Rabbit reticulocyte lysates were incubated under standard conditions for protein synthesis (Hunt et al., 1972; Ernst et al., 1978): in the presence (hemin-supplemented lysates) or absence (heme-deficient lysates) of 20 μM hemin; in the presence or absence of 25 μM BSA or RCM-BSA in hemin supplemented lysates; in the presence of 25 μM Hg^{++} or 20 μM NEM in hemin-supplemented lysates for 20 min at 30 °C; or in the presence of 20 μM hemin-HCl for 20 min at 42 °C. The protein synthesis mixes were then treated with or without apyrase (one unit of apyrase per 10 μl of protein synthesis mix) on ice for 15 min. Apyrase treatment was found to be necessary for stabilization of hsp70-protein complexes (Beckmann et al., 1990; Palleros et al., 1991; Matts et al., 1992). After the removal of excess adsorption buffer from the GAMG-, or GAMM-, or GARG-agarose pellets containing the bound antibodies, 10 μl of protein synthesis mixes were added directly for the adsorption of hsp90, the EC1 antigen, HRI, BSA or RCM-BSA, and their associated proteins. Samples were incubated at 4 °C for 90 min with continuous mixing. The samples were then diluted 10-fold with 90 μl of adsorption buffer, and the non-adsorbed proteins (supernatants) were separated from the proteins adsorbed to the agarose (pellets) by centrifugation. For the analysis of hsp70 associated with HRI, it was necessary to dilute the protein synthesis

mixes 10-fold with adsorption buffer prior to the 90-min adsorption of the proteins, to reduce the nonspecific binding of hsp70.

The pellets were then washed five times with 500 µl of the buffers described below to remove nonadsorbed proteins. For the adsorption of hsp90 or the EC1 antigen, and their associated proteins with the 8D3 anti-hsp90 or the EC1 mAbs, the pellets were washed sequentially: once with TB, twice with TB/50, and twice with TB. For the adsorption of HRI, BSA or RCM-BSA and their associated proteins with the anti-HRI mAb F or the rabbit anti-BSA antiserum, the pellets were washed five times with TBS. For the analysis of hsp70 associated with HRI, the pellets were washed sequentially: once with TBS, three times with TBS containing 1% sodium deoxycholate, 1% Triton X-100, and 0.1% SDS, and once with TBS. Pellets were diluted with an equal volume of water, and stored at -70 °C prior to analysis. Samples were prepared for SDS-PAGE by the addition of one volume of 2XSDS sample buffer followed by heating in a boiling water bath for 2.5 min.

Western Blot Analysis

Samples were separated by SDS-PAGE in 10% or 8% gels (9x14x0.15 cm; 37.5:1 acrylamide:Bis) for analysis (as noted in the figure legends), and proteins were transferred to a PVDF membrane (Biorad) at a current density of 2.5 mA/cm^2 for 40 min for Western blot analysis as described previously (Matts and Hurst, 1989; Matts et al., 1992). Blots were blocked with TBS/5% NFDM for one hour at room temperature. The primary antibody was then reacted with the blots at 4 °C overnight. Blots were washed twice with TBS containing 0.5% Tween-20, once with TBS, and then blocked with TBS/5% NFDM prior to reaction with alkaline phosphatase-conjugated rabbit anti-mouse IgG or goat anti-rabbit IgG (diluted 1:1000 in TBS/1% NFDM) for two hours at room temperature. Blots were washed twice with TBS containing 0.5% Tween-20, and twice with TBS. Proteins were then detected by incubating blots in alkaline phosphatase reaction buffer (100 mM

Tris-HCl, pH9.5, containing 100 mM NaCl, 100 mM $Mg(OAc)_2$, 300 μg/ml NTB and 150 μg/ml BCIP) at 35 °C for 1 to 5 min.

For detecting HRI, the ascites fluid containing the anti-HRI mAb F was diluted 1:250 in TBS/1% NFDM. For detecting hsp90, the 4322 anti-hsp90/hsp70 antiserum was diluted 1:50 in TBS/1% NFDM. For detecting hsp70, the N27 anti-hsc73/hsp 72 mAb was diluted 1:1000 in TBS/1% NFDM. For detecting the EC1 antigen, the EC1 mAb was diluted 1:1000 in TBS/1% NFDM.

Reconstitution Reactions

Hemin-supplemented reticulocyte lysates were incubated under standard conditions for protein synthesis (Hunt et al., 1972; Ernst et al., 1978) at 30 °C (control) or 42 °C (heat shock) for 20 min. The protein synthesis mixes were then treated with apyrase at a concentration of one unit of apyrase per 10 μl of protein synthesis mixture to stabilize the hsp70-protein complexes. The HRI-free reconstitution reaction supernatants (control and heat shocked) were prepared by quantitatively removing HRI with the anti-HRI mAb F, but with no apyrase treatment. For reconstitution, HRI was then immunoprecipitated from these apyrase treated lysates by the anti-HRI mAb F as described above. After washing with TBS five times, the HRI immune pellets were then incubated with the reconstitution reaction supernatants as described in the legends of the figures. The reconstitution reactions were carried out at 30 °C for 5 min. The pellets were then washed twice with TBS, three times with TBS containing 1% DOC, 1% Triton 100, and 0.1% SDS, and twice with TBS. The adsorbed proteins were then extracted into SDS-PAGE sample buffer and separated on a 10% SDS-PAGE gel followed by Western blot analysis as described above. The HRI-associated hsp70 was then detected by the N27 anti-hsp70 mAB, or the 4322 anti-hsp90/hsp70 serum.

Results

The constitutively expressed form of hsp70 (hsc73) interacts with HRI.

The N27 anti-hsp70 monoclonal antibody (mAb) is able to recognize both hsc73, the constitutively expressed form of hsp70, and hsp72, the inducible form of hsp70, respectively (Kost et al., 1989). However, the C92 anti-hsp70 mAb can recognize only hsp72 (Welch and Suhan, 1986). By using the C92 and the N27 anti-hsp70 mAbs, both hsc73 and hsp72 were found to be present in the rabbit reticulocyte lysates by Western blot analysis (Fig. 12). When the immune pellets of the anti-HRI mAb F were analyzed by the C92 mAb, no hsp70 band was observed to be coadsorbed with HRI (Fig. 12A). However, when the immune pellets were analyzed by the N27 mAb, a hsp70 band was clearly observed to be coadsorbed with HRI from both hemin-supplemented and heme-deficient reticulocyte lysates (Fig. 12B). The immunological properties of the hsp70 band coadsorbed with HRI indicates that the constitutively expressed form of hsp70 is associated with HRI in reticulocyte lysates. More hsc73 was observed to associate with HRI from heme-deficient lysates than that from hemin-supplemented lysates (Fig. 12B).

The Release of Hsp70 from HRI Is Dependent upon ATP-Hydrolysis.

Hsp70 binds to ATP and has a weak ATPase activity (Zylicz et al., 1983; Welch and Feramisco, 1985). Release of hsp70 from its substrates is dependent on ATP-hydrolysis (Pelham, 1986; Clarke et al., 1988; Flynn et al., 1989; Kost et al., 1989; Beckmann et al., 1990; Palleros et al., 1991). These observations prompted us to determine whether the release of hsp70 from HRI requires the hydrolysis of ATP. HRI was immunoprecipitated from hemin-supplemented and heme-deficient lysates by the anti-HRI mAb F. The immunoprecipitates were then incubated with TBS containing 5 mM Mg^{++} or TBS containing 5 mM Mg^{++} and 1 mM ATP. Western blot analysis indicated that hsp70 dissociated from hsp70-HRI complexes when ATP was present in the incubation

buffer, while no hsp70 was released of from HRI-hsp70 complexes if no ATP was present in the incubation buffer (Fig. 13).

Changes in the Levels of Hsp70 Associated with HRI During Activation of HRI in Heme-Deficiency.

In heme-deficiency, HRI is activated and protein synthesis shuts off after 5 min of incubation (Fig. 15). Both hsp90 and the EC1 antigen disassociate from HRI in heme-deficient reticulocyte lysates, while hsp70 remains bound to HRI (Matts et al., 1992). To understand the behavior of hsp70 during the process of HRI activation in heme-deficient lysates, the interaction of HRI with hsp70 was examined after various times of incubation. The amount of hsp70 coadsorbed with HRI was observed to vary with the incubation time (Fig. 14). The highest amount of hsp70 was observed to be associated with HRI prior to the incubation of the heme-deficient lysates (0 min) (lane 2); little hsp70 was observed to be associated with HRI after 5 min of incubation (lane 3); after 10 min of incubation, the amount of hsp70 associated with HRI increased (lane 4); no further increase in the amount of hsp70 bound to HRI was observed after 15 min of incubation (lanes 5 & 6). The amount of hsp70 coadsorbed with HRI (Fig. 14) was quantitated by scanning of Western blots with the Discovery Series TM PDI Densitometer (Model DNA 35) (Table I and Fig. 15). We noted that protein synthesis in heme-deficient lysates shut off after 5 min incubation at 30 °C, the time at which little hsp70 was bound to HRI.

Denatured Proteins Sequester Hsp70 Causing the Activation of HRI.

Denatured Proteins Inhibit Protein Synthesis in Rabbit Reticulocyte Lysates. Ananthan et al. (1986) reported that microinjection of denatured proteins, RCM-BSA or RCAM-LG, into frog oocytes triggered the activation of heat shock genes. Reduced carboxymethylated bovine α-lactabumin (RCM-LA) has been used as a model substrate to study the interactions of hsp70 with proteins *in vitro* (Palleros et al., 1991). The effect of

denatured proteins on protein synthesis in hemin-supplemented reticulocyte lysates was examined. Protein synthesis in hemin-supplemented lysates was inhibited by the addition of the denatured proteins (RCM-BSA, RCM-LA, and RCAM-LG), while little or no inhibitory effect of the native proteins was observed (Fig. 16A). The concentrations of RCM-BSA, RCM-LA and RCM-LG that inhibited protein synthesis by 50% relative to the control (IC50) were estimated to be 27, 23 and 17 μM, respectively (Fig. 16B).

Denatured Proteins Activate HRI in Hemin-supplemented Lysates. The capacity of lysate to phosphorylate eIF-2α in situ was examined to determine whether the inhibition of protein synthesis in hemin-supplemented reticulocyte lysates caused by the addition of the denatured proteins was due to the activation of HRI. An increase in eIF-2α phosphorylation in the lysates containing the 25 μM RCM-BSA was observed, while no increase in eIF-2α phosphorylation above the control was observed in lysates containing 25 μM native BSA (Fig. 17). The increase in eIF-2α phosphorylation caused by the RCM-BSA was suppressed by the anti-HRI mAb F (Fig. 17). Similarly, RCM-LA and RCM-LG, but not native LA or LG, also stimulated eIF-2α phosphorylation (data not shown). These observations suggest that denatured proteins induce the activation of HRI in hemin-supplemented lysates.

Denatured proteins bind hsp70 and sequester hsp70 from HRI. The observations that the sensitivity of protein synthesis to stress-induced inhibition is inversely correlated with the levels of hsp70 present in the lysate (Matts and Hurst, 1992) and that denatured proteins trigger the inhibition of protein synthesis in lysates suggest a relationship among the activation of HRI, denatured proteins, and hsp70. To determine whether hsp70 binds to denatured proteins, an anti-BSA antibody was used to determine whether hsp70 could be co-adsorbed from the lysates with RCM-BSA or BSA. Hsp70 from hemin-supplemented lysates was immune-specifically coadsorbed with the RCM-BSA, but not native BSA, by the anti-BSA antibody (Fig. 18). Western blot analysis of immunoprecipitated RCM-BSA

detected no bound HRI (data not shown), indicating that: (i) RCM-BSA binds to hsp70 directly; and (ii) RCM-BSA does not bind to HRI and affect its activity. HRI from control lysates (no additions) or from the lysates containing BSA or RCM-BSA was adsorbed using the anti-HRI mAb F. Western blot analysis indicated that hsp70 was coadsorbed with HRI from both the control lysate and the lysate containing the native BSA, but not from the lysates containing the RCM-BSA (Fig. 18B).

The data suggest that the mechanism by which denatured proteins trigger the inhibition of protein synthesis in hemin-supplemented lysates is through the capacity of denatured proteins to bind and sequester hsp70, blocking the interaction of HRI with hsp70, which subsequently activates HRI.

Heat Shock Blocks the Interaction of Hsp70 with HRI and Activates HRI.

Heat Shock Inhibits Protein Synthesis in Hemin-supplemented Lysates. Protein synthesis in hemin-supplemented lysates was observed to become inhibited in response to a heat shock (42 °C), confirming previous reports (Bonanou-Tzedaki et al., 1978; Ernst et al., 1982) (Fig. 19). The heat shock-induced inhibition of protein synthesis in hemin-supplemented lysates occurred more slowly and was not as severe as the inhibition observed in heme-deficient lysates. An increase in eIF-2α phosphorylation was observed in response to heat shock in hemin-supplemented lysate (Fig. 20, lane 3). The heat induced eIF-2α phosphorylation was significantly reduced by preincubation of the lysates with the anti-HRI mAb F (Fig. 20, lane 5), indicating that heat shock induces the activation of HRI which results in shut-off of protein synthesis.

Hsp90 and the EC1 Antigen Remain Bound to HRI During Heat Shock. Hsp90 and the EC1 antigen associate with inactive HRI in hemin-supplemented lysates and dissociate from HRI in conjunction with activation of HRI during heme-deficiency (Matts et al., 1992). Therefore, it was of interest to determine whether the activation of HRI in

hemin-supplemented lysates in response to heat shock occurs in conjunction with the dissociation of hsp90 or the EC1 antigen. The hsp90 from the hemin-supplemented lysates incubated at 30 °C control) or at 42 °C (heat shocked) was immunoadsorbed by the 8D3 anti-hsp90 mAb. Western blot analysis indicated that HRI was quantitatively coadsorbed with hsp90 (pellets) from both control and heat shocked lysates (Fig. 21). The data indicate that no dissociation of hsp90 from HRI is required for the activation of HRI in response to heat shock. Similarly, using the 4322 anti-hsp90/hsp70 polyclonal antibody, hsp90 was detected to be coadsorbed with HRI by the anti-HRI mAb F from both heat shocked and control lysates (Fig. 22).

