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21. All assays were performed on a ^{32}P–end-labeled restriction fragment (Eco RI–Pvu II) from the plasmid pARE/GCRE, which contains an AP1 binding site (ARE) and a GCN4 binding site (GCRE) (boxes on right) (*18*). DNase I footprinting (*22*) reaction conditions were 10 m*M* tris·HCl, 10 m*M* KCl, 10 m*M* $MgCl_2$, 5 m*M* $CaCl_2$, 200 μ*M* dithiothreitol (DDT), 0,33 μg/ml DNase I, 200 μ*M* calf thymus DNA (bp), and 30,000-cpm end-labeled DNA, *p*H 7.0. Reactions were initiated with the addition of DNase and DTT, then allowed to proceed at room temperature for 3.5 min. The MPE footprinting (*23*) reaction conditions were 20 m*M* phosphate, 20 m*M* NaCl, 5 m*M* DTT, 5 μ*M* Fe(II)·MPE, 100 μ*M* calf thymus DNA (bp), and 30,000-cpm labeled DNA, *p*H 7.5. Reactions were initiated by the addition of DTT and Fe(II)·MPE and allowed to proceed 10 min at room temperature. Affinity cleaving reaction conditions were 30 m*M* tris·HCl, 3 m*M* sodium acetate, 20 m*M* NaCl, 5 m*M* DTT, 100 μ*M* calf thymus DNA, and 30,000-cpm labeled DNA, *p*H 7.9. After an equilibration period of 30 min, the reactions were initiated with the addition of DTT and allowed to proceed at room temperature for 30 min. Cleavage products were analyzed on an 8%, 1:20 cross-linked denaturing polyacrylamide wedge-shaped gel.
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Interaction of Hsp 70 with Newly Synthesized Proteins: Implications for Protein Folding and Assembly

RICHARD P. BECKMANN, LEE A. MIZZEN, WILLIAM J. WELCH

The 70-kilodalton family of heat shock proteins (Hsp 70) has been implicated in posttranslational protein assembly and translocation. Binding of cytosolic forms of Hsp 70 (Hsp 72,73) with nascent proteins in the normal cell was investigated and found to be transient and adenosine triphosphate (ATP)–dependent. Interaction of Hsp 72,73 with newly synthesized proteins appeared to occur cotranslationally, because nascent polypeptides released prematurely from polysomes in vivo can be isolated in a complex with Hsp 72,73. Moreover, isolation of polysomes from short-term [^{35}S]Met-labeled cells (pulsed) revealed that Hsp 72,73 associated with nascent polypeptide chains. In cells experiencing stress, newly synthesized proteins co-immunoprecipitated with Hsp 72,73; however, in contrast to normal cells, interaction with Hsp 72,73 was not transient. A model consistent with these data suggests that under normal growth conditions, cytosolic Hsp 72,73 interact transiently with nascent polypeptides to facilitate proper folding, and that metabolic stress interferes with these events.

IN MOST ORGANISMS ADVERSE changes in the local environment result in increased expression of heat shock or stress proteins (*1*). Most inducers of the stress response represent treatments that perturb protein structure and folding (*2*). The Hsp 70 family of stress proteins consists of at least four major members, all of which are related in structure and bind ATP. These include (i) constitutively expressed Hsp 73, present in cytosol and nucleus; (ii) the highly stress-inducible Hsp 72, present in cytosol, nucleus, and nucleolus; (iii) the constitutive glucose-regulated protein (Grp) 78 kD (or BiP) present in endoplasmic reticulum (ER); and (iv) the recently identified, constitutive Grp 75 kD protein present in mitochondria (*3*). Studies indicate that Hsp 70 family members interact transiently with various cellular proteins that are in the process of maturation. For example, in yeast, the cytosolic Hsp 70 proteins are essential in facilitating translocation of proteins across ER or mitochondrial membranes (*4*). Hsp 72,73 also facilitate disassembly of clathrin-coated vesicles (*5*). Finally, BiP interacts with proteins that are translocated into and assembled within the ER lumen (*6*). The interaction of the Hsp 70 family member with its target proteins is transient, with release of the target dependent on ATP hydrolysis. We present data that are consistent with the idea that most, if not all, proteins transiently interact with cytosolic Hsp 70 during their normal maturation, and we discuss how these observations relate to the stress response.

