Biochemistry

 Volume 32, Number 29 July 27, 1993

Accelerated Publications

Denatured Proteins Inhibit Translation in Hemin-Supplemented Rabbit Reticulocyte Lysate by Inducing the Activation of the Heme-Regulated eIF-2α Kinase†

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Received April 1, 1993; Revised Manuscript Received May 20, 1993

ABSTRACT: The heme-regulated inhibitor (HRI) of protein synthesis becomes activated in rabbit reticulocyte lysates in response to a variety of conditions including heme-deficiency, addition of oxidants, and heat shock. Activated HRI inhibits translation by catalyzing the phosphorylation of the α-subunit of eukaryotic initiation factor eIF-2. The molecular nature of the "signal" that leads to the activation of HRI in response to heat shock has not been characterized. We have recently reported that HRI interacts with the 90- and 70-kDa heat shock proteins (hsp) and a 56-kDa protein in hemin-supplemented lysates [Matts, R. L., Xu, Z., Pal, J. K., & Chen, J.-J. (1992) *J. Biol. Chem. 267*, 18160-18167]. In this report, we demonstrate that addition of denatured proteins, bovine serum albumin (BSA), β-lactoglobulin, or α-lactalbumin, but not the addition of the native proteins, inhibits protein synthesis in hemin-supplemented reticulocyte lysates. The inhibition was reversed upon the addition of 10 mM cAMP or purified eIF-2B, classical criteria for HRI-mediated translational inhibition. Denatured BSA, but not native BSA, stimulated the phosphorylation of the α-subunit of eIF-2. This stimulation of eIF-2α phosphorylation was inhibited by a monoclonal antibody to HRI, confirming that denatured BSA was causing the activation of HRI. The concentration of denatured BSA required to inhibit protein synthesis by 50% correlated with the levels of hsp70 present in each lysate preparation. Lysate hsp70 co-immunoadsorbed with denatured BSA, but not with native BSA. Hsp70 was co-adsorbed with HRI from lysate in the presence of native BSA, but not in the presence of denatured BSA. These observations indicate that the dissociation of hsp70 from HRI is strongly correlated with the activation of HRI caused by denatured proteins and suggest that denatured protein, through its capacity to sequester hsp70, is the "signal" that leads to activation of HRI in response to heat shock.

The heat shock response is induced in cells in response to a variety of environmental and pathophysiological stresses (Craig, 1985; Lanks, 1986; Lindquist, 1986). The 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 synthesis of a family of heat shock proteins (hsp)[1] (Craig, 1985; Lindquist, 1986). The level of hsp70[2] 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 & Welch, 1988). Hsp gene transcription subsequently becomes refractory for a period to induction by an ensuing stress.

† This work was supported by National Institutes of Health Grant ES-04299 from the NIEHS and by the Oklahoma State University Agricultural Experiment Station of which this is Journal Article No. J-6270.

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0006-2960/93/0432-7323$04.00/0

The heat shock response has been proposed to be autoregulated at the transcriptional level through the ability of hsp70 to bind and maintain the heat shock transcription factor (HSF) in an inactive conformation (Craig, 1991; Sorger, 1991; Morimoto et al., 1992). Members of the hsp70 family bind to nascent polypeptides during their synthesis and to aberrantly folded or processed proteins (Beckman et al., 1990; Schlesinger, 1990; Ang et al., 1991; Ellis & van der Vies, 1991; Georgopoulos, 1992). Hsp70s may also prevent the improper aggregation of newly synthesized polypeptides, mediate the renaturation of proteins, and facilitate the dissociation of polypeptides from aggregates of denatured proteins. Since most agents or conditions that induce the heat shock response likely cause the denaturation or oxidation of proteins (Hightower, 1980; Ananthan et al., 1986; Craig, 1991; Hightower, 1991), it has been proposed that accumulation of denatured protein would sequester hsp70, leading to the activation of the HSF, and thus transcriptional activation of hsp genes (Craig, 1991; Sorger, 1991; Morimoto et al., 1992).