HRI was also co-adsorbed with the EC1 antigen by the EC1 mAb from control and heat shocked hemin-supplemented lysates (Fig. 23, pellets). The quantitative removal of HRI by the EC1 mAb (Fig. 23, supernatant) indicated that activation of HRI in hemin-supplemented lysates in response to heat shock is not accompanied with its dissociation of the EC1 antigen.

Hsp70 Dissociates from HRI During Heat Shock. Hsp70 was found to associate with HRI in both heme-deficient and hemin-supplemented lysates (Matts et al., 1992). HRI was adsorbed from control and heat shocked hemin-supplemented lysates with the anti-HRI mAb F. Western blot analysis indicated that hsp70 was coadsorbed with HRI from the control lysate, but not from the heat shocked lysate (Fig. 24). The results indicate that hsp70 dissociates from HRI during heat shock. This observation was consistent with the observation that hsp70 is released from HRI upon its activation in the presence of denatured proteins.

Hsp70 Can Bind Back to HRI when Heat Shock Is Removed. The loss of HRI bound hsp70 in response to heat shock could be due to the accumulation of the heat-induced denatured proteins, which bind and sequester hsp70, or due to a heat-induced change in the conformation of HRI, which makes HRI be unable to associate with hsp70.

Therefore, we examined whether hsp70 could reassociate with HRI that had been activated by heat stress. HRI was immunoprecipitated from the control or heat shocked lysate by the anti-HRI mAb F, and subsequently reincubated with HRI-free control or heat-shocked HRI-free lysates (reconstitution reaction supernatants). The reassociation of hsp70 with HRI was observed when the control pellet was incubated with the control or heat shocked reconstitution reaction supernatant at 30 °C (Fig. 25, lanes 1 & 2). No hsp70 was observed to bind back to HRI when the heat shocked pellet was incubated with the heat shocked reconstitution reaction supernatant (Fig. 25, lane 4). In contrast, when the heat shocked pellet was incubated with the control reconstitution mixture, hsp70 was observed to bind back to HRI (Fig. 25, lane 3). The data indicate that the loss of HRI bound hsp70 in hemin-supplemented lysates in response to heat shock is not due a heat-induced conformational change in HRI, which results in the loss of the hsp70 binding site on HRI. The data support the hypothesis that heat shock induces the denaturation of proteins in lysates, which bind and sequester free hsp70. Subsequently, the interaction of hsp70 with HRI is blocked, which activates HRI and leads to the inhibition of protein synthesis in hemin-supplemented lysates.

Oxidants Activate HRI Through A Pathway Differed from That of Heat Shock.

HRI is also activated in hemin-supplemented lysates upon the addition of oxidants, such as N-ethylmaleimide (NEM) or heavy metal ions (Hg^{++}) (Hunt, 1979; Hurst et al., 1987; Matts et al., 1991). Preincubation of hemin-supplemented lysates with the anti-HRI mAb F did not prevent the activation of HRI caused by the addition of Hg^{++} (Fig. 26) or NEM (Fig. 27). In addition, the activation of HRI in heme-deficient lysates was not blocked by the preincubation of the lysates with the anti-HRI mAb F (Fig. 26).

The activation of HRI in heme-deficient lysates correlates with the dissociation of hsp90 and the EC1 antigen from HRI with hsp70 remaining bound (Matts et al., 1992), while the activation of HRI in response to heat shock correlates with the dissociation of

hsp70 with hsp90 and the EC1 antigen remaining bound. Therefore, we examined the interactions of HRI with its associated heat shock proteins during the process of its activation upon the addition of the oxidants. Western blot analysis indicated that HRI was coadsorbed with hsp90 by the 8D3 anti-hsp90 mAb from hemin-supplemented lysates incubated with 10 mM of NEM or 25 μM of Hg^{++} (Fig. 28). Similarly, the coadsorption of HRI with the EC1 antigen by the EC1 mAb from the NEM or Hg^{++} treated reticulocyte lysates was observed (Fig. 29). Furthermore, Western blot analysis indicated that hsp70 remained bound to HRI adsorbed from hemin-supplemented lysates after its activation in response to the addition of NEM or Hg^{++} (Fig. 30).

Discussion

HRI interacts with hsp90, hsp70, and the EC1 antigen in rabbit reticulocyte lysates. Activation of HRI in reticulocyte lysates correlates with alterations of these interactions. By characterizing the composition of HRI/hsps complexes during the activation of HRI and the effect of the anti-HRI mAb on HRI activation, three different pathways for activating HRI have been distinguished. These three pathways are summarized in Table 2.

Pathway I refers the activation of HRI in heme-deficiency. In heme-deficient lysates, activation of HRI correlates with the dissociation of hsp90 and the EC1 antigen from HRI, while hsp70 remains bound to HRI. The activation of HRI through this pathway cannot be prevented by pre-incubation with the anti-HRI mAb. We noted that although hsp70 remains bound to HRI in pathway I, changes in the interaction of hsp70 with HRI during heme-deficiency have been observed. A transient decrease was observed in the amount of hsp70 bound to HRI after 5 min of incubation. This transient decrease was observed when lysates were treated with apyrase. However, without apyrase treatment, this decrease in the amount of HRI-bound hsp70 in heme-deficient lysates was not observed. Interestingly, more hsp70 was observed to bind to HRI at 5 min compared to longer incubation in the presence of ATP in heme-deficient lysates (data not shown).

We are currently trying to determine the basis for the apparently contradictory results. Hsp70 binds ADP more tightly than ATP to form a binary complex, which binds to the unfolded protein more rapidly that free hsp70 (Greene and Eisenberg, 1990; Palleros et al., 1992). Dissociation of hsp70 from its bound proteins requires ATP-hydrolysis, which involves an ADP/ATP exchange reaction (Sadis and Hightower, 1992). In heme-deficient lysates, protein synthesis occurs at a normal rate before the reversing factor is sequestered by phosphorylated eIF-2 (eIF-2(αP)). During that period, usually about 5 min, a large amount of nascent polypeptides is produced. With the apyrase treatment, hsp70 only binds ADP since no ATP is available. These hsp70/ADP binary complexes may bind to the nascent polypeptides, resulting in a loss of hsp70 from HRI. With a longer incubation during heme-deficiency, the nascent polypeptides may fold or assemble with the assistance of hsp70. Hsp70 would be released from the polypeptides, upon the exchange of ATP for ADP, and hsp70 would bind back to HRI. If ATP is present during immunoprecipitation, free hsp70 would be present in lysates upon the hydrolysis of ATP. Therefore, the association of HRI with hsp70 could be observed at any time point of incubation in heme-deficient lysates.

Pathway II represents the pathway for the activation of HRI in response to the addition of denatured proteins or heat shock in hemin-supplemented lysates. In this pathway, HRI remains associated with hsp90 and the EC1 antigen, but not with hsp70. This finding suggests that hsp70 may play a role in keeping HRI in an inactive form in hemin-supplemented lysates. The activation of HRI that occurs due to the loss of bound hsp70 can be reversed by pre-incubation of the anti-HRI mAb. However, once HRI is activated by heat shock, the anti-HRI mAb F has little effect on the HRI activity (data not shown). Therefore, it appears as if the antibody affects the activation of HRI but not the catalytic activity of activated HRI. This observation suggests that HRI undergoes a conformational change during its activation in response to heat shock, which correlates with the release of hsp70 from HRI. This conformation change probably involves amino

acids present in the "kinase insertion sequence" in HRI, which contains the antigenic site recognized by the mAb F (Chen et al., 1991a,b). This region represents a unique insertion of approximately 140 amino acids located between the conserved domains V and VI present in all known protein kinases (Chen et al., 1991a,b).

Evidence suggests that the rate-limiting steps for the expression of heat-induced damage in mammalian cells involve protein denatration (Lepock, 1987; Lepock et al., 1988; Pinto et al., 1991). The release of hsp70 from HRI during heat shock may be the result of the accumulation of denatured proteins in lysates caused by heat shock. When the concentration of denatured proteins reaches at a certain level, the free hsp70 in the lysate would be sequestered in complexes with denatured proteins, which blocking the interaction of hsp70 with HRI. In the absence of bound hsp70, HRI would be activated, leading to the shutdown of the protein synthesis.

The proposed mechanism would also ensure that the rate of protein synthesis within a cell could not exceed the capacity of the cell to transport, fold, assemble or otherwise process newly synthesized proteins properly. Nacent polypeptides that are destined to enter the lumen of the endoplasmic reticulum (ER) or the mitochondrial matrix are thought to be maintained in a conformation that is competent of undergoing transport by cytosolic hsp70 (Lindquist and Craig, 1988; Schlesinger, 1990; Welch, 1990). Mitochondrial hsp70 is reuired for the translocation of polypeptides and their folding in the mitochondrial matrix (Linduist and Craig, 1988; Ang et al., 1991; Ellis and van der Vies, 1991). The hsp70 homolog of the ER (Bip or grp78) is essential for protein transfer into the lumen of the ER. Secretory proteins accumulate in the cytosol in yeast in the absence of functional Bip (Vogel et al., 1990). The inability of proteins to be translocated into the ER or mitochondria, or to be properly folded or assembled within these organelles, may lead to the accumulation of nascent polypeptide precursors within the cytoplasm. These nascent polypeptides would compete with HRI (or a homologous kinase) for the binding of hsp70. The subsequent activation of the eIF-2α kinase would inhibit protein synthesis. Therefore,

nascent polypeptides probably represent a physiologically relevant pool of hsp70 binding substrates. A global inhibition of protein synthesis, through the activation of HRI or a homologous eIF-2α kinas, may be important for maintaining the viability of stressed cells that do not constitutively express sufficient levels of hsp70 to protect cells from damage.

Pathway III describes the activation of HRI in response to the addition of oxidants, such as NEM and Hg^{++}, to hemin-supplemented lysates. Similar to heme-deficiency (pathway I), the activation of HRI induced by oxidants cannot be prevented by pre-incubation with the anti-HRI mAb F and hsp70 remains bound to HRI. On the other hand, HRI remains associated with hsp90 and the EC1 antigen, which is similar to the mechanism proposed for pathway II. However, hsp70 is observed to remain bound to HRI in pathway III, which distinguishes this pathway from that in pathway II. Oxidative conditions have been proposed to possibly cause a rearrangement of disulfide bonds within HRI, which may lead to the stable binding of hsp70 to HRI.

Figure 12. Immunological Properties of Hsp70 Associated with HRI in Rabbit Reticulocyte Lysates.

Proteins from 10 µl of hemin-supplemented (*+H*) and heme-deficient (*-H*) protein synthesis mixes were adsorbed with non-immune control (*C*) or the anti-HRI (*I*) antibody bound to GAG-agarose in the presence of TBS as described under "Experimental Procedures". The adsorbed prtoteins were Western blotted with the C92 anti-hsp72 (*Upper*) or with the N27 anti-hsp72/hsc73 (*Lower*) antibody to detect the presence of hsp70. *L*, 1.25 µl of unincubated whole reticulocyte lysates.

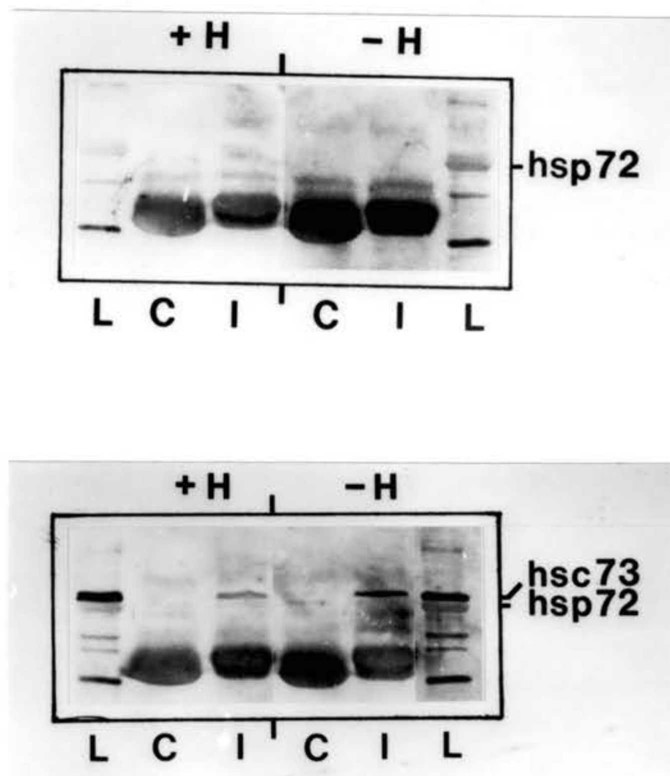

+H
-H
hsp72
L C I C I L
+H
-H
hsc73
hsp72
L C I C I L

Figure 13. Requirement for ATP-Hydrolysis in Releasing of Hsp70 from HRI.

Proteins from 10 μl of apyrase treated (10 μl protein synthesis mixes per unit apyrase, on ice for 15 min) hemin-supplemented (*+H*) and heme-deficient (*-H*) protein synthesis mixes were adsorbed with non-immune (*C*) or the anti-HRI (*I*) antibody in the presence of TBS, as described under "Experimental Procedures". The adsorbed proteins were then eluted with 5 mM Mg^{++} (*- ATP*) or with 5 mM Mg^{++} and 1 mM ATP (*+ ATP*) at 30 °C for 5 min. The eluted portion (*Sup*) and non-eluted portion (*Pel*) were then Western blotted with the N27 anti-hsp70 mAb to detect the presence of hsp70, respectively. The non-eluted portion were also blotted with the anti-HRI mAb F to show the presence of HRI. *L*, 1.25 μl of unincubated whole reticulocyte lysates; *HC*, heavy chain of IgG.