Interaction of Hsp 70 with intracellular proteins was examined by immunoprecipitation with monoclonal antibodies specific for Hsp 72,73 (Fig. 1). Duplicate plates of HeLa cells growing at 37°C were labeled with [^{35}S]Met for 20 min. One group of cells was immediately harvested (pulsed), while a second group was incubated 2 hours more without label and then harvested (pulse-chased). After cells were lysed, ATP was added to half of the lysate, while the other half was treated with apyrase to degrade endogenous ATP. In the pulse-labeled cell lysates depleted of endogenous ATP, immunoprecipitation with antibodies to Hsp 72,73 (anti-Hsp 72,73) resulted in the coprecipitation of many newly synthesized polypeptides ranging in size from 20 to 200 kD (Fig. 1B). Proteins of defined size were coprecipitated along with a background smear of labeled material, perhaps representing unfinished nascent polypeptide chains. In contrast, few proteins coprecipitated when the pulse-labeled cell lysate was incubated with exogenous ATP. Cells that were pulse-labeled with [^{35}S]Met and then incubated for 2 hours without label exhibited a different pattern of coprecipitating proteins (Fig. 1B). Specifically, fewer proteins coprecipitated with Hsp 72,73 in lysates treated with apyrase. Again, fewer proteins coprecipitated when ATP was added to the cell lysate.

We next investigated the interaction of Hsp 70 with newly synthesized proteins in HeLa cells treated with the proline analog, L-azetidine 2-carboxylic acid (Azc) to induce stress. Cells were treated with Azc for 4 hours, labeled for 15 min with [^{35}S]Met while still in the presence of the analog, and immediately harvested (pulsed) or further incubated in the absence of label and Azc for 2 hours (pulsed-chased) (Fig. 1C). Significant amounts of labeled proteins coprecipitated with anti-Hsp 72,73 in apyrase-treated lysates. Addition of ATP to immunoprecipitation yielded considerably fewer coprecipitating proteins. However, contrary to results obtained for normal (unstressed) cells (Fig. 1B), the amount of coprecipitating material was not reduced after a subsequent chase period (Fig. 1C). Instead, most of the precipitating proteins were still complexed with Hsp 72,73 after the 2-hour chase period.

We also examined the effects of no prior treatment or addition of a nonhydrolyzable ATP analog (AMPPNP) to the cell lysates prior to immunoprecipitation. In either case, an intermediate amount of newly synthesized proteins coprecipitated with Hsp 72,73. Partial release was probably due to endogenous ATP in untreated lysates. Moreover, results with AMPPNP indicate that ATP hydrolysis is required for complete release of coprecipitating proteins (*7*).

Exposure of cells to the antibiotic, puromycin, in concentrations sufficient to release nascent polypeptides from polysomes but

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

not to inhibit translation completely, induces the stress response (*8*). Because Hsp 72,73 interacted with newly synthesized proteins (Fig. 1), we sought to determine whether Hsp 72,73 associated with nascent peptides prematurely released from polysomes. Because exposure of HeLa cells to puromycin at 1 to 4 μg/ml induced the stress response (*7*), we labeled cells for 15 min with [^{35}S]Met in the presence of puromycin (4 μg/ml). Puromycin treatment resulted in the generation of incomplete polypeptides, as demonstrated by the absence of high molecular weight proteins and the smear of labeled material in the cell lysate (Fig. 2). Apyrase treatment and subsequent immunoprecipitation with anti-Hsp 72,73 resulted in coprecipitation of a smear of labeled polypeptides, while treatment with ATP reduced the amount of coprecipitating material. Cells labeled in the presence of puromycin and further incubated in the absence of the drug revealed a reduction in coprecipitating material, perhaps because of degradation of the prematurely released nascent peptides.