Like the heat-induced transcriptional effects, recovery of protein synthesis in heat stressed cells and the acquisition of resistance to stress-induced translational inhibition also correlate with the synthesis and accumulation of hsp70 within a variety of cell types (DiDomenico et al., 1982; Laszlo, 1988; Mizzen & Welch, 1988). The molecular events that lead to the arrest and recovery of normal protein synthesis in response to heat shock are not well defined. We have used rabbit reticulocyte lysate as a model system to study the effects of heat and oxidative stress on protein synthesis *in situ*. The heme-regulated eIF-2α kinase (HRI) becomes activated in hemin-supplemented rabbit reticulocyte lysates in response to a variety of agents (London et al., 1987; Jackson, 1991) that induce the heat shock response in living cells (Craig, 1985; Lanks, 1986; Lindquist, 1986). HRI phosphorylates the α-subunit of eukaryotic initiation factor eIF-2, which then blocks polypeptide chain initiation by sequestering the initiation factor (eIF-2B) responsible for eIF-2 recycling in an inactive complex (London et al., 1987; Jackson, 1991). The molecular nature of the "signal" that induces HRI activation in response to heat and oxidative stress is not known.

HRI interacts with hsp90, hsp70, and p56 in hemin-supplemented reticulocyte lysate (Matts & Hurst, 1989; Matts et al., 1992). The extent of HRI activation in response to heat or oxidative stress correlates inversely with the levels of hsp70 present in lysates (Matts & Hurst, 1992). The similarities between the transcriptional and translational changes induced by heat shock have lead us to propose that heat stress causes denatured protein to accumulate and sequester hsp70, resulting in the loss of HRI-bound hsp70 and the subsequent activation of HRI (Matts & Hurst, 1992). This model predicts that the addition of denatured proteins to hemin-supplemented reticulocyte lysates should activate HRI and inhibit protein synthesis. In this report we demonstrate that denatured, but not native, proteins activate HRI and inhibit protein synthesis in hemin-supplemented reticulocyte lysates, apparently by competing with HRI for the binding to the available hsp70.

EXPERIMENTAL PROCEDURES

Materials. Bovine serum albumin (fraction V), bovine β-lactoglobulin A, bovine α-lactalbumin, reduced carboxymethylated α-lactalbumin, and rabbit anti-BSA were purchased from Sigma. L-[^{14}C]Leucine (184 mCi/mmol) was obtained from ICN, and [γ-^{32}P]ATP (1000–3000 Ci/mmol) was obtained from DuPont–New England Nuclear. eIF-2 and eIF-2·eIF-2B were purified as reported (Hurst et al., 1987). N27F3-4 anti-hsp70 mAb (Kost et al., 1989) and bovine brain hsp70 were purchased from StressGen. The anti-HRI mAb F (Pal et al., 1991) was provided by Dr. Jane-Jane Chen (MIT).

Buffers. Buffers were as follows: 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; adsorption buffer, TBS containing 20 μM hemin-HCl.

Protein Synthesis and Phosphorylation in Reticulocyte Lysates. Reticulocyte lysates were prepared as described (Matts et al., 1991). Protein synthesis mixes containing 20 μM hemin-HCl (40 μL) were incubated at 30 °C, and the incorporation of [^{14}C]leucine into acid precipitable protein (5-μL aliquots) was measured as described (Hunt et al., 1972; Ernst et al., 1978). For immunoadsorptions and phosphoprotein profiles, the [^{14}C]leucine was omitted. Proteins were phosphorylated by pulsing protein synthesis mixes with [γ-^{32}P]-ATP (0.5 μCi/μL of protein synthesis mix), and samples were analyzed by SDS/PAGE on 8% gels (9 × 14 × 0.15 cm, 37.5:1 acrylamide:bis) as previously described (Matts et al., 1991). Gels were run until the dye front was ~1 cm from the bottom of the gel. We have found that this protocol gives the best resolution of the eIF-2α band from phosphorylated polypeptides that migrate just above and just below eIF-2α. Other gels percentages or cross-linking ratios result in the eIF-2α band being poorly resolved from or comigrating with other phosphopeptide bands.[3] This comigration often complicates the quantitation of changes in the phosphorylation state of eIF-2α. The extent of eIF-2α phosphorylation was quantitated using a PDI Discovery Series (model DNA 35) scanning densitometer, after correcting lanes on the autoradiograms for background density. Statistical analysis was carried out using Microsoft Excel version 4.