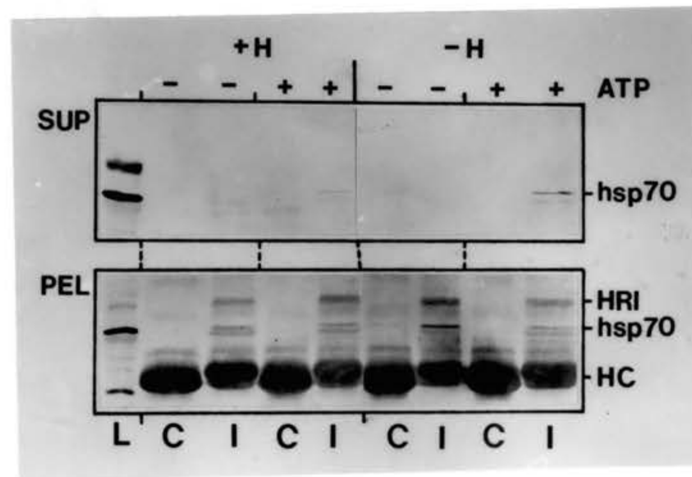

+H
-H
- - + + - - + + ATP
SUP
hsp70
PEL
HRI
hsp70
HC
L C I C I C I C I

Figure 14. Interaction of HRI with Hsp70 in Heme-Deficient Lysates.

Protein synthesis mixes were incubated at 30 °C for 0 min (*lane2*), 5 min (*lane3*), 10 min (*lane 4*), 15 min (*lane 5*), and 20 min (*lanes 1, 6, & 7*), respectively. After incubation, these mixes were immediately treated with apyrase (one unit of apyrase per 10µl of protein synthesis mix) on ice for 15 min. Proteins from 10 µl of the apyrase treated heme-deficient (*lanes 1 to 6*) and hemin-supplemented (*lane 7*) protein synthesis mixes were adsorbed with non-immune control (*lane 1*) or the anti-HRI (*lanes 2 to 7*) antibody bound to GAG-agarose in the presence of TBS, as described under "Experimental Procedures". The adsorbed proteins were separated by 10% SDS-PAGE followed by Western blot analysis, probing with the N27 anti-hsp70 mAb, to detect the interaction of HRI with hsp70. *HC*, heavy chain of IgG.

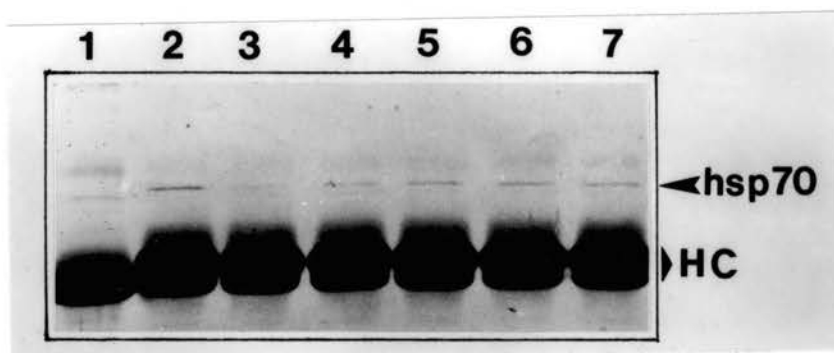
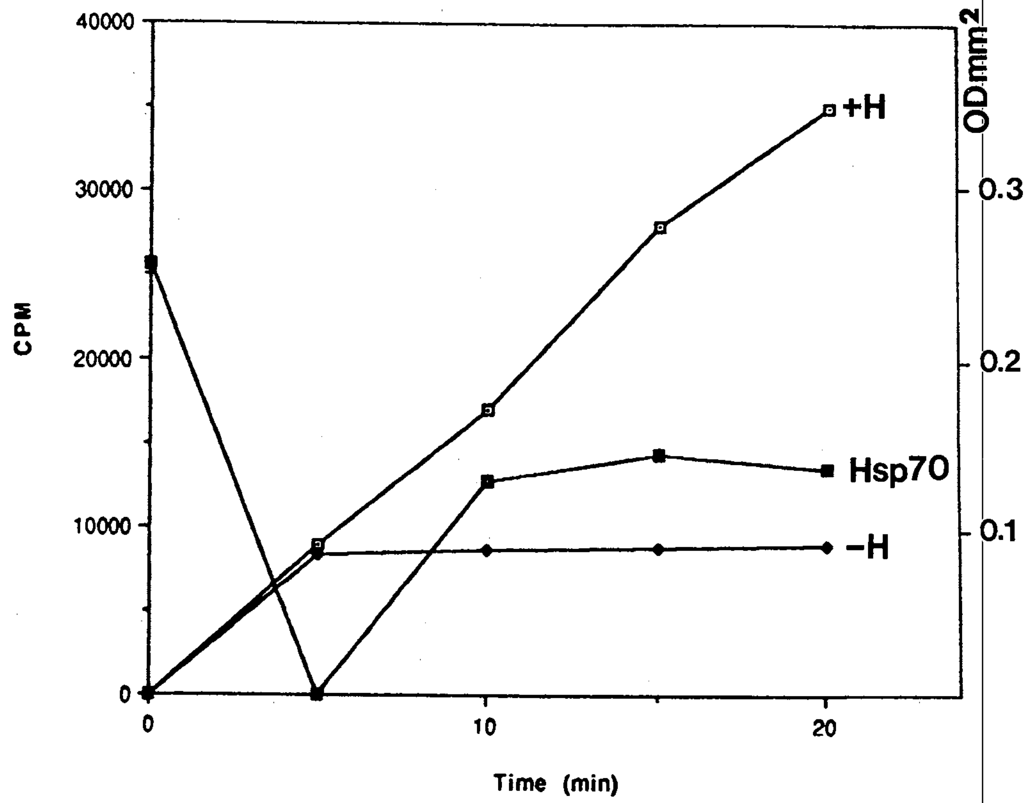

1 2 3 4 5 6 7
hsp70
HC

Table I The Amount of Hsp70 Associated with HRI During the Activation Process in Heme-Deficient Reticulocyte Lysate.

Band No.	Antibody Added	Hemin Added	Incubation (30 °C)	Quantity $ODxMM^2$
1	Non	No	0 min	-------
2	mAb F	No	0 min	0.257
3	mAb F	No	5 min	0
4	mAb F	No	10 min	0.127
5	mAb F	No	15 min	0.143
6	mAb F	No	20 min	0.135
7	mAb F	Yes	20 min	0.125

Figure 15. Inhibition of Protein Synthesis and the Amount of Hsp70 Associated with HRI in Heme-Deficient Lysates.

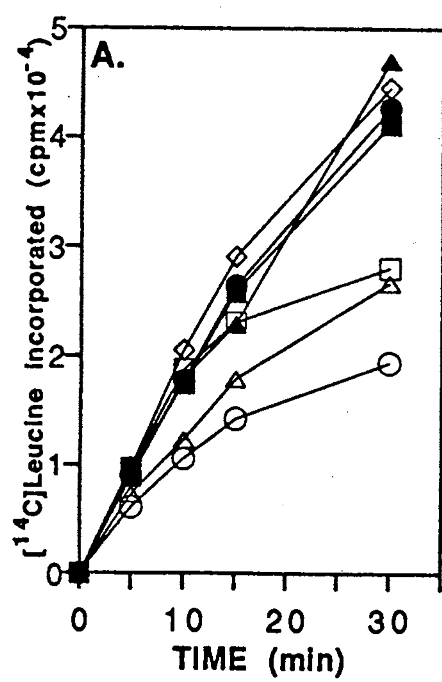
Protein synthesis was measured by the incorporation of ^{14}C-leucine into acid precipitable protein at 30 °C in the hemin-supplemented (+*H*) and heme-deficient (-*H*) protein synthesis mixes, as described under "Experimental Procedures". The amount of hsp70 associated with HRI was determined by a densitometer and the result was shown in Figure 14 and Table 1.



40000
30000
20000
10000
0
CPM
0
10
20
Time (min)
ODmm2
0.3
0.2
0.1
+H
Hsp70
-H

Figure 16. Effect of Denatured Proteins on Protein Synthesis in Hemin-Supplemented Reticulocyte Lysates.

(**A**) Protein synthesis was measured at 30 °C in standard (40 μl) rabbit reticulocyte lysate reaction mixtures containing 20 μM hemin with no additions (◇-◇) or with the addition of 20 μM BSA (■-■), RCM-BSA (□-□), LA (●-●), RCM-LA (○-○), LG (▲-▲), or RCAM-LG (△-△). The amount of [^{14}C]leucine incorporated into acid precipitable protein in a 5 μl aliquot was determined at the times indicated in the figure. (**B**) Hemin-supplemented protein synthesis mixtures were incubated for 30 min at 30 °C with no additions (controls), or with varying concentrations of RCM-BSA (□-□), RCM-LA (○-○), or RCAM-LG (△-△). The amount of protein synthesis is reported as the amount of [^{14}C]leucine incorporated into acid-precipitable protein in 30 min relative to the amount incorporated in the control (% control). Values are the average of three titrations.


A.
[^{14}C]Leucine Incorporated (cpmx10^{-4})
0
1
2
3
4
5
0
10
20
30
TIME (min)

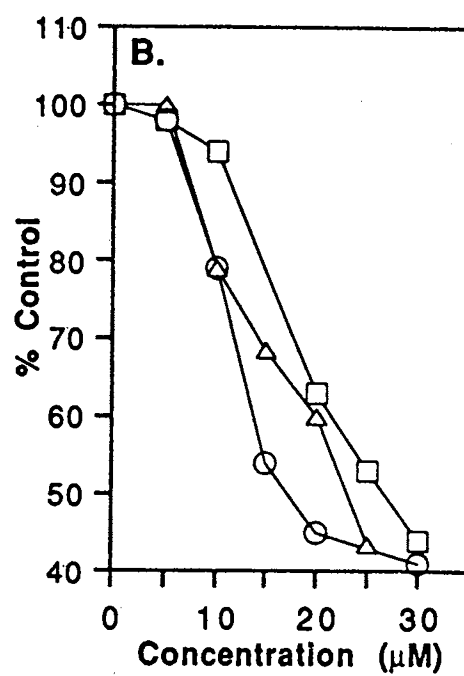

B.
% Control
40
50
60
70
80
90
100
110
0
10
20
30
Concentration (μM)

Figure 17. Addition of the Anti-HRI mAb F Blocks the Phosphorylation of eIF-2α Induced by RCM-BSA.

Hemin-supplemented protein synthesis mixes were pre-incubated on ice for 15 min in the presence of no additions (NONE, lanes 1-3) or in the presence of ~1 µg IgG/10 µl protein synthesis mix of control monoclonal IgG (MOPC 21 mouse IgG ascites, Sigma) (lanes 4-6) or anti-HRI mAb F (lanes 7-9). Samples were subsequently incubated at 30 °C for 12 min in the presence of no additions (lanes 1,4 & 7), or in the presence of 25 µM BSA (lanes 2, 5 &8) or RCM-BSA (lanes 3, 6 & 9), and then pulsed with [γ-^{32}P]ATP for 4 min. Samples (2.5 µl) were analyzed by SDS/PAGE, as described under "Experimental Procedures", followed by autoradiography.

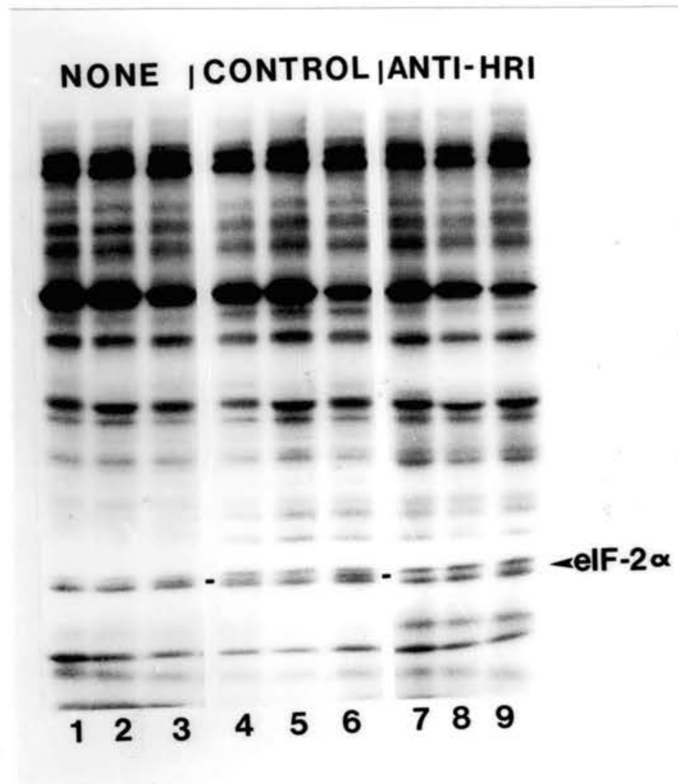
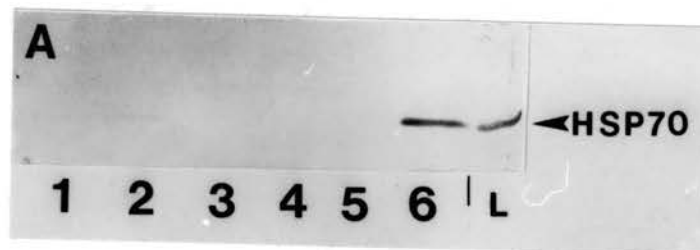

NONE
CONTROL
ANTI-HRI
eIF-2α
1 2 3 4 5 6 7 8 9

Figure 18. Analysis of the Capacity of RCM-BSA to Bind Hsp70 and Block the Interaction of Hsp70 with HRI in Hemin-Supplemented Lysates.