We next wanted to determine whether interactions between Hsp 72,73 and nascent peptides were occurring cotranslationally or only after release of the unfinished polypeptide from the polysome. If the interaction was indeed cotranslational, then a portion of Hsp 72,73 should fractionate with polysomes through association with the nascent polypeptides. For testing this possibility, cells were pulse-labeled with [^{35}S]Met and lysed with nonionic detergent in the presence of apyrase and the translation elongation inhibitor, cycloheximide (CHX). A 20,000*g* supernatant was prepared, and polysomes were isolated by velocity sedimentation through sucrose. Although the majority of Hsp 72,73 was in the top portion of the gradient, a small fraction comigrated with polysomes (Fig. 3A). Peak polysome-containing fractions were pooled and polysomes were reisolated by high-speed centrifugation. Immunoprecipitations were then performed with either the intact polysomes or polysomes digested exhaustively with ribonuclease (RNase) and treated with EDTA. In either case, immunoprecipitation with anti-Hsp 72,73 (Fig. 3B, lane 4) resulted in the coprecipitation of a smear of labeled peptides similar to those present in the polysome fraction (Fig. 3B, lane 1). Therefore, Hsp 72,73 cosedimented with polysomes, probably through association with nascent polypeptides.

Because the interaction of Hsp 72,73 with newly synthesized proteins persisted significantly longer in cells exposed to an amino acid analog, we suspected that one primary effect of stress was to interfere with the proper maturation of newly synthesized proteins. Therefore, reducing the overall amount of new protein synthesis (or assembly) might reduce the severity of the stress event. Indeed, incubation of cells with CHX prior to and during heat shock treatment reduces cell lethality (*9*). As a test of this hypothesis, cells were incubated at 37°C in the presence of CHX for 2 hours to completely inhibit new protein synthesis and to allow for assembly of proteins synthesized before the drug was added. While still in the presence of CHX, cells were subjected to heat shock or exposure to sodium arsenite. After the stress treatment, CHX was removed and cells were returned to normal growth conditions, then labeled with [^{35}S]Met to determine whether induction of stress protein synthesis had occurred (Fig. 4). Cells stressed in the absence of CHX exhibited a vigorous stress response, as evidenced by induction of stress protein synthesis. In contrast, cells treated with CHX prior to and during stress exhibited reduced Hsp 72,73 synthesis. Finally, cells to which CHX was added only during stress (but not

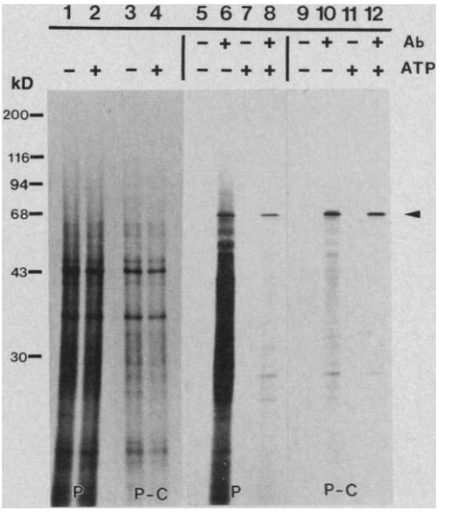

Fig. 2. Interaction of Hsp 72,73 with prematurely released peptides. To detect interaction of Hsp 72,73 with peptides prematurely released by puromycin treatment, HeLa cells were incubated with puromycin (4 μg/ml) for 30 min and labeled with [^{35}S]Met for 20 min in the presence of the drug. One plate of cells was immediately harvested (pulsed, P) while a second plate was harvested 2 hours after the removal of both the label and puromycin (pulsed-chased, P-C). Cell lysates were divided and incubated with either apyrase (ATP−) or 2.5 m*M* ATP (ATP+). Immunoprecipitations were performed with either control antibodies (Ab−) or anti-Hsp 72,73 (Ab+) as described in Fig. 1 and analyzed by SDS-PAGE. The pattern of proteins synthesized in either the pulsed (P, lanes 1 and 2) or pulsed-chased cell lysates (P-C, lanes 3 and 4) are shown. Lanes 5 to 8 and 9 to 12 show immunoprecipitates from the pulsed and pulsed-chased cells, respectively. The position of Hsp 72,73 is indicated by an arrowhead.