Preparation of Denatured Protein Substrates. BSA and LG were reduced and carboxymethylated or carboxyamidomethylated, respectively, as described (Ananthan et al., 1986). The denatured proteins were then dialyzed against six changes (2 L) of TBS. Solutions of the native proteins (~10 mg/mL) were prepared fresh in TBS.

Immunoadsorptions. Goat anti-rabbit IgG was cross-linked to agarose (GaR-agarose) as previously described (Matts et al., 1992). Antibody from 75 μL of rabbit anti-BSA antiserum or nonimmune control antiserum was bound to 75 μL of GaR-agarose (1:1 in TBS) for 2 h on ice. The GaR-agarose was then washed with 500 μL each of TBS, TB/500, TBS, and adsorption buffer. Hemin supplemented lysates (20 μL) were incubated at 30 °C for 30 min in the presence or absence of 25 μM BSA or RCM-BSA. After treatment with 2 units of apyrase on ice for 15 min to stabilize hsp70·protein complexes

[1] Abbreviations: BSA, bovine serum albumin; LG, bovine β-lactoglobulin A; LA, bovine α-lactalbumin; RCM-BSA, reduced carboxymethylated BSA; RCAM-LG, reduced carboxamidomethylated LG; RCM-LA, reduced carboxymethylated LA; eIF-2, eukaryotic initiation factor 2; eIF-2α, α-subunit of eIF-2; HRI, heme-regulated eIF-2α kinase; hsp, heat shock protein; hsc, heat shock cognate protein; p56, 56-kDa protein; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; mAb, monoclonal antibody; GaR, goat anti-rabbit IgG; ER, endoplasmic reticulum.

[2] The terms hsp90 and hsp70 are used to refer to members of these heat shock protein families in general, and the terms are not meant to refer to the actual molecular weights of the proteins. While we generally refer to hsp70 in this manuscript, the hsp70 that associates with HRI in reticulocyte lysate has immunological properties similar to those of hsc70 (Matts et al., 1992).

[3] Z. Xu, R. Hurst, and R. L. Matts, unpublished observations.

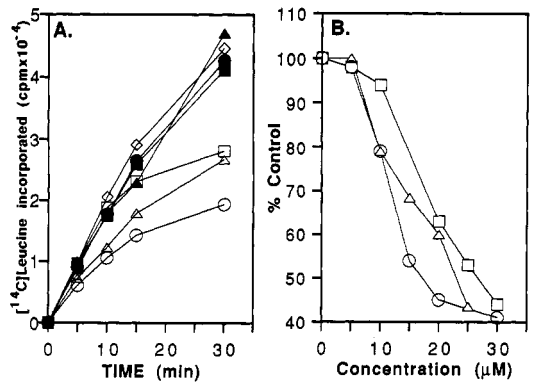

FIGURE 1: Effect of denatured proteins on protein synthesis in hemin-supplemented lysates. (A) Hemin-supplemented protein synthesis mixes were incubated at 30 °C with no additions (◇) or with the addition of 20 μM BSA (■), RCM-BSA (□), LA (●), RCM-LA (○), LG (▲), or RCAM-LG (△) as described under Experimental Procedures. (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. The activation of HRI occurs somewhat slowly in response to the addition of denatured proteins. Because of the biphasic nature of the translational inhibition, the maximm observed inhibition was about 60% in the presence of 30 μM RCM-LA (B), which corresponds to ~40% of control at 30 min. This represents a nearly complete arrest of initiation and ribosome run-off occurring between 10 and 15 min (data not shown).