(**A**) Western blot analysis comparing the capacity of hsp 70 to bind to RCM-BSA versus BSA. (**B**) Western blot analysis comparing the capacity of RCM-BSA versus BSA to block the interaction of hsp 70 with HRI. Hemin-supplemented protein synthesis mixes (20 μl for **A**, or 10 μl for **B**) were incubated at 30 °C for 30 min in the presence of no additions (lanes 1 & 2), or in the presence of 25 μM BSA (lanes 3 & 4) or RCM-BSA (lanes 5 & 6). Proteins adsorbed by rabbit anti-BSA (**A**, lanes 2, 4 & 6), anti-HRI mAb F (**B**, lanes 2, 4 & 6), or non-immune control antibodies (**A** & **B**, lanes 1, 3, & 5) were resolved by SDS/PAGE and hsp 70 was detected by Western blotting as described under "Experimental Procedures". *HC*, heavy chain of anti-HRI mAb; *L*, 1.25 μl of unincubated whole reticulocyte lysate.


A
HSP70
1
2
3
4
5
6
L

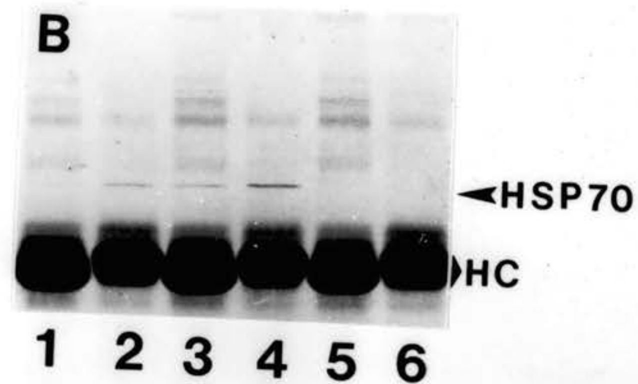

B
HSP70
HC
1
2
3
4
5
6

Figure 19. Effect of Heat Shock on Protein Synthesis in Hemin-Supplemented Lysates.

Protein synthesis was measured in standard reaction mixtures at 30 °C in the absence (-h) or presence (+h) of 20 μM hemin-HCl, or at 42 °C in the presence of hemin (42 °C). The amount of ^{14}C-leucine incorporated into acid precipitable protein in a 5 μl aliquot was determined at the times indicated in the figure.

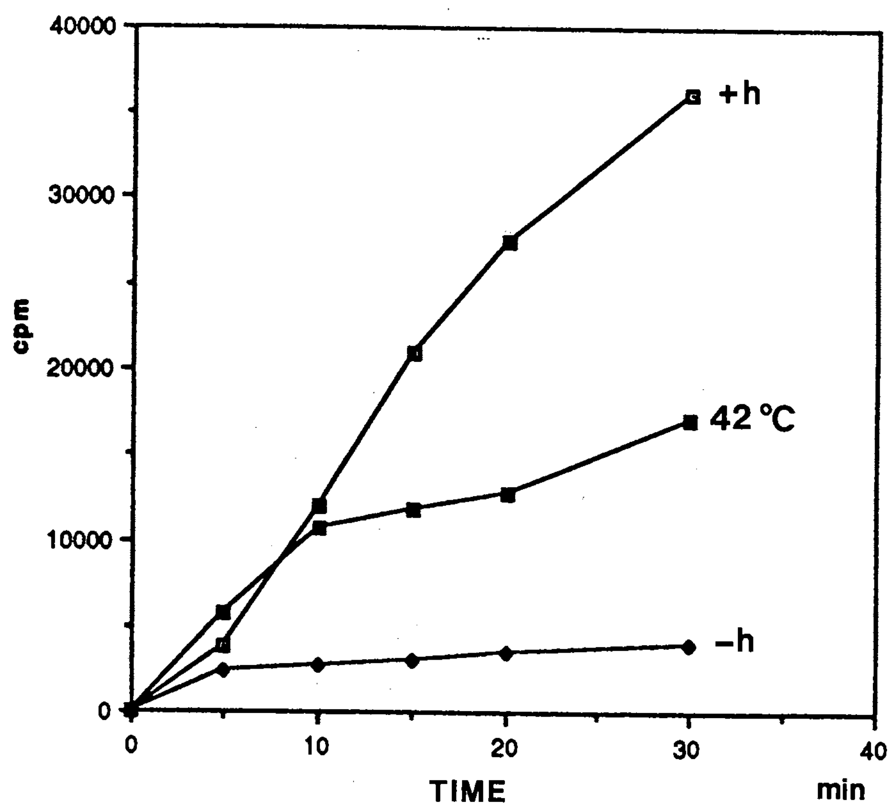

40000
30000
20000
10000
0
cpm
0
10
20
30
40
TIME
min
+h
42 °C
-h

Figure 20. Heat Shock Induces the Activation of HRI in Hemin-Supplemented Lysates.

Hemin-supplemented (*lanes 1 & 3-5*) and heme-deficent (*lane 2*) protein synthesis mixes were pre-incubated on ice for 15 min in the presence of no additions (*lanes 1 to 3*) or in the presence of ~ 1 μg IgG per 10 μl of protein synthesis mix of control monoclonal IgG (MOPC 21 mouse IgG ascites, Sigma) (*lane 4*) or anti-HRI mAb F (*lane 5*). Samples were subsequently incubated at 30 °C (*lanes 1 & 2*) or at 42 °C (*lanes 3 to 5*) for 20 min, and then pulsed with [γ-^{32}P]ATP for 4 min. Samples (2.5 μl) were analyzed by 8% SDS-PAGE followed by autoradiography.

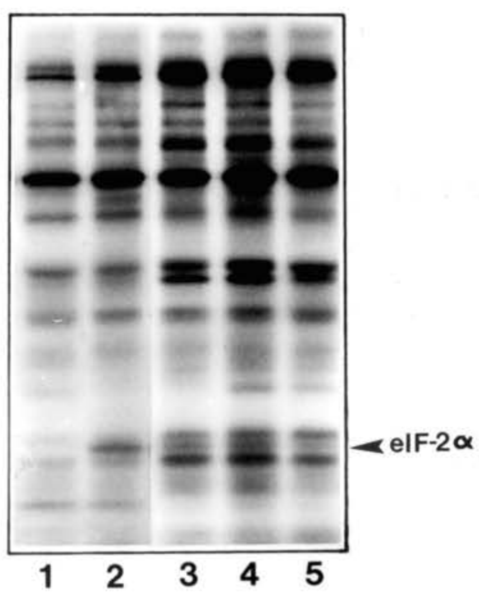

eIF-2α
1
2
3
4
5

Figure 21. Coadsorption of HRI with Hsp90 by the 8D3 Anti-hsp90 mAb in Heat Shocked Hemin-supplemented Lysates.

Hemin-supplemented protein synthesis mixes were incubated at 30 °C or 42 °C for 20 min. Proteins from 2.5 µl of the incubated protein synthesis mixes were adsorbed with the non-immune control (*C*) or the 8D3 anti-hsp90 (*I*) antibody bound to GAM-agarose in the presence of TB, as described under "Experimental Procedures". The adsorbed (*Pel*) and non-adsorbed (*Sup*) fractions were immunoblotted with the anti-HRI mAb F for the presence of HRI.

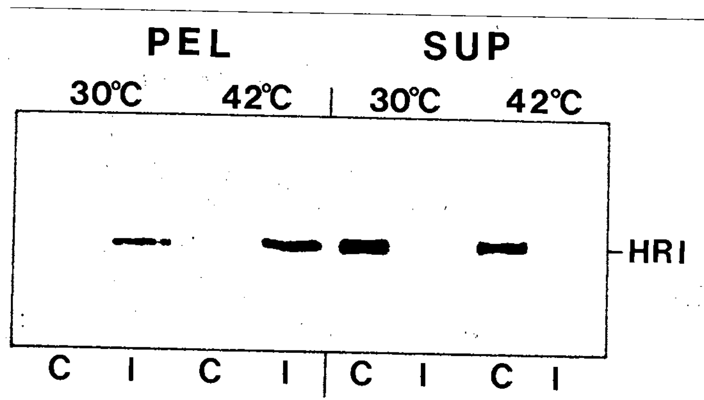

PEL
SUP
30°C
42°C
30°C
42°C
HRI
C
I
C
I
C
I
C
I

Figure 22. Coadsorption of Hsp90 with HRI by the Anti-HRI mAb F in Heat Shocked Hemin-Supplemented Lysates.

Hemin-supplemented protein synthesis mixes were incubated at 30 °C or 42 °C for 20 min. Proteins from 10 μl of the incubated protein synthesis mixes were adsorbed with the non-immune control (*C*) or the anti-HRI (*I*) antibody bound to GAG-agarose.in the presence of TBS, as described under "Experimental Procedures". The adsorbed fractions were Western blotted with the 4322 anti-hsp90/hsp70 antibody to detect the presence of hsp90. *HC*, heavy chain of IgG.

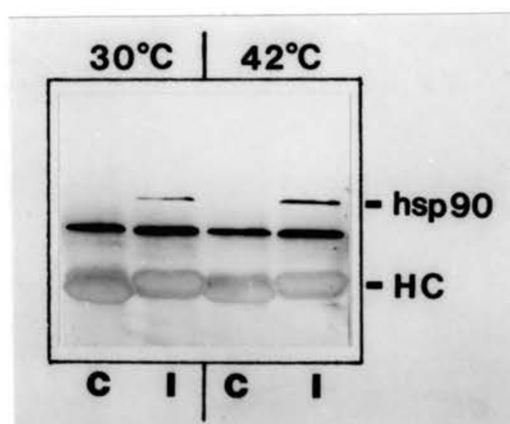

30°C
42°C
hsp90
HC
C
I
C
I

Figure 23. Interaction of HRI with the EC1 Antigen in Heat Shocked Hemin-Supplemented Lysates.

Hemin-supplemented protein synthesis mixes were incubated at 30 °C or 42 °C for 20 min. Proteins from 2.5 μl of the incubated protein synthesis mixes were adsorbed with the non-immune control (*C*) or the EC1 (*I*) antibody bound to GAG-agarose in the presence of TB as described under "Experimental Procedures". The nonadsorbed (*Sup*) and adsorbed (*Pel*) fractions were Western blotted for the presence of HRI. *HC*, heavy chain of IgG.

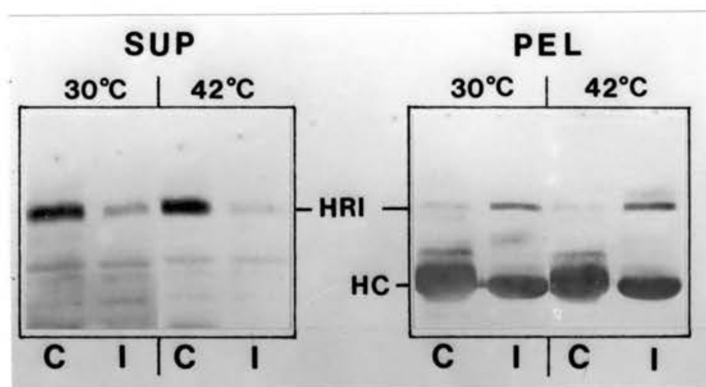

SUP
PEL
30°C
42°C
30°C
42°C
HRI
HC
C
I
C
I
C
I
C
I

Figure 24. Heat Shock Blocks the Interaction of HRI with Hsp70.

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 10 µl 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 as described under "Experimental Procedures". The adsorbed fractions were Western blotted with the 4322 anti-hsp90/hsp70 antibody to detect the presence of hsp70. *HC*, heavy chain of IgG.

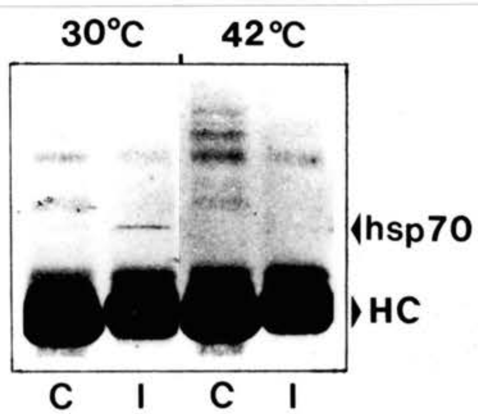

30°C
42°C
hsp70
HC
C
I
C
I

Figure 25. Reassociation of HRI with Hsp70 After Heat Shock Is Removed.

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 10 μl of protein synthesis mixes) on ice for 15 min. Proteins from 10 μl of protein synthesis mixes were adsorbed with the non-immune control (*C*) or the anti-HRI (*I*) antibody bound to GAG-agarose in the presence of TBS under "Experimental Procedures". After washing five times with TBS, the pellets were then reincubated with the HRI-free hemin-supplemented protein synthesis supernatants (which had been preincubated at 30 °C or 42 °C for 20 min) at 30 °C for 5 min. After incubation, the pellets were washed with TBS containing 0.1% SDS, 1% DOC, and 1% Triton 100 to remove the non-specific binding. The adsorbed fractions were separated by SDS-PAGE followed Western blotting with the 4322 anti-hsp90/hsp70 antibody to detect the presence of hsp70. *Lane 1*, 30 °C pellet incubated with 30 °C supernatant; *lane 2*, 30 °C pellet incubated with 42 °C supernatants; *lane 3*, 42 °C pellet incubated with 30 °C supernatant; and *lane 4*, 42 °C pellet incubated with 42 °C supernatant. *HC*, heavy chain of IgG.

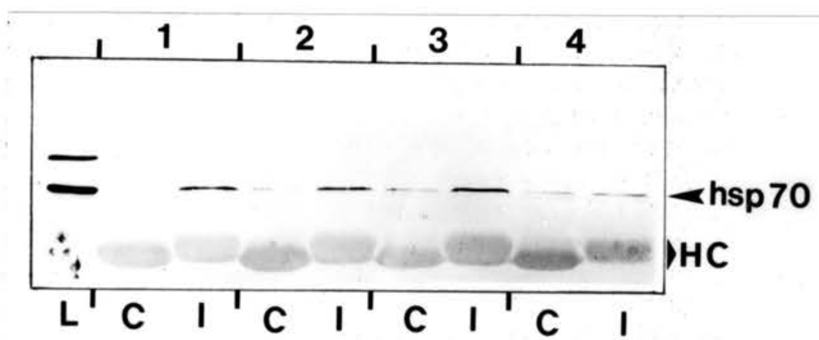

1
2
3
4
hsp70
HC
L
C
I
C
I
C
I
C
I

Figure 26. The Ability of Anti-HRI mAb F to Prevent the Activation of HRI.