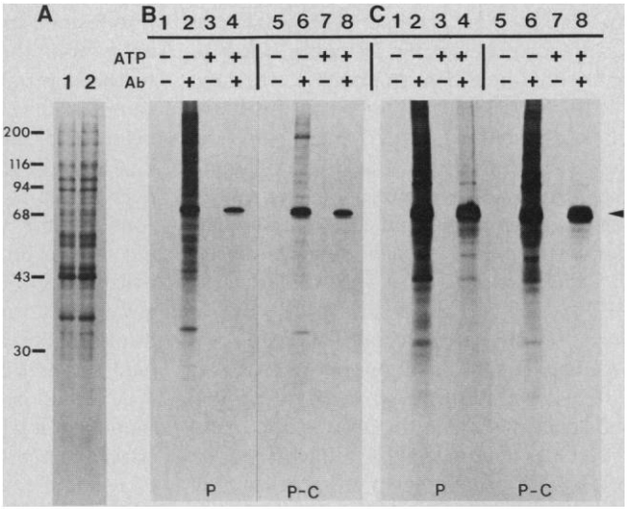

Fig. 1. Interaction of Hsp 72,73 with newly synthesized proteins. (**A** and **B**) Duplicate HeLa monolayer cultures at 37°C were labeled for 20 min with [^{35}S]Met (65 μCi/ml). One plate of labeled cells was immediately harvested (pulsed, P) while a second plate was harvested 2 hours after the label had been removed (pulsed-chased, P-C). Cells were lysed by the addition of phosphate buffered saline (PBS) containing 0.1% Triton X-100 (TX-100) and 5 m*M* $MgCl_2$, and the lysates were divided. The ATP hydrolytic enzyme, apyrase (10 units/ml), was added to one portion, while 2.5 m*M* ATP was added to the other. After a 15-min incubation at 4°C, the lysates were clarified by centrifugation (5 min, 16,000*g*), and the supernatants were adjusted to 1% TX-100, 1% sodium deoxycholate (DOC), and 0.1% SDS in PBS for immunoprecipitation. Immunoprecipitations were performed with either rabbit antibodies to mouse IgG (control) or anti-Hsp 72,73 (*21*). Equal amounts of incorporated radioactivity (as determined by trichloracetic acid precipitation) from the lysates were used in each immunoprecipitation and analyzed by SDS–polyacrylamide gel electrophoresis (PAGE). (A) Proteins present in [^{35}S]Met pulse-labeled cells incubated with either apyrase (lane 1) or ATP (lane 2). (B) Immunoprecipitates from either the pulsed (P) cell lysates (lanes 1 to 4) or the pulsed-chased (P-C) cell lysates (lanes 5 to 8) were depleted of ATP with apyrase treatment (ATP−) or incubated with 2.5 m*M* ATP (ATP+) prior to the immunoprecipitation with either the control (Ab−) or the anti-Hsp 72,73 (Ab+). (**C**) Duplicate cultures of HeLa cells at 37°C were incubated with Azc (5 m*M*) for 4 hours. In the presence of Azc, cells were pulse-labeled with [^{35}S]Met for 20 min and immediately harvested or further incubated for 2 hours in the absence of label and Azc. Lysates were treated with either apyrase or ATP and immunoprecipitated as described in (B).