(Beckman et al., 1990; Palleros et al., 1991; Matts et al., 1992), samples were diluted 10-fold with adsorption buffer and added to the GaR-agarose containing the bound antibodies. Samples were incubated at 4 °C for 90 min with continuous mixing. The unadsorbed proteins (supernatants) were separated from adsorbed proteins (pellets) by centrifugation. Pellets were washed five times with 500 μL of TBS. This method was determined to quantitatively adsorb BSA or RCM-BSA. HRI was quantitatively adsorbed with the anti-HRI mAb F from 10 μL of protein synthesis mixes in the presence or absence of 25 μM BSA or RCM-BSA as previously described (Matts et al., 1992).

Western Blot Analysis. Samples were prepared for SDS–PAGE, separated in 8% gels, and transferred to a PVDF membrane (Bio-Rad) as previously described (Matts et al., 1992). Hsp 70 was detected by western blotting with the N27F3-4 anti-hsp70 mAb as previously described (Matts et al., 1992).

RESULTS

Denatured Proteins Inhibit Protein Synthesis in Rabbit Reticulocyte Lysates. The effects of native BSA, LA, or LG on protein synthesis in hemin-supplemented reticulocyte lysates were compared to the effects of denatured RCM-BSA, RCM-LA, or RCAM-LG. These proteins were selected because microinjection of denatured BSA or LG into frog oocytes triggers the activation of heat shock genes (Ananthan et al., 1986), and RCM-LA has been used as a model substrate to study hsp70/protein interactions *in vitro* (Palleros et al., 1991). Addition of the denatured proteins inhibited protein synthesis, while the addition of the native proteins had little or no inhibitory effect (Figure 1A). The concentrations of RCM-BSA, RCM-LA, and RCAM-LG that inhibited protein synthesis by 50% of the maximally induced inhibition (IC_{50}) were estimated to be 18, 12, and 14 μM (~1.2, 0.22, and 0.20 mg/mL), respectively (Figure 1B).

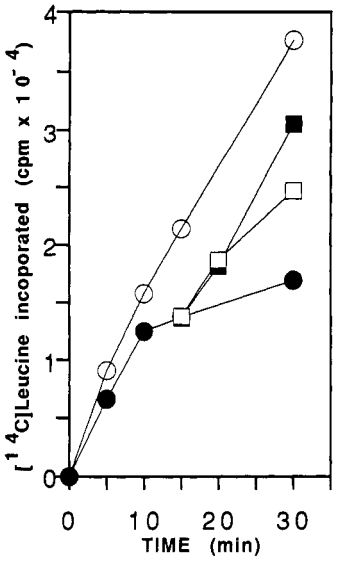

FIGURE 2: Cyclic AMP and eIF-2B reverse the inhibition of protein synthesis in hemin-supplemented lysate induced by RCM-BSA. Hemin-supplemented protein synthesis mixtures were incubated at 30 °C with no additions (○), with 20 μM RCM-BSA (●), or with 20 μM RCM-BSA and 10 mM cAMP (■) or 2 μg eIF-2B (□) added at 15 min as described under Experimental Procedures.

Denatured Proteins Activate HRI in Hemin-Supplemented Lysates. Because the addition of purified eIF-2B or high nonphysiological levels of cAMP reverse HRI-mediated translational inhibition (Hunt, 1979; London et al., 1987; Jackson, 1991), we tested the effects of these agents on the shutoff of protein synthesis caused by RCM-BSA. Addition of cAMP completely reversed the inhibition of protein synthesis in hemin-supplemented lysates brought about by the presence of RCM-BSA (Figure 2). eIF-2B restored protein synthesis to control rates for the first 5 min after its addition to lysates inhibited by RCM-BSA. The rate of protein synthesis at longer incubation times subsequently declined, as the added eIF-2B is sequestered by the phosphorylated eIF-2 present in the RCM-BSA inhibited lysate (see below).