Hemin-supplemented (+H, 42 °C, and Hg^{++}) and heme-deficient (-H) protein synthesis mixes were pre-incubated on ice for 15 min in the presence of no addition (-) or in the presence of ~ 1 µg IgG per 10 µl of protein synthesis mix of control monoclonal IgG (MOPC 21 mouse IgG ascites, Sigma) (*C*) or anti-HRI mAb F (*I*). Samples were subsequently incubated at 30 °C (+H, -H, and Hg^{++}) or 42 °C for 20 min, and then pulsed with [γ-^{32}P]ATP for 4 min. Samples (2.5 µl) were analyzed by 8% SDS-PAGE followed by autoradiography.

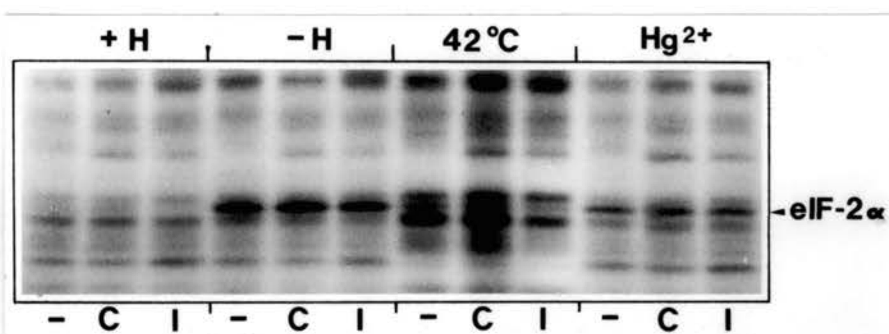

+ H
− H
42 °C
Hg 2+
eIF-2α
− C I − C I − C I − C I

Figure 27. The Anti-HRI mAb Cannot Prevent the Activation of HRI Induced by NEM.

Hemin-supplemented (*lanes 1 & 3-5*) and heme-deficient (*lane 2*) protein synthesis mixes were pre-incubated on ice for 15 min in the presence of no additions (*lanes 1-3*) or in the presence of ~ 1 µg IgG per 10 µl of protein synthesis mix of control monoclonal IgG (MOPC 21 mouse IgG ascites, Sigma) (*lane 4*) or anti-HRI mAb F (*lane 5*). Samples were subsequntly incubated at 30 °C in the presence of no additions (*lanes 1 & 2*) or in the presence of 10 mM of NEM (*lanes 3-5*) for 15 min, and then pulsed with [γ-^{32}P]ATP for 4 min. Samples (2.5 µl) were analyzed by 8% SDS-PAGE followed by autoradiography.

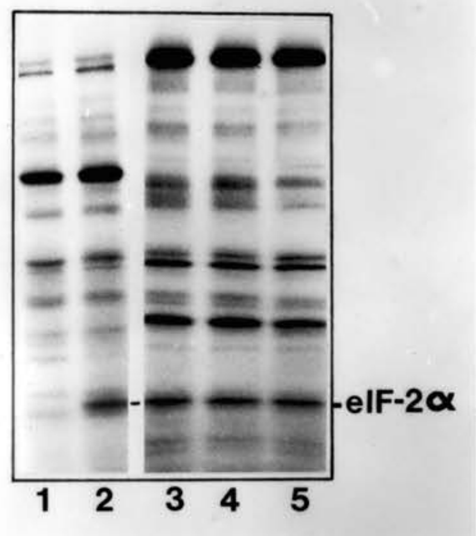

eIF-2α
1
2
3
4
5

Figure 28. Interaction of HRI with Hsp90 in Hemin-Supplemented Lysates Treated with NEM or Hg^{++}.

Proteins from 2.5 μl of hemin-supplemented protein synthesis mixes (*a*) with no addition, (*b*) with 25 μM Hg^{++}, (*c*) with 5 mM NEM, or (*d*) at 37 °C for six hours, were adsorbed with non-immune control (*C*) or the 8D3 (*I*) antibody bound to GAM-agarose. The non-adsorbed (*Sup*) and adsorbed (*Pel*) fractions were immunoblotted with the anti-HRI mAb to detect the presence of HRI. *HC*, heavy chain of IgG.

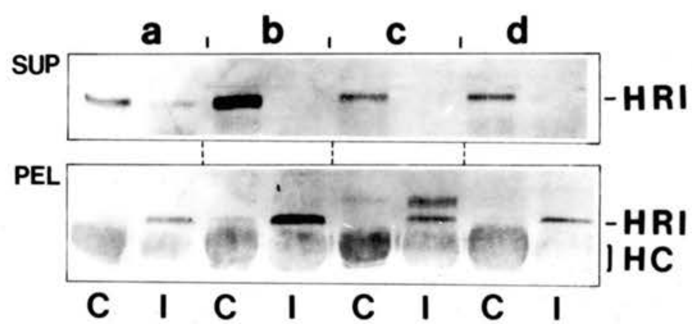

a
b
c
d
SUP
PEL
-HRI
-HRI
HC
C
I
C
I
C
I
C
I

Figure 29. Interaction of HRI with the EC1 Antigen in Hemin-Supplemented Lysates Treated with NEM or Hg^{++}.

Proteins from 2.5 µl of hemin-supplemented protein synthesis mixes (*a*) with no addition, (*b*) with 5 mM NEM, (*c*) with 25 µM Hg^{++}, or (*d*) at 42 °C for 30 min, were adsorbed with non-immune control (*C*) or the EC1 (*I*) antibody bound to GAG-agarose. The non-adsorbed (*Sup*) and adsorbed (*Pel*) fractions were immunoblotted with the anti-HRI mAb to detect the presence of HRI.

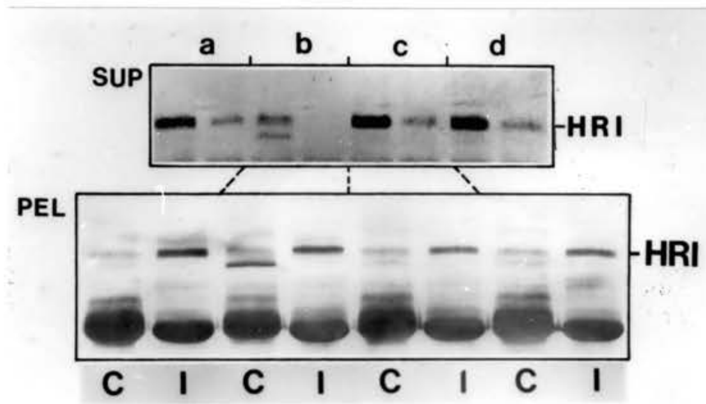
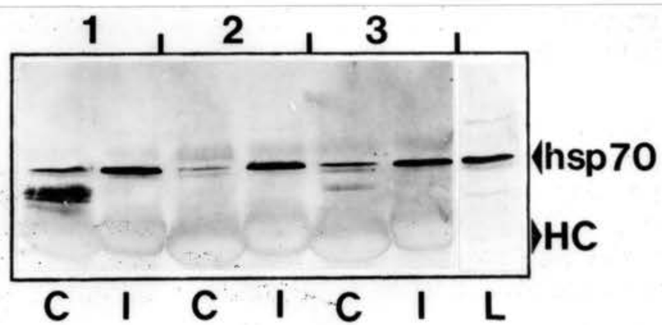

a
b
c
d
SUP
HRI
PEL
HRI
C
I
C
I
C
I
C
I

Figure 30. Interaction of HRI with Hsp70 in Hemin-Supplemented Lysates Treated with NEM or Hg^{++}.

Proteins from 10 μl of hemin-supplemented protein synthesis mixes with no addition (*lane 1*) or with 5 mM NEM (*lane 2*) or with 25 mM Hg^{++} (*lane 3*), were adsorbed with non-immune control (*C*) or the anti-HRI (*I*) antibody bound to GAG-agarose. The adsorbed fractions were immunoblotted with the N27 anti-hsp70 mAb to detect the presence of hsp70. *L*, 1.25 μl of unincubated whole reticulocyte lysate; *HC*, heavy chain of IgG.



1
2
3
hsp70
HC
C I C I C I L

Table II Three Pathways for Activation of HRI in Rabbit Reticulocyte Lysates

Pathway	Activating Conditions	Sensitivity to the anti-HRI mAb	Composition of HRI-hsps Complex		
			hsp90	hsp70	hsp56
I	Heme-Deficiency	Resistance	no	yes	no
II	Heat Stress	Sensitive	yes	no	yes
III	Oxidative Stress	Resistance	yes	yes	yes

CHAPTER IV

RECONSTITUTION OF HRI-HSPS COMPLEX

Introduction

The heme-regulated inhibitor (HRI) in rabbit reticulocytes and their lysates is a cAMP-independent protein kinase participating in the control of protein synthesis at the initiation level (Howard et al., 1970; Maxwell et al., 1971; Hunt et al., 1972; Hunt, 1979; Ochoa, 1983; London et al., 1987; Jackson, 1991). Activated HRI specifically phosphorylates the 38 kDa α-subunit of the eukaryotic initiation factor 2 (eIF-2α) (Levin et al., 1976; Ranu and London, 1976; Kramer et al., 1976; Farrell et al., 1977). The phosphorylated eIF-2(αP) binds and sequesters the reversing factor RF, also termed eIF-2B or GEF, guanine nucleotide exchange factor, which is required for eIF-2 recycling (Matts et al., 1983; Pain and Clemens, 1983; Panniers and Henshaw, 1983; Siekierka et al., 1984). The unavailability of RF results in the cessation of the initiation of protein synthesis in reticulocyte lysates (Matts et al., 1983; Matts and London, 1984; Thomas et al., 1984; Matts et al., 1986). Although the mechanism for the control of translation by HRI has been extensively studied, little is known about the regulation of HRI itself.

Heat shock proteins (hsp) are a small number of highly conserved proteins whose synthesis is dramatically induced at high temperatures (Lindquist and Craig, 1988; Schlesinger, 1990). Some major heat shock proteins, such as hsp90, hsp70 and hsp60, are members of multi-gene families which include proteins expressed in cells at normal temperature (Lindquist, 1986; Lanks, 1986, Bond and Schlesinger, 1988; Schlesinger, 1990; Morimoto et al., 1992). Hsp90 is an abundantly expressed, vital protein that is found complexed with various biologically important proteins, such as the steroid hormone

receptors and pp60^{v-src} (Courtneidge and Bishop, 1982; Brugge et al., 1983; Pratt, 1990; Smith et al., 1990; Abravay et al., 1992; Edwards et al., 1992). The EC1 antigen, which has been identified as a 56 kDa heat shock protein (Sanchez, 1990), is often associated with hsp90-substrate complexes (Smith et al., 1990) and binds the immunosuppressant FK506 (Lebeau et al., 1992; Yem et al., 1992; Tai et al., 1992). Hsp70 participates in protein folding, the translocation of proteins across membranes, the assembly of monomeric proteins to larger macromolecular complexes, and the disassembly of protein aggregates (reviewed by Pelham, 1990; Gething and Sambrook, 1992). Hsp70 also interacts with a number of cellular proteins, such as steroid hormone receptors, heat shock factors, and the mutant form of p53 (Kost et al., 1989; Sanchez et al., 1990b; Smith et al., 1990a; Edwards et al., 1992; Smith et al., 1992; Scherrer et al., 1992; Abravaya et al., 1992; Hainaut and Milner, 1992). Recently, hsc70 (the constitutively expressed form of hsp70) has been reported to interact with the immunosuppressant deoxyspergualin (Nadler et al., 1992). However, the functional significance of these associations between heat shock proteins and cellular proteins remains to be established.

Previous work has demonstrated that HRI associates with hsp90, hsp70, and hsp56 (the EC1 antigen) in rabbit reticulocyte lysates (Matts and Hurst, 1989; Matts et al., 1992). The interactions of HRI with hsp90 and the EC1 antigen are stabilized by hemin in lysates (Matts et al., 1992). Hemin has been reported to bind to HRI, suppressing its activation (London et al., 1985). Activation of HRI in heme-deficiency, which is a multistage process (Hunt, 1979; Jackson, 1991), has been observed to occur in conjunction with the dissociation of hsp90 and the EC1 antigen, while hsp70 remains bound to HRI (Matts et al., 1992). In contrast, the activation of HRI in hemin-supplemented lysates in response to heat shock and other forms of stress is not accompanied by the dissociation of hsp90 and the EC1 antigen. However, the dissociation of hsp70 from HRI has been observed during the activation of HRI in response to heat shock in hemin-supplemented lysates (Chapter III). Furthermore, the ability of hemin to

restore protein synthesis in heme-deficient lysates correlated with the levels of hsp90 present, while the ability of lysate to resist stress-induced inhibition of protein synthesis correlated with the levels of hsp70 present (Matts and Hurst, 1992). These observations support the notion that hsp90, hsp70, and the EC1 antigen play a fundamental role in regulating the activation and the activity of HRI, although the molecular mechanism for this regulation is unclear.

In order to determine the significance of the associations of hsps with HRI and to better understand the mechanism for the assembly of the HRI-hsps multiprotein complex *in vivo*, we have examined the requirements for reconstituting the HRI-hsps complex in the rabbit reticulocyte lysate system. This system has been successfully applied to the reconstitution of complexes of hsps with glucocorticoid receptor, progesterone receptor, and $pp60^{src}$, respectively (Scherrer et al., 1990, 1992; Smith et al., 1992; Hutchison et al., 1992). The data presented here indicate that: (i) the binding of hsp90 to HRI requires the presence of hemin and elevated incubation temperature (30 °C); (ii) the EC1 antigen is not required for the binding of hsp90 to HRI; (iii) the EC1 antigen can bind to HRI in the absence of hsp90 and the binding is also hemin dependent; (iv) ATP and Mg^{++} facilitate the binding hsp70 to HRI; (v) the binding of hsp90 to HRI-hsp70 complexes does not require ATP-hydrolysis, and Mg^{++} facilitates this reaction; and (vi) if hsp70 is not associated with HRI, then the binding of hsp90 to HRI requires both ATP and Mg^{++}.