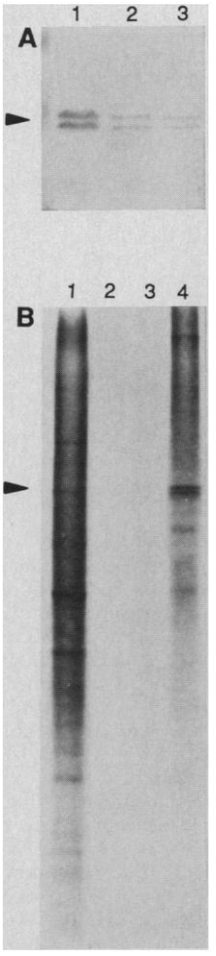

Fig. 3. Hsp 72,73 cosediment with polysomes in apparent association with nascent chains. HeLa cells were pulse-labeled with [^{35}S]Met (1.5 mCi per 10-cm dish) for 15 min at 37°C and lysed as in Fig. 1 in the presence of apyrase (20 units/ml) and CHX (100 μg/ml). Following incubation (15 min, 0°C), the harvesting buffer was adjusted to 1% TX-100 and 1% DOC, and the monolayer was scraped from the dish and clarified by centrifugation (20,000*g*, 5 min). Polysomes were prepared by sedimentation of the 20,000*g* supernatant through a sucrose gradient (*22*) and analyzed by SDS-PAGE, immunoblotting, and immunoprecipitation. (**A**) For immunoblotting, equal amounts of protein (*23*) were resolved by SDS-PAGE, transferred to nitrocellulose, and probed with anti-Hsp 72,73. Bound antibody was visualized by alkaline phosphatase–mediated color development (*24*). (Lane 1) 20,000*g* supernatant from the detergent-lysed cells; (lane 2) 80*S* ribosome peak from the sucrose gradient; and (lane 3) polysome peak from the sucrose gradient. (**B**) For immunoprecipitation, peak fractions of the polysomes were pooled, pelleted by centrifugation, resuspended in immunoprecipitation buffer (see Fig. 1), and then incubated with RNAse (20 μg/ml) (Worthington) for 15 min at 30°C. The sample was adjusted to 20 m*M* EDTA and incubated at 0°C for 10 min prior to immunoprecipitation. Immunoprecipitates were analyzed by SDS-PAGE. (Lane 1) Total labeled protein present in the polysome fraction; (lane 2) immunoprecipitate with control antibodies; (lane 3) immunoprecipitate with antibody to β-tubulin (control); and (lane 4) immunoprecipitate using anti-Hsp 72,73. Arrowheads indicate the position of Hsp 72,73.

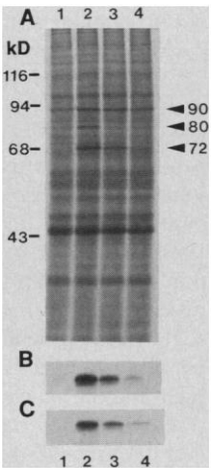

Fig. 4. Treatment of cells with cycloheximide (CHX) prior to and during stress results in a reduction of Hsp 72,73 synthesis. (**A**) HeLa cells were incubated at 37°C with 100 μg/ml of CHX for 15 min or 2 hours. While still in the presence of CHX, cells were subjected to a 43°C/90 min heat shock treatment or exposure to 200 μ*M* sodium arsenite for 90 min. Cells in the absence of CHX were similarly stressed. After stress treatment, cells were returned to normal growth conditions (removal of CHX and arsenite) and steady-state labeled with [^{35}S]Met (4 hours). After labeling, cells were lysed in SDS sample buffer and labeled proteins were analyzed by SDS-PAGE (A). Shown are the control or heat shock–treated cells. (Lane 1) 37°C cells; (lane 2) cells subjected to heat shock in the absence of CHX; (lane 3) cells incubated with CHX for 15 min prior to and during the heat shock treatment; and (lane 4) cells incubated with CHX for 2 hours before and during the heat shock treatment. (**B**) For a more direct examination of relative amounts of Hsp 72, immunoprecipitation with a mouse monoclonal antibody specific to Hsp 72 was performed under denaturing conditions. Lane designation is the same as in (A). (**C**) Immunoprecipitation of Hsp 72 was performed from cells exposed to sodium arsenite in the presence or absence of CHX as described above. (Lane 1) 37°C cells; (lane 2) cells exposed to 200 μ*M* arsenite in the absence of CHX; (lane 3) cells exposed to CHX 15 min prior to and during exposure to 200 μ*M* arsenite; and (lane 4) cells exposed to CHX for 2 hours prior to and during exposure to 200 μ*M* arsenite.