Addition of RCM-BSA to hemin-supplemented protein synthesis mixes induced the phosphorylation of the α-subunit of eIF-2 concomitant with the shut-off protein synthesis (Figure 3). The extent of eIF-2α phosphorylation in RCM-BSA inhibited lysates was 12-fold higher than the level of eIF-2α phosphorylation observed in control lysates containing no additions (Table I). No significant increase in eIF-2α phosphorylation above the control was observed in lysates containing native BSA. Ascites fluid containing anti-HRI mAb blocked the induction of eIF-2α phosphorylation by RCM-BSA, while the addition of ascites fluid containing nonimmune control mAb in most cases increased the level of eIF-2α phosphorylation above that observed in its absence (Figure 3 and Table I). Similarly, RCM-LA and RCAM-LG, but not native LA or LG, also stimulated eIF-2α phosphorylation (data not shown). These observations demonstrate that denatured proteins inhibit protein synthesis in hemin-supplemented lysate by causing the activation of HRI.

RCM-BSA Blocks the Interaction of Hsp70 with HRI. Our model predicts that denatured proteins should bind to hsp70, inhibiting its ability to associate with HRI. Hsp70 from hemin-supplemented lysate was co-adsorbed with RCM-BSA, but not with BSA, in an immune specific fashion (Figure 4A). Western blot analysis of immunoprecipitated RCM-BSA detected no bound HRI (data not shown), indicating that the RCM-BSA is not binding directly to HRI and affecting its activity. When the anti-HRI mAb was used to

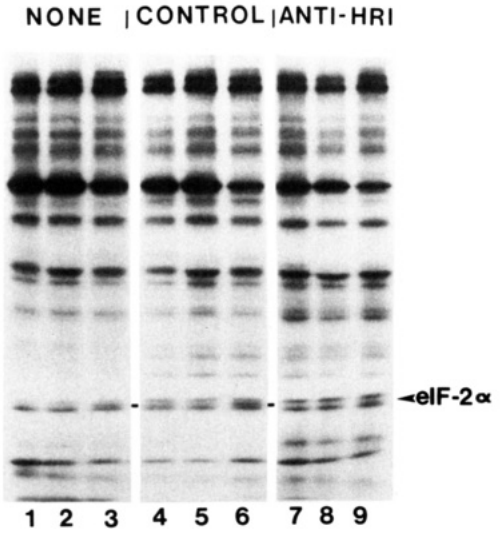

FIGURE 3: Addition of an anti-HRI monoclonal antibody blocks the phosphorylation of eIF-2α induced by RCM-BSA. Hemin-supplemented protein synthesis mixes were preincubated on ice for 15 min in the presence of no additions (NONE, lanes 1–3) or in the presence of ~1 μg IgG/10 μL of protein synthesis mix of control MOPC 21 mouse IgG ascites (Sigma) (CONTROL, lanes 4–6) or anti-HRI monoclonal antibody F ascites (ANTI-HRI, lanes 7–9). Samples were subsequently incubated at 30 °C for 12 min in the presence of no additions (lanes 1, 4, and 7) or in the presence of 25 μM BSA (lanes 2, 5, and 8) or RCM-BSA (lanes 3, 6, and 9). Following a 4-min pulse with [γ-^{32}P]ATP, samples (2.5 μL) were separated by SDS–PAGE. Phosphorylated eIF-2α was identified by the comigration of the phosphorylated protein band with a purified eIF-2α standard. The figure is an autoradiogram.

Table I: Quantitation of the Extent of eIF-2α Phosphorylation[a]

additions to lysate	relative level of eIF-2α phosphorylation (OD × mm^2)		
	none[b]	control[b]	anti-HRI[b]
none	0.028 ± 0.018	0.28 ± 0.25	0.051 ± 0.023
+25 μM BSA	0.056 ± 0.019	0.38 ± 0.25	0.021 ± 0.026
+25 μM RCM-BSA	0.34 ± 0.20†	0.78 ± 0.35*	0.018 ± 0.027

[a] The extent of eIF-2α phosphorylation in samples prepared and analyzed as described in the legend to Figure 3 was quantitated by scanning densitometry of the autoradiograms. Values represent the mean ± standard deviations of determinations made from autoradiograms of four separate experiments. Significance levels of a value's difference from control samples containing no antibody (paired two-tailed *t*-test: †, $p < 0.05$; *, $p < 0.02$). [b] Antibody present during preincubation.

adsorb HRI from hemin-supplemented lysates that were incubated in the presence or absence of BSA or RCM-BSA, western blot analysis indicated that hsp70 was specifically associated with HRI in control lysates (no additions) and in lysates containing BSA, but hsp70 was not co-adsorbed with HRI from lysates containing RCM-BSA (Figure 4B). Thus, RCM-BSA competed with HRI for the binding of the available hsp70.