Experimental Procedures

Materials

Goat anti-mouse IgM and IgG were obtained from Jackson Immunoresearch Laboratories. Apyrase (grade VIII), N-ethylmaleimide (NEM), nitro blue tetrazolium, 5-bromo-4-chloro-3-indolyl phosphate, p-nitrophenyl-agarose, mouse IgM (TEPC 183), and mouse IgG (MOPC 21) were obtained from Sigma. Alkaline phosphatase conjugated to

rabbit anti-mouse IgG or to goat anti-rabbit IgG was obtained from ICN. The 8D3 anti-hsp90 IgM monoclonal antibody (mAb) (Perdew, 1988; Perdew and Whitelaw, 1991) was provided by Dr. Gary Perdew (Purdue University); the KN382/EC1 IgG mAb (EC1) (Tai et al., 1986) was provided by Dr. Lee Faber (Medical College of Ohio, OH); the N27F3-4 (N27) anti-hsp70 IgG mAb (Kost et al., 1989; Welch and Suhan, 1986) was purchased from StresGen; the anti-HRI IgG mAb F (Pal et al., 1991) was provided by Dr. J.J. Chen (Massachusetts Institute of Technology). The 4322 rabbit anti-hsp90/hsp70 antiserum (Erhart et al., 1988) was provided by Dr. Stephen Ullrich (National Cancer Institute).

Buffers

TB, 10 mM Tris-HCl (pH7.5); TB/50, 10 mM Tris-HCl (pH 7.5) containing 50 mM NaCl; TBS, 10 mM Tris-HCl (pH 7.5) containing 150 mM NaCl; TB/500, 10 mM Tris-HCl (pH 7.5) containing 500 mM NaCl; TBS/5% NFDM, TBS containing 5% (w/v) non-fat dry milk; 4X Sample Buffer, 250 mM Tris-HCl (pH 6.8) containing 16% (v/v) glycerol, 5% (w/v) SDS, 0.01% (w/v) bromophenyl blue (BPB), and 0.055% (w/v) dithiothreitol (DTT) (fresh DTT is added just before use).

Rabbit Reticulocyte lysates and Protein Synthesis Mixtures.

Reticulocyte lysates were prepared from anemic rabbits as described previously (Hunt et al., 1972), using buffered saline containing 5 mM glucose to wash the reticulocytes prior to their lysis (Matts et al., 1991). Protein synthesis mixtures with or without 20 μM hemin were constructed under the standard conditions, including an ATP regenerating system, as described previously (Hunt et al., 1972; Ernst et al., 1978).

Immunoprecipitation

p-Nitrophenyl-agarose was rinsed free of isopropanol, and was coupled to goat anti-mouse IgG (GAG), or goat anti-mouse IgM (GAM) as described previously (Matts et

al., 1992). The preparations contained approximately 1.6 mg of antibody coupled per ml of packed agarose. GAG-agarose and GAM-agarose were used as a 1:1 slurry in TBS. The following general procedure describes the quantities of antibodies that were determined to quantitatively adsorb hsp90, the EC1 antigen, or HRI from 10 μl of reticulocyte lysate protein synthesis mix: for the adsorption of hsp90, 8D3 anti-hsp90 mAb from 62.5 μl of ascites fluid was bound to 87.5 μl of GAM-agarose; for the adsorption of the EC1 antigen, 62.5 μl of EC1 mAb (2.8 mg/ml) was bound to 100 μl of GAG-agarose; for the adsorption of HRI, anti-HRI mAb F from 15 μl of anti-HRI mAb F ascites fluid was bound to 25 μl of GAG-agarose. Non-immune control incubations contained equivalent volumes of TEPC 183 mouse IgM or MOPC 21 mouse IgG ascites with a concentration of approximately 5 mg of antibody per ml.

Antibodies were bound to the GAG- or GAM-agarose for two hours on ice. The antibody-bound GAG- or GAM-agarose was then washed sequentially with 500 μl each of: TBS; TB/500; TBS; and adsorption buffer (either TBS or TB as indicated in the legends of the figures). The GAG- or GAM-agarose was pelleted by a 10 sec centrifugation (full speed burst) in a Brinkman model 5415 Microfuge. The heme-deficient protein synthesis mixes were incubated at 30 °C for 10 min. These mixes were then treated with or without apyrase (one unit of apyrase per 10 μl of protein synthesis mix) on ice for 15 min. After the removal of excess adsorption buffer from the GAG- or GAM-agarose pellets containing the bound antibodies, 10 μl of protein synthesis mixes were added directly for the adsorption of hsp90, the EC1 antigen, or HRI. Samples were incubated at 4 °C for two hours with continuous mixing. The samples were then diluted 10-fold with 90 μl of adsorption buffer, and the non-adsorbed proteins (supernatants) were separated from the proteins adsorbed to the agarose (pellets) by centrifugation.

The pellets were then washed five times with 500 μl of the buffers described below to remove nonadsorbed proteins. For the adsorption of hsp90 or the EC1 antigen, and their associated proteins with the 8D3 anti-hsp90 or the EC1 mAbs, the pellets were

washed sequentially: once with TB, twice with TB/50, and twice with TB. For the adsorption of HRI and its associated proteins with the anti-HRI mAb F, the pellets were washed five times with TBS. For the analysis of hsp70 associated with HRI, the pellets were washed sequentially: once with TBS, three times with TBS containing 1% sodium deoxycholate, 1% Triton X-100, and 0.1% SDS, and once with TBS.

Reconstitution Reactions

Hsp90-free lysates were prepared by preincubating heme-deficient lysates with the 8D3 anti-hsp90 mAb adsorbed to the GAM-agarose pellets at 4 °C for 90 min followed by brief centrifugation to pellet the resin as described above. The supernatants were the hsp90-free lysates. Similarly, the EC1-antigen-free lysates and the HRI-free lysates were prepared by using the EC1 mAb and the anti-HRI mAb F preadsorbed to GAG-agarose, respectively. These hsp90-free, EC1 antigen-free, or HRI-free lysates were referred to reconstitution supernatants. For reconstitution, the immune pellets, isolated as described above, were incubated with specific reconstitution supernatants (detailed in the legends of the figures) at 30 °C for 10 min. The immune pellets were then washed, as described above, to remove nonspecifically bound proteins.

Gel Electrophoresis and Western Blot Analysis

The immune pellets were extracted with 80 µl of SDS sample buffer and the proteins in the samples were resolved on 10% or 8% SDS-polyacrylamide gels (9x14x0.15 cm; 37.5:1 acrylamide:Bis) using the procedure of Laemmli (Laemmli, 1970). Proteins were transferred to a PVDF membrane (Biorad) at a current density of 2.5 mA/cm^2 for 40 min for Western blot analysis as described previously (Matts and Hurst, 1989; Matts et al., 1992). Blots were blocked with TBS/5% NFDM for one hour at room temperature. The primary antibody was then reacted with the blots overnight at 4 °C. Blots were washed twice with TBS containing 0.5% Tween-20, once with TBS, and then blocked with

TBS/5% NFDM prior to reaction with alkaline phosphatase-conjugated rabbit anti-mouse IgG or goat anti-rabbit IgG (diluted 1:1000 in TBS/1% NFDM) for two hours at room temperature. Blots were washed twice with TBS containing 0.5% Tween-20, and twice with TBS. Proteins were then detected by incubating blots in alkaline phosphatase reaction buffer (100 mM Tris-HCl, pH9.5, containing 100 mM NaCl, 100 mM $Mg(OAc)_2$, 300 μg/ml NTB and 150 μg/ml BCIP) at 35 °C for 1 to 5 min.

For detecting HRI, the ascites fluid containing the anti-HRI mAb F was diluted 1:250 in TBS/1% NFDM. For detecting hsp90, the 4322 anti-hsp90/hsp70 antiserum was diluted 1:50 in TBS/1% NFDM. For detecting hsp70, the N27 anti-hsc73/hsp 72 mAb was diluted 1:1000 in TBS/1% NFDM. For detecting the EC1 antigen, the EC1 mAb was diluted 1:1000 in TBS/1% NFDM.

Results and Discussion

Hemin and Elevated Temperature Facilitate the Reconstitution of HRI with Hsp90.

Hsp90 in rabbit reticulocyte lysate was immunoprecipitated from the heme-deficient lysates by the 8D3 anti-hsp90 mAb. Hsp90-free lysate was then added to these HRI-free 8D3 anti-hsp90 pellets. No HRI was coadsorbed with the 8D3 anti-hsp90 mAb under this condition (Fig. 31, none). In the presence of 20 μM hemin, HRI from the hsp90-free lysate bound back to the hsp90 pellet (Fig. 31, +H, Pel.). No HRI was observed remaining in the supernatant after the reconstitution reaction (Fig. 31, +H, Sup.). However, much less HRI was observed to bind back to hsp90 in the heme-deficient hsp90-free lysate (Fig. 31, -H), suggesting that the reassembly of HRI with hsp90 was facilitated by hemin.

Compared to the non reconstituted condition (Fig. 31, none), in which no HRI was coadsorbed with hsp90 from the heme-deficient lysates by the 8D3 anti-hsp90 mAb, a significant amount of HRI reassociated with hsp90 under the reconstitution conditions in

the absence of hemin (Fig. 31, -H). This observation suggests that the affinity of the immunoprecipitated hsp90 for HRI was altered. This alteration may have been caused by the loss of some hsp90-associated proteins or factors during the washing of the 8D3 immune pellets. The lost proteins may play a role in mediating the binding of hsp90 to HRI or to other proteins.

Elevated temperature (30 °C) was required for the reconstitution of functional glucocorticoid receptor-hsp90 and progesterone receptor-hsps complexes in rabbit reticulocyte lysates (Scherrer et al., 1990; Smith et al., 1990, 1992). HRI-free 8D3 pellets were incubated with the hsp90-free lysates in the presence of hemin at 0 °C or 30 °C. HRI was observed to rebind quantitatively to hsp90 after incubation at 30 °C for 5 min (Fig. 32). However, if the reconstitution reaction was carried out at 4 °C, only about 50% of the HRI was observed to rebind to hsp90 after a two hour incubation (Fig. 32). This observation suggests that the assembly of hsp90-HRI complex was a temperature and time dependent process.

The EC1 Antigen Is Not Required for the Assembly of the HRI-Hsp90 Complex.

We have previously reported that the interactions of HRI with hsp90 and the EC1 antigen are dependent upon the presence of hemin in reticulocyte lysates (Matts et al., 1992). The association of hsp90 with the EC1 antigen has been observed in cytosol prepared from human cells, rabbit uteri, calf uteri, chicken oviducts and rabbit reticulocytes (Renoir et al., 1990; Matts et al., 1992). Both free hsp90 and free EC1 antigen exist in reticulocyte lysates (Matts et al., 1992). To determine whether the interaction of hsp90 with HRI is dependent upon the presence of the EC1 antigen in reticulocyte lysates, we examined whether the hemin-dependent association of hsp90 with HRI could be reconstituted in EC1 antigen-free reconstitution supernatants. Western blot analysis of 8D3 anti-hsp90 pellets indicated that HRI quantitatively reassociated with hsp90 in the presence

of hemin, even though no EC1 antigen was presented (Fig. 33, +H). No HRI was observed to associate with hsp90 in the absence of hemin (Fig. 33, -H).

Although hsp90 is frequently observed to associate with the EC1 antigen, the data suggest that the EC1 antigen is not required for the hemin-dependent interaction of hsp90 with HRI in reticulocyte lysates. The EC1 antigen has been demonstrated to bind the immunosuppressant FK506 (Yem et al., 1992; Tai et al., 1992), suggesting that the EC1 antigen may have the peptidylproline *cis-trans* isomerase (PPI) activity. Therefore, the EC1 antigen may play a role in regulating the activity of proteins with which it is associated through its PPI activity. While hsp90 was observed to bind to HRI in absence of the EC1 antigen in lysates, the EC1 antigen could still play a role in regulating the effect that the binding of hsp90 has on HRI activity.

Hsp90 Is Not Required for the Binding of the EC1 Antigen to HRI.

To test whether the EC1 antigen interacts with HRI via its interaction with hsp90, we examined whether the HRI-EC1 antigen complex reassembled in reticulocyte lysates in the absence of hsp90. In the presence of hsp90, the EC1 antigen was observed to reassociate with HRI in hemin-supplemented lysate, but not in heme-deficient lysate (Fig. 34C, with hsp90). This was consistent with previous observations (Matts et al., 1992). Surprisingly, we found that the EC1 antigen could interact with HRI in the absence of hsp90 in lysates, and that this interaction was also hemin-dependent (Fig. 34C, without hsp90). This observation is the first evidence which shows that the EC1 antigen can associate with its binding substrate, bypassing its interaction with hsp90. Compared to the lysate containing hsp90, only about 50% of the HRI was observed to reassociate with the EC1 antigen in the hsp90-free lysate in the presence of hemin. This suggests that hsp90 may facilitate the interaction between HRI and the EC1 antigen. However, another explanation for this finding may be that less EC1 antigen is available in such lysate, since

hsp90 associates with the EC1 antigen (Matts et al., 1992). The depletion of hsp90 might result in decreasing the amount of the EC1 antigen present in the lysates.

The data show that the interaction of the EC1 antigen with HRI does not require the presence of hsp90. It creates an opportunity for us to study the biochemical function of the EC1 antigen in regulating the activities of its substrates. Its potential peptidylproline *cis-trans* isomerase activity suggests that the EC1 antigen may play a role in altering the conformation of its substrates, which may result in changing the activities of the proteins with which it interacts.

The Requirement of ATP and Mg^{2+} for the Binding of Hsp90 to HRI.

HRI associates with hsp90 and hsp70 in hemin-supplemented lysates (Matts and Hurst, 1989; Matts et al., 1992). Previous experiments indicated that the non-specific binding of hsp70 could not be eliminated unless the washing buffer contained detergents (0.1% SDS, 1% DOC, and 1% Triton 100) (data not shown). We subsequently found that incubation of anti-HRI immune pellets for 5 min at 30 °C in the presence of high salt and ATP completely stripped hsp90 and hsp70 from HRI (Fig. 35, lane 3).