2 hours prior) responded with an intermediate induction of stress protein synthesis. These results imply that a major target affected in cells experiencing stress are proteins in the process of synthesis.

Despite their designation, most stress proteins are expressed constitutively and appear to be essential to the life-style of normal cells. Most information concerning the functions of individual stress proteins comes from studies of normal cellular events in which Hsp 70–related proteins are involved. Available evidence has implicated these related proteins in various aspects of protein maturation. For example, BiP, present in ER, facilitates assembly of monomeric proteins into their final multimeric structure. BiP binds to the monomeric forms of the immunoglobulin G (IgG) heavy (H) and light (L) chains prior to their assembly into the mature H_2L_2 structure, as well as to influenza hemagglutinin (HA) monomers during the course of their trimerization (*6, 10*). The cytosolic Hsp 70 proteins (Hsp 72,73) facilitate translocation of proteins across the ER or mitochondrial membranes (*4*). Thus, the prevailing notion is that Hsp 70 proteins function to stabilize protein substrates that are in the process of maturation. Our data suggest that the participation of Hsp 70 proteins in protein maturation may be more general than previously suspected.

We suggest that during synthesis (and perhaps posttranslational assembly) most proteins interact transiently with Hsp 72,73 (Fig. 5). Through such an interaction, the nascent protein may be maintained in a stable conformation until its translation is completed; the protein may then begin to fold into its final conformation with a concomitant release of Hsp 72,73 (Fig. 5, pathway 1). We envision that the interaction of Hsp 72,73 with the maturing polypeptides is a dynamic one in which Hsp 72,73 bind to and stabilize peptide domains as they emerge from the ribosome. Whether folding occurs entirely posttranslationally or cotranslationally followed by an orderly release of Hsp 72,73 from the individual peptide domains as they fold, is difficult to distinguish. Both mechanisms may be occurring for any given polypeptide. Release of Hsp 70 from a target peptide is probably determined by its relative affinity for that peptide versus the affinity of the neighboring peptide domains for one another as they fold. Consistent with this scenario, recent studies demonstrate that Hsp 70 proteins are promiscuous in their ability to bind a variety of synthetic peptides (*11*). Moreover, the apparent binding constants (in the micromolar range) indicate that interactions of Hsp 72,73 with such peptides in vitro are relatively weak. It is possible that the binding constants for adjacent peptide domains in the mature protein would be greater than that for Hsp 70. As domains fold into their mature conformation, Hsp 72,73 would be displaced, perhaps because of their ability to bind and hydrolyze ATP.

As would be predicted by this model, we found that interaction of Hsp 70 with newly synthesized proteins was transient. However, some newly synthesized proteins remained complexed with Hsp 72,73 for a considerable period of time following their synthesis (Fig. 1). Hence, Hsp 72,73 may also stabilize completed proteins that require additional time to reach their final conformation (Fig. 5, pathways 2 and 3). Examples include proteins that are assembled into multimeric structures, proteins that are posttranslationally translocated to organelles, or proteins that exist in equilibrium between monomeric and polymeric states (clathrin, cytoskeletal proteins).