Western blotting also detected several additional polypeptides with molecular masses of greater than 90 kDa that were present in the pellets containing the nonimmune control IgG or the anti-HRI mAb F (Figure 4B). These polypeptides were not detected in western blots of reticulocyte lysate (Figure 4A, lane L). Western blot analysis of samples containing nonimmune control IgG or anti-HRI mAb F that were adsorbed by the immobilized anti-mouse IgG, but not incubated with reticulocyte lysate, confirmed that the source of the polypeptides was the ascites fluid (data not shown). The proteins are detected because of their reaction with the secondary anti-mouse IgG antibody.

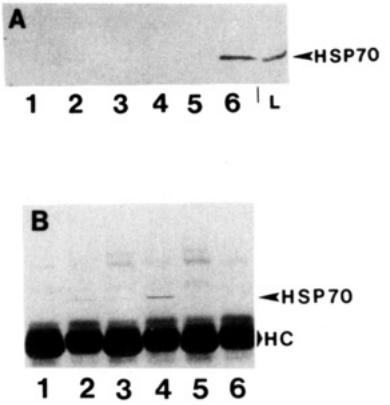

FIGURE 4: RCM-BSA binds to hsp70 and blocks the interaction of hsp70 with HRI. (A) Western blot analysis comparing the capacity of hsp70 to bind to RCM-BSA versus BSA. (B) Western blot analysis comparing the capacity of RCM-BSA versus BSA to block the interaction of hsp70 with HRI. Hemin-supplemented protein synthesis mixes were incubated in the presence of no additions (lanes 1 and 2) or in the presence of 25 μM BSA (lanes 3 and 4) or RCM-BSA (lanes 5 and 6) as described under Experimental Procedures. Proteins adsorbed by rabbit anti-BSA (A, lanes 2, 4, and 6), anti-HRI mAb F (B, lanes 2, 4, and 6), or nonimmune control antibodies (A and B, lanes 1, 3, and 5) were resolved by SDS–PAGE, and hsp70 was detected by Western blotting as described under Experimental Procedures. (L) 1 μL of unfractionated reticulocyte lysate: (HC) heavy chain of anti-HRI mAb detected through its reaction with the secondary anti-mouse IgG antibody.

In the experiment shown in Figure 4B, more hsp70 was nonspecifically adsorbed from reticulocyte lysate in the presence of BSA. Scanning densitometry indicated that the amount of hsp70 that was coadsorbed with HRI was approximately 3-fold higher than the levels of nonspecific binding of hsp70 to the nonimmune controls. When the level of hsp70 bound to HRI is corrected for the amount of hsp70 that is nonspecifically bound, the amount of hsp70 coadsorbed with HRI from lysate in the presence of BSA was approximately double the amount bound to HRI in the absence of BSA. However, this difference in the binding of hsp70 to HRI was not consistently observed in repeats of the experiment.

The Sensitivity of Protein Synthesis to RCM-BSA Induced Inhibition Varies Inversely with Hsp70 Levels Present in Reticulocyte Lysates. Since the sensitivity of protein synthesis in hemin-supplemented lysates to stress-induced inhibition correlates inversely with the levels of hsp70 present (Matts & Hurst, 1992), we compared the sensitivity of protein synthesis to RCM-BSA induced inhibition in three different lysate preparations (Table II). The degree to which protein synthesis was inhibited in the presence of 10 μM RCM-BSA varied inversely with the levels of hsp70. The IC_{50} values for the inhibition of protein synthesis by RCM-BSA were proportional to the amount of hsp70 present in the preparation; the 4.4-fold difference in IC_{50} values corresponded to a 4.5-fold difference in hsp70 levels. These observations suggest that there is a relationship between the sensitivity of HRI to activation in hemin-supplemented lysate in response to denatured proteins and the level of hsp70 present in the lysate.