To determine the requirement for ATP and Mg^{++} for the binding of hsp90 to HRI, the salt-stripped HRI-resin was incubated with HRI-free hemin-supplemented lysates at different conditions. In the presence of both ATP and Mg^{++}, hsp90 was observed to bind back to HRI (Fig. 36, lane 5). Hsp90 did not bind to HRI in the absence of Mg^{++}, although ATP was present in the reconstitution reaction mixture (Fig. 36, lane 6). No hsp90 was observed to reassociate with HRI in the apyrase treated reconstitution reaction mixtures (Fig. 36, lanes 7 and 8). Although non-specific binding of hsp70 was observed in this experiment (Fig. 36, lane 4), the binding of hsp70 to HRI was reduced under conditions that either lacked Mg^{++} (lane 6) or ATP (lane7) or both (lane 8), compared to conditions where both ATP and Mg^{++} were presented.

The data described above indicated that both ATP and Mg^{++} are required for the binding of hsp90 to HRI, if hsp70 has also been stripped from HRI. Subsequently, the ATP requirement for the binding of hsp90 to HRI was examined under conditions where hsp70 remains bound to HRI. The 8D3 anti-hsp90 mAb was used to immunoadsorb hsp90 and its associated proteins from heme-deficient lysates. The 8D3 anti-hsp90 immunoprecipitate was incubated with hemin-supplemented hsp90-free lysates Western blot analysis indicated that no HRI was reassociated with hsp90 in the absence of heme (Fig. 37, lane 1). In contrast, HRI quantitatively rebound to hsp90 in the presence of hemin (Fig. 37, lane 2). When the reconstitution reaction mixture was treated with apyrase, the association of HRI with hsp90 was still observed (Fig. 37, lane 3). However, the binding of HRI to hsp90 was significantly decreased upon the addition of EDTA (5 mM) to the reconstitution reaction mixture (Fig. 37, lane 4). This effect could be reversed by the addition of excess Mg^{++} (10 mM) (Fig. 37, lane 5). We have not examined the efficacy of Mn^{++} or other divalent actions in reversing the EDTA blockage of protein binding.

From these data, it appears that the requirement of ATP and Mg^{++} for the binding of hsp90 to HRI is affected by whether hsp70 is bound to HRI. If hsp70 is not associated with HRI, the binding of hsp90 to HRI requires both ATP and Mg^{++}. If hsp70 is bound to HRI, the binding of hsp90 to HRI requires only Mg^{++}, but not ATP. The data suggest that Mg·ATP is required for the binding of hsp70 to HRI, while Mg^{++} is needed for the binding of hsp90 to the HRI-hsp70 complex. Once hsp70 is associated with HRI, the binding of hsp90 to HRI is not ATP dependent.

The interactions of HRI with hsp90, hsp70, and the EC1 antigen are summarized in a model described in Figure 38. This model is based on several observations: (i) the interaction of HRI with hsp90 and the EC1 antigen is hemin dependent (Matts et al., 1992); (ii) the interaction of HRI with hsp70 is not dependent on hemin (Matts et al., 1992); (iii) hsp90, hsp70, and the EC1 antigen form a hsps complex in the lysates (Matts et al., 1992);

(iv) the EC1 antigen is not required for binding of hsp90 to HRI; (v) although hsp90 interacts with the EC1 antigen (Matts et al., 1992), the binding of the EC1 antigen to HRI is not dependent upon the presence of hsp90; (vi) the binding of hsp70 to HRI requires ATP, and Mg^{++} facilitates this interaction; and (vii) the binding of hsp90 to HRI requires Mg^{++}, but not ATP.

Figure 31. Effect of Hemin on Reconstitution of HRI-Hsp90 Complex

The non-immune control (*C*) or the 8D3 (*I*) resin was incubated with the hsp90-free reconstitution reaction supernatants in the presence of hemin (*+H*) or in the absence of hemin (*-H*) at 30 °C for 5 min. The reassociated (*Pel*) and non-reassociated (*Sup*) fractions were immunoblotted with the anti-HRI mAb F to detect the presence of HRI. *None*, shows both the control or immune pellet and the reconstitution reaction supernatants before reconstitution assay.

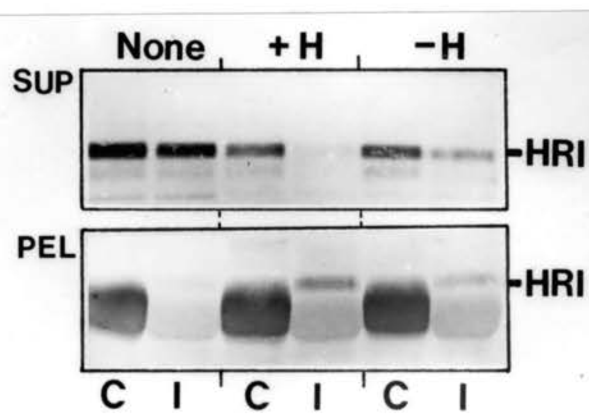

None
+H
−H
SUP
HRI
PEL
HRI
C
I
C
I
C
I

Figure 32. Effects of Time and Temperature on the Reconstitution of HRI-Hsp90 Complex.

Proteins from 2.5 μl of heme-deficient protein synthesis mixes were adsorbed with non-immune control (lanes 1) or the 8D3 anti-hsp90 (lanes 2 & 3) antibody bound to GAM-agarose as described under "Experimental Procedures". Reconstitution reactions were incubated at 0 °C (lanes 2) or at 30 °C (lanes 1 & 3) for the times indicated in the figure. Hemin was added back during the incubation. After incubation, the reassociated fractions were analyzed by SDS-PAGE and Western blotting with the anti-HRI mAb F to detect the presence of HRI. HC, heavy chain of IgM.

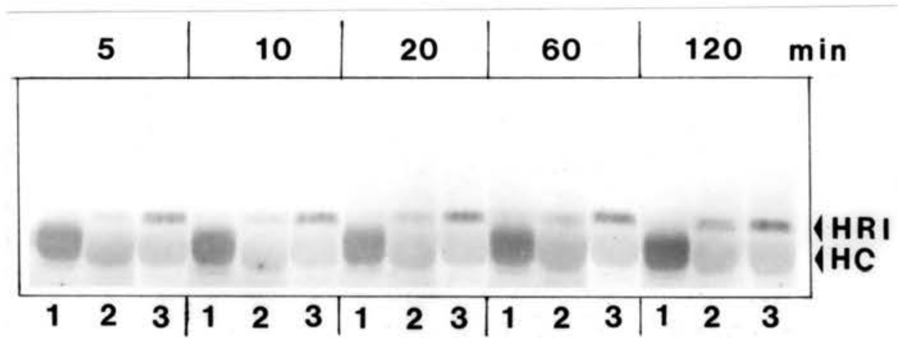

5
10
20
60
120
min
HRI
HC
1 2 3 1 2 3 1 2 3 1 2 3 1 2 3

Figure 33. Effect of the EC1 Antigen on the Reconstitution of HRI-Hsp90 Complex.

Proteins from 2.5 μl of heme-deficient protein synthesis mixes were first adsorbed with the EC1 mAb to generate the EC1 antigen-free lysate. This EC1 antigen-free lysate was then used for the second immunoprecipitation of hsp90 by the non-immune control (C) or the 8D3 anti-hsp90 (I) antibody bound to GAM-agarose in the presence of 20 μM of hemin (+H) or in the absence of hemin (-H) as described under "Experimental Procedures". The reassociated (Pel) and non-associated (Sup) fractions were separated by SDS-PAGE and Western blotted with the anti-HRI mAb F to detect the presence of HRI. HC, heavy chain of IgM.

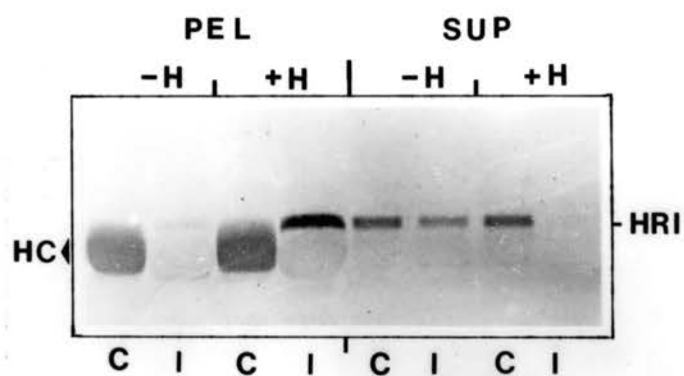

PEL
SUP
-H
+H
-H
+H
HC
HRI
C I C I C I C I

Figure 34. Effects of Hsp90 and Hemin on the Reconstitution of HRI-EC1 Antigen Complex.

(**A**) Hsp90 from 2.5 μl of heme-deficient protein synthesis mixes were removed with non-immune control (C) or the 8D3 anti-hsp90 (I) antibody bound to GAM-agarose. The adsorbed (Pel) and non-adsorbed (Sup) fractions were Western blotted with the 4322 anti-hsp90/hsp70 antibody to detect the presence of hsp90 and hsp70. (**B**) The supernatants of (**A**), containing hsp90 (lane 2) or containing no hsp90 (lane 3), were secondly immunoprecipitated with the non-immune control (lane 1) or with the EC1 (lanes 2 & 3) antibody bound to GAG-agarose. The non-adsorbed fractions were immunoblotted with the EC1 mAb to detect the presence of the EC1 antigen. Lane 4, 1.25 μl of unincubated whole lysate. (**C**) 15 μl of the diluted (1:10) supernatants of (**A**) (with hsp90 or without hsp90) were then used for the second immunoprecipitation of the EC1 antigen by the non-immune control (C) or the EC1 (I) antibody bound to GAG-agarose in the presence of 20 mM of hemin (+H) or in the absence of hemin (-H) at 30 °C for 10 min. The reassociated fractions were separated by SDS-PAGE and Western blotted with the anti-HRI mAb F to detect the presence of HRI. L, 1.25 μl of unincubated whole lysate. HC, heavy chain of IgG.

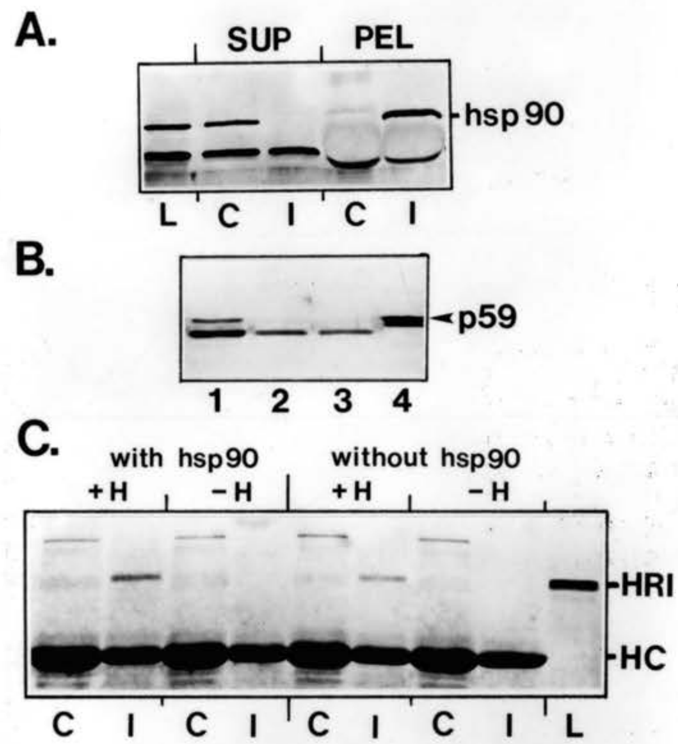

A.
SUP
PEL
hsp 90
L
C
I
C
I
B.
p59
1
2
3
4
C.
with hsp90
without hsp90
+H
−H
+H
−H
HRI
HC
C
I
C
I
C
I
C
I
L

Figure 35. Conditions for Stripping Heat Shock Proteins from HRI.

Proteins from 10 µl of hemin-supplemented protein synthesis mixes were adsorbed with non-immune control (C) or the anti-HRI (I) antibody bound to GAG-agarose. The resin was washed with TBS at 0 °C (lane 1) or with TB containing 0.5 M NaCl, 1 mM ATP, and 5 mM Mg^{++} either at 0 °C (lane 2) or at 30 °C (lane 3). After washing, samples were analyzed by SDS-PAGE and Western blotting with the 4322 anti-hsp90/hsp70 antibody to detect the presence of hsp90 and hsp70. HC, heavy chain of IgG.

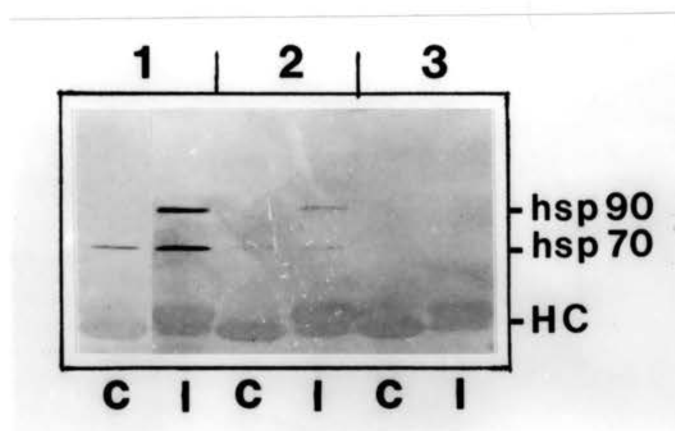

1
2
3
hsp 90
hsp 70
HC
C I C I C I

Figure 36. Requirement for ATP and Mg^{++} in Hsp90 Binding to HRI if Hsp70 Is not Pre-associated with HRI.