In cells exposed to Azc, newly synthesized

proteins were still bound to Hsp 72,73 2 hours after synthesis (Fig. 2). Perhaps proteins containing an amino acid analog cannot fold correctly, and Hsp 72,73 release does not occur. With time, these abnormal proteins are degraded by the cell (*12*). The abnormal proteins might remain bound to Hsp 72,73 in order to maintain their solubility until presentation to the appropriate proteolytic system. Our observations that indicate a stable association of Hsp 72,73 with Azc-containing proteins may be analogous to previous observations with BiP. Specifically, when immunoglobulin producing cells are treated with inhibitors of glycosylation, under-glycosylated IgG H chains are unable to properly associate with L chains and remain stably bound to BiP (*13*). Similarly, mutant forms of the influenza HA that are unable to correctly trimerize within the membrane of the ER also remain bound to BiP (*10*). Hence, one function suggested for BiP is to recognize abnormal proteins that are unable to correctly mature and fail to move properly along the secretory pathway (*10, 13–15*). We suggest instead that BiP binds to and facilitates the folding and assembly of normal proteins. When assembly cannot occur due to an abnormality in the substrate protein, BiP remains bound to its target as did Hsp 72,73 (Fig. 2). Thus, we suggest that release of Hsp 70 from target proteins is dictated by the structure of the target protein itself and that failure of the target to fold properly results in a stable complex between the target and Hsp 70.

Our results also may bear on the regulation of stress protein synthesis. Hsp 72,73 likely exists in equilibrium between free and substrate-bound forms. When the equilibrium is shifted toward the substrate-bound forms (as in Azc-treated cells), the corresponding reduction in amounts of free Hsp 72,73 may induce Hsp 72,73 synthesis. When the free pool of Hsp 72,73 is restored to some critical concentration, synthesis is halted. This mode of regulation is consistent with previous observations: (i) Hsp 72 synthesis appears to be autoregulated primarily at a posttranslational level (*16*); (ii) Hsp 72 synthesis increases in direct proportion to the severity of the stress (*16, 17*); (iii) preexisting amounts of Hsp 72,73 influence Hsp 72,73 synthesis following a second stress event (*17*); (iv) microinjection of denatured proteins into frog oocytes is sufficient to activate synthesis of Hsp 72, presumably due to binding of Hsp 72,73 to the denatured proteins (*18*); and (v) microinjection of anti-Hsp 72,73 into cells results in an immediate induction of Hsp 72,73 synthesis (*19*). Each of these observations is consistent with our model in which the concentration of free versus substrate-bound Hsp 70 is carefully monitored. Such regulation also appears to occur with BiP (*20*).

Our results obtained with puromycin and CHX are also consistent with the proposed model (Fig. 5). Exposure of cells to low concentrations of puromycin sufficient to generate unfinished polypeptides resulted in increased Hsp 72,73 synthesis, and the unfinished peptides were stably bound to Hsp 70. Reduction in the pool of free Hsp 70 probably results in increased synthesis of Hsp 72,73. Data with CHX support the idea that proteins in the process of synthesis represent a major labile target in cells exposed to stress. By reducing the amount of such targets (with CHX), subsequent exposure of the cells to heat shock or sodium arsenite did not result in significantly increased synthesis of Hsp 72,73. With fewer labile targets to be affected by the stress event, the available free pool of Hsp 72,73 would not be significantly reduced, and the cell, therefore, would not require significant new Hsp 72,73 synthesis. Studies that support this hypothesis show that cells treated with glycerol or D_2O, agents known to protect proteins against thermal denaturation, exhibit little heat-induced stress protein synthesis (*2*).

Our model implying that Hsp 70 proteins participate in protein folding and assembly are in contrast to earlier models suggesting that protein folding is a spontaneous event dictated entirely by the primary amino acid sequence. We suggest that folding requires the participation of accessory molecules, like members of the stress protein family.