DISCUSSION

Protein Denaturation in Heat-Stressed Cells. Several observations suggest that the concentrations of denatured proteins (mg/mL) which were found to be required to inhibit translation in lysate are within the range that denatured proteins could be expected to accumulate in cells during a heat stress. The evidence suggests that the expression of heat-induced damage in mammalian cells involves protein dena-

Table II: Relationship between the Relative Quantities of Hsp70s Present in Lysate Preparations and the Extent of Translational Inhibition Induced by RCM-BSA

lysate preparation[a]	Hsp70[b] (mg/mL)	protein synthesis[c] (% control)	IC_{50}[d] (μM)	IC_{50}[d] (mg/mL)
lysate 2	0.13	36	5	0.33
lysate 3	0.35	50	10	0.66
lysate 5	0.58	89	22	1.5

[a] Designations of lysate preparations were those reported previously (Matts & Hurst, 1992). [b] Hsp70 levels were determined by scanning densitometry of Western blots as described previously (Matts & Hurst, 1992) using varying amounts of purified bovine brain hsc70 as a standard. [c] The rate of protein synthesis was measured as the amount of [^{14}C]leucine incorporated into acid-precipitable protein in 30 min at 30 °C. The values are reported as the percent incorporation in lysates containing 10 μM RCM-BSA relative to the incorporation in hemin-supplemented control incubations containing no additions. [d] Values are reported as the concentration of RCM-BSA that inhibited protein synthesis by 50% relative to the control incubations of hemin-supplemented lysates containing no additions.

turation (Lepock, 1987; Lepock et al., 1988). It has been estimated that 5.2% of cell protein represents the fraction of noncrucial thermolabile protein that denatures at temperatures below the denaturation temperature of the critical target for cell killing (Lepock et al., 1988). The concentrations of total soluble proteins in different cell types have been estimated to be between 200 and 320 mg/mL (Srivastava & Bernhard, 1986). Therefore, the concentrations of denatured proteins that were found to be required to cause the activation of HRI are well within the range that denatured proteins could be expected to accumulate physiologically in cells in response to heat shock.

Model for Heat Stress-Induced Activation of HRI. We have proposed that the interaction of hsp70 with HRI is required to maintain HRI in an inactive conformation in hemin-supplemented reticulocyte lysates (Matts & Hurst, 1992) and that the activation of HRI occurs in response to stress when a concentration of denatured protein accumulates that is sufficient to sequester hsp70, blocking its interaction with HRI. This model is analogous to the model proposed for the regulation of the HSF (Craig, 1991; Sorger, 1991; Morimoto et al., 1992). The predictions suggested by this model, that denatured protein should (i) compete with HRI for the binding of hsp70, (ii) cause the activation of HRI, and (iii) inhibit translation at concentrations that vary inversely with the amount of hsp70 present in the lysate, were upheld.

The addition of nonimmune control ascites fluid alone to hemin-supplemented lysate was observed to be sufficient to induce eIF-2α phosphorylation (Table I), particularly if phosphorylations were carried out after longer incubation times (e.g., 30 min).[3] This observation suggests that whole ascites fluid contains an agent, possibly polypeptides that bind hsp70, that is capable of causing the activation of HRI. Therefore, we suggest that one should be cautious when interpreting results from experiments in which whole ascites fluid or antiserum are used to demonstrate immune specific stimulation of HRI activity in reticulocyte lysate, since hsp70 "promiscuously" binds to many polypeptides (Beckman et al., 1990). Care should be taken to determine that the activation of HRI has not occurred simply because one has added polypeptides to which hsp70 binds.