The salt stripped HRI resin was incubated with HRI-free reconstitution supernatants at 30 °C for 5 min followed by apyrase treatment. The samples were then washed with TBS and analyzed by SDS-PAGE and Western blotting with the anti-hsp90/hsp70 antibody to detect the presence of hsp90 and hsp70. Lane 1, HRI resin, without salt stripping; lane 2, control IgG resin, with salt stripping; lane 3, HRI resin, with salt stripping; lane 4, salt stripped control IgG resin incubated with reconstitution supernatant in the presence of ATP and Mg^{++}; salt stripped HRI resin incubated with the reconstitution supernatant in the presence of both ATP and Mg^{++} (lane 5) or in the presence of only ATP (lane 6) or in the presence of only Mg^{++} (lane 7) or in the absence both ATP and Mg^{++} (lane 8).

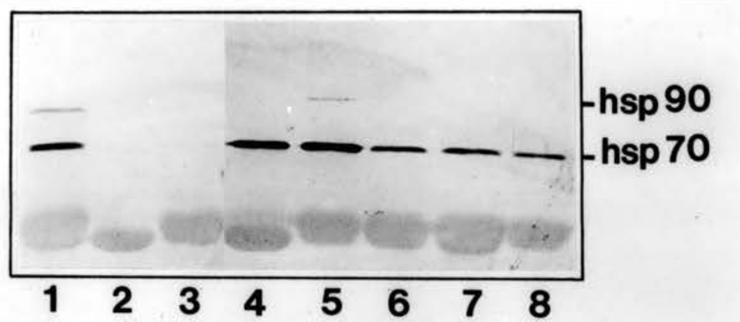

hsp 90
hsp 70
1
2
3
4
5
6
7
8

Figure 37. The Requirement of ATP and Mg^{++} for the Binding of Hsp90 to the HRI-Hsp70 Complex.

Proteins from 2.5 μl of heme-deficient protein synthesis mixes were adsorbed with non-immune control (C) or the 8D3 anti-hsp90 (I) antibody bound to GAM-agarose. After incubation of the reconstitution reaction mixes with hsp90-free supernatants for 5 min at 30 °C, the reassociated (Pel) and non-associated (Sup) fractions were analyzed by SDS-PAGE and Western blotting with the anti-HRI mAb F to detect the presence of HRI. The reconstitution conditions are: without hemin (lane 1), with hemin (lanes 2-5), with ATP and Mg^{++} (lanes 1-2), without ATP, but with Mg^{++} (lane 3), and with ATP and 5 mM EDTA (lane 4), and with ATP, 5 mM EDTA, and 10 mM Mg^{++}.

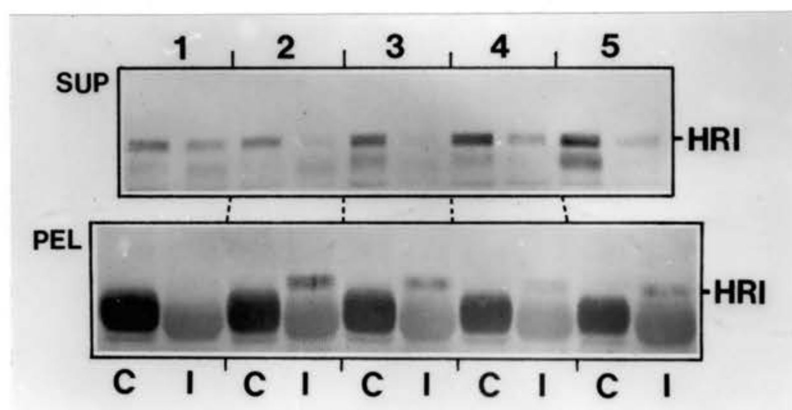
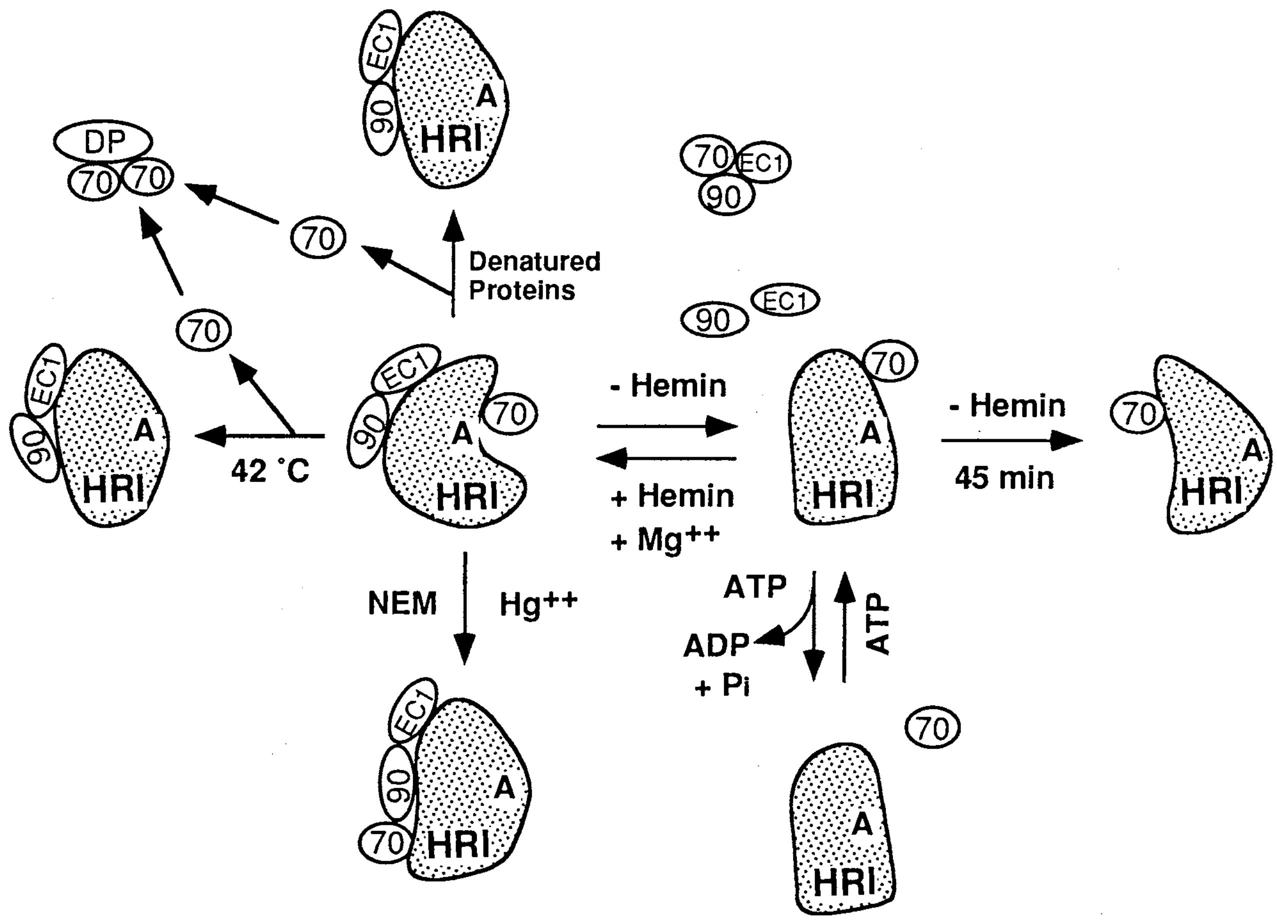

1
2
3
4
5
SUP
HRI
PEL
HRI
C I C I C I C I C I

Figure 38. The Model for Translational Regulation by Heat Shock Proteins.

HRI, hemin-regulated eIF-2a kinase; *A*, the active site within HRI; *90*, thsp90; *70*, hsp70; *EC1*, the EC1 antigen; *DP*, denatured proteins; *NEM*, N-ethylmaleimide.



EC1
90
A
HRI
DP
70 70
70
Denatured
Proteins
70 EC1
90
70
90 EC1
EC1
90
A
HRI
42 ˚C
EC1
90
70
A
HRI
- Hemin
+ Hemin
+ Mg++
70
A
HRI
- Hemin
45 min
70
A
HRI
NEM
Hg++
ATP
ADP
+ Pi
ATP
EC1
90
70
A
HRI
70
A
HRI

CHAPTER V

SUMMARY

Concluding Remarks

The translational regulation by heat shock proteins (hsps) at the initiation stage in rabbit reticulocyte lysates has been studied. Hsp90, hsp70 and hsp56 (the EC1 antigen) interact with the heme-regulated eIF-2α kinase (HRI) and form a HRI-hsps complex in rabbit reticulocyte lysates. The association of HRI with hsp90 and the EC1 antigen is dependent upon the presence of hemin at a concentration of at least 5 μM in reticulocyte lysates. Hsp70 associates with HRI in both hemin-supplemented and heme-deficient lysates. It is the constitutively expressed form of hsp70, hsc70, that associates with HRI in reticulocyte lysates.

Three different pathways for activation of HRI in reticulocyte lysates have been distinguished. Activation of HRI in heme-deficient lysates correlates with the dissociation of hsp90 and the EC1 antigen, while hsp70 remains bound to HRI. This type of activation is not prevented by the anti-HRI monoclonal antibody (mAb) F. In addition, an alteration of the levels of hsp70 bound to HRI is observed. The release of hsp70 from HRI requires the hydrolysis of ATP.

In contrast, activation of HRI in hemin-supplemented lysates in response to stress conditions does not require the dissociation of hsp90 and the EC1 antigen. Addition of denatured proteins into the hemin-supplemented lysates activates HRI. This kind of activation can be prevented by the anti-HRI mAb F. Denatured proteins bind to hsp70 and sequester hsp70 from HRI. Similarly, heat shock induces the activation of HRI in hemin-supplemented lysates and inhibits protein synthesis. The anti-HRI mAb F can also reverse

the activation of HRI caused by heat shock. Heat shock blocks the interaction of HRI with hsp70. Interestingly, the activation of HRI caused by addition of oxidants into the hemin-supplemented lysates cannot be prevented by the anti-HRI mAb F. Hsp90, hsp70, and the EC1 antigen remain bound to HRI in such a case.

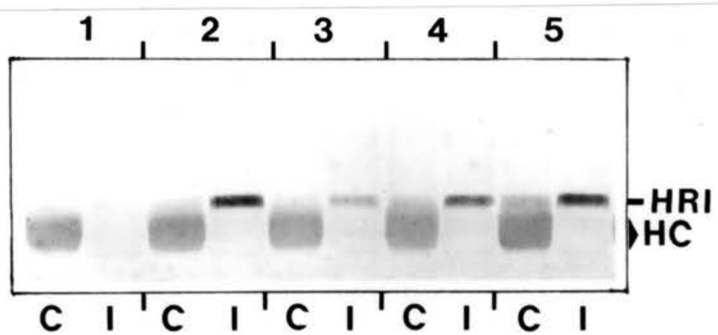
The HRI-hsps complex can be reconstituted *in vitro*. The reassembly of hsp90 with HRI is hemin-dependent. Elevated temperature facilitates the reconstitution of the HRI-hsps complex. The EC1 antigen is not required for the binding of hsp90 to HRI. In the absence of hsp90, the EC1 antigen can associate with HRI. If hsp70 is pre-associated with HRI, the reconstitution of hsp90 with HRI does not require ATP, but needs Mg^{++}. However, both ATP and Mg^{++} are required for the reassembly of hsp90 with HRI if hsp70 is not pre-associated with HRI. Based on the observations described above, a model is presented for translational regulation by heat shock proteins in rabbit reticulocyte lysates (Fig. 38).

The Carboxyl Terminal of Hsp90 Binds to HRI

To determine the binding site of hsp90 to HRI, we have employed the AC88 anti-hsp90 mAb (Riehl et al., 1985) and the D7α anti-hsp90 mAb (Brugge et al., 1983). The AC88 anti-hsp90 mAb recognizes the carboxyl terminal of hsp90, while the D7α anti-hsp90 mAb recognizes the amino terminal of hsp90 (Riehl et al., 1985; Brugge et al., 1983; Smith at al., 1992). When the heme-deficient lysates were preincubated with the AC88 mAb, the interaction of HRI with hsp90 was reduced to about 50% (Fig. 39, lane 3). In contrast, very little inhibition of the binding of hsp90 to HRI was observed when the lysates were preincubated with the D7α anti-hsp90 mAb (lane 4) or with the non-immune control antibody (lane 5). The decrease in the binding of hsp90 to HRI caused by the preincubation with the AC88 anti-hsp90 mAb suggests that the AC88 mAb competes with HRI for the binding to hsp90. We also observed that the AC88 mAb band was present in the 8D3 immune pellets after incubation of heme-deficient lysates with the AC88

Figure 39. The Abilities of the AC88 and the D7α Anti-hsp90 mAbs to Prevent the Binding of HRI to Hsp90.

Heme-deficient protein synthesis mixes were preincubated on ice for 15 min in the presence of no additions (lanes 1 & 2) or in the presence of ~1μg IgG/10 μl protein synthesis mix of the AC88 anti-hsp90 mAb (lane 3) or the D7α anti-hsp90 mAb (lane 4) or the non-immune control monoclonal IgG (MOPC 21 mouse IgG ascites, Sigma) (lane 5). Proteins from 2.5 μl of the preincubated heme-deficient protein synthesis mixes were adsorbed with the non-immue control (C) or the 8D3 anti-hsp90 (I) antibody bound to GAM-agarose in the absence of hemin (lane 1) or in the presence of 20 μM of hemin (lanes 2-5). The adsorbed fractions were analyzed by SDS-PAGE and Western blotting with the anti-HRI mAb F to detect the presence of HRI. HC, the heavy chain of IgM.


1
2
3
4
5
HRI
HC
C
I
C
I
C
I
C
I
C
I

mAb (data not shown). The data suggest that the carboxyl terminal of hsp90 is involved in the interaction with HRI. Preliminary studies using deletion mutants of HRI (provided by Dr. J.-J. Chen, M.I.T.) have been carried out to begin mapping regions of HRI that are responsible for its interaction with hsp90 and the EC1 antigen. The laboratory plans future studies in conjunction with Dr. Chen's laboratory to further elucidate the domains present in each protein that are involved in the interaction of these proteins and the regulation of HRI activity.

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