The observations that heat shock blocks the binding of hsp70 to HRI[3] and that the addition of purified hsp70 to reticulocyte lysate protects protein synthesis, to an extent, from inhibition caused by heat shock (Mivechi & Ogilvie, 1989) support the proposed model. While the interactions of hsp90 and p56 with HRI are heme-dependent, the interaction of hsp70 with HRI is not (Matts & Hurst, 1989; Matts et al., 1992), indicating that the binding of hsp70 to HRI alone is not sufficient to suppress HRI activity. Recently, we have found that heat shock stabilizes the interaction of hsp90 with HRI and that hsp90 stimulates the activity of purified HRI *in vitro* in the presence of hemin.[4] The interaction of hsp70 with HRI may function to suppress the ability of hsp90 to enhance HRI activity that we[4] and others have observed (Szyszka, 1989). How hsp90, hsp70, and p56 interact to regulate HRI activation in response to heme deficiency, heat, and oxidative stress is currently under study.

The model proposed above for the regulation of HRI in response to stress may also be applicable to other cell types. Phosphorylation of eIF-2α and dephosphorylation of eIF-4E occur in a number of cultured cell lines in response to physiological stresses (London et al., 1987; Hershey, 1989; Jackson, 1991; Pain & Clemens, 1991). The extent of eIF-2α phosphorylation in HeLa and Ehrlich ascites cells was proportional to the severity of the heat stress (Rowlands et al., 1988; Duncan & Hershey, 1989). While the eIF-2α kinases present in these cells have yet to be characterized extensively, the HeLa eIF-2α kinase has been reported to have antigenic properties similar to HRI (DeBenedetti & Baglioni, 1986). The observation that suppression of eIF-2α phosphorylation by expression of nonphosphorylatable mutants of eIF-2α in HeLa cells mitigates the inhibition of translation during heat shock (Murta-Riel et al., 1991), indicates that the phosphorylation of eIF-2 contributes to the translational shut-off induced by heat shock. If the eIF-2α kinases present in these cells are regulated in a manner analogous to HRI, stress-induced accumulation of denatured protein could be the signal that causes the activation of the kinases, resulting in the inhibition of protein synthesis. The ability of hsp70 to suppress the activation of the eIF-2α kinase in cells following recovery from the stress could account for the observation that the refractoriness of translation to stress-induced inhibition correlates with the synthesis and accumulation of hsp70 in cells (DiDomenico et al., 1982; Laszlo, 1988; Mizzen & Welch, 1988).

Regulation of eIF-2α phosphorylation in this manner would also provide a cell with an elegant feedback control mechanism that links protein maturation with protein synthesis. Cytosolic hsp70s interact with nascent polypeptides that are destined to enter the lumen of the ER or the mitochondrial matrix (Lindquist & Craig, 1988; Schlesinger, 1990; Ang et al., 1991; Ellis & van der Vies, 1991; Hightower, 1991). ER and mitochondrial homologs of hsp70 are also required for the translocation the nascent polypeptides into these organelles and for their subsequent folding. Conditions that would overload these transport and folding systems with an excess of nascent or denatured polypeptides would lead to an accumulation of nascent polypeptide precursors within the cytoplasm. These would compete with HRI (or an analogous kinase) for the binding of hsp70, resulting in the activation of the eIF-2α kinase and inhibition of protein synthesis. Consistent with this model, agents that disrupt the proper processing or folding of proteins in the ER have been observed to stimulate eIF-2α phosphorylation and inhibit protein synthesis in GH_3 cells (Prostko et al., 1992). Protein synthesis rates returned to normal together with the dephosphorylation of eIF-2 upon the accumulation of newly synthesized BiP, the

[4] J. K. Pal, Z. Xu, R. L. Matts, H. P. Hahn, W. J. Welch, I. M. London, and J.-J. Chen, manuscript submitted.

ER homolog of hsp70. This mechanism would 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.

ACKNOWLEDGMENT

We thank Dr. Jane-Jane Chen for providing us with the anti-HRI mAb, Drs. Jane-Jane Chen, Franklin Leach, and Steve Hartson for their critical reading of the manuscript, and Janet Rogers for her technical assistance.

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