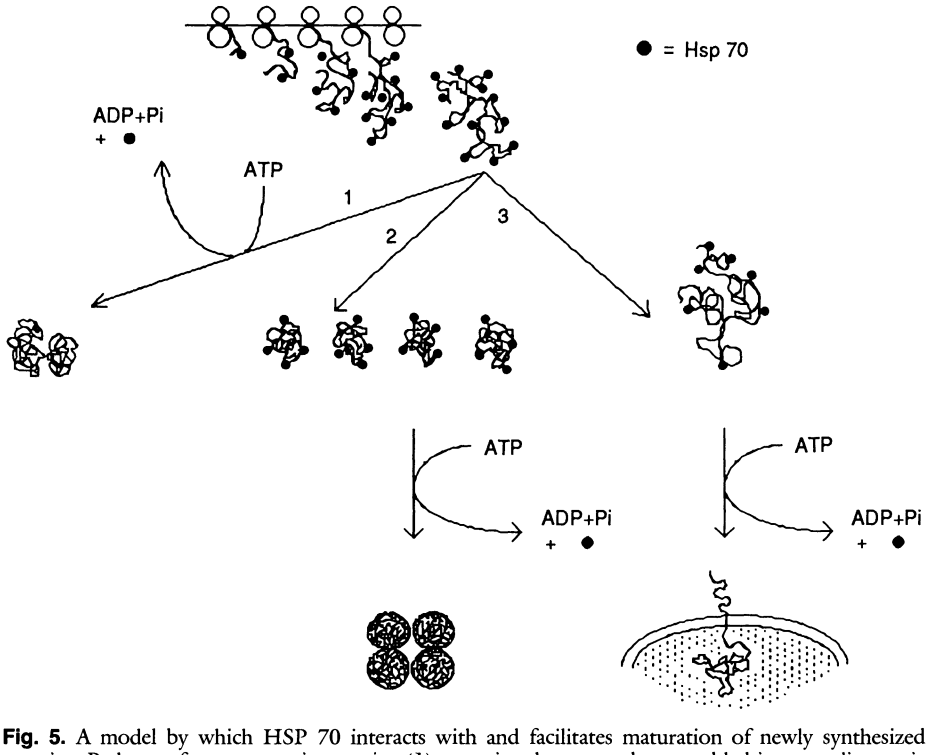

Fig. 5. A model by which HSP 70 interacts with and facilitates maturation of newly synthesized proteins. Pathways for monomeric proteins (1), proteins that are to be assembled into an oligomeric structure (2), and proteins that are translocated from the cytosol into organelles (3). Subsequent folding of the protein occurs within the organelle, perhaps facilitated by chaperonin-like molecules (*25*).

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22. For preparation of polysomes, each 10-cm dish of [^{35}S]Met-labeled HeLa cells was harvested as in Fig. 3 in a total volume of 1 ml. The 20,000*g* supernatant was layered onto an 11.5-ml continuous gradient of 0.5*M* to 1.5*M* sucrose in 10 m*M* Hepes-KOH *p*H 7.4, 5 m*M* $MgCl_2$ and 100 m*M* KCl. Gradients were centrifuged in an SW41 rotor (Beckman) at 100,000*g* for 2 hours at 8°C, and fractionated into 24 samples (0.5 ml). Absorbance at 254 nm was determined for each fraction in a model DU-65 spectrophotometer (Beckman).
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Table 1. Patient information. Abbreviations: AD, Alzheimer's disease; PD, idiopathic Parkinson's disease; and MID, multi-infarct dementia.

Case number	Age	Sex	Diagnosis
1	90	F	AD
2	85	F	AD
3	87	M	AD
4	82	M	AD
5	81	F	AD
6	70	F	AD
7	78	M	AD/PD
8	88	M	PD
9	69	M	PD
10	78	M	PD/MID
11	66	M	Normal

Relation of Neuronal APP-751/APP-695 mRNA Ratio and Neuritic Plaque Density in Alzheimer's Disease

STEVEN A. JOHNSON, THOMAS MCNEILL, BARBARA CORDELL, CALEB E. FINCH

An ongoing controversy concerns the cellular distribution of the differentially spliced forms of the amyloid protein precursor (APP) mRNAs and changes in prevalence of these transcripts during Alzheimer's disease. In situ hybridization on serial sections was used to prove that most hippocampal pyramidal neurons contain both APP-751 and APP-695 mRNA species. The APP-751/APP-695 mRNA ratio is generally increased during Alzheimer's disease, as shown by RNA gel blot analysis. Moreover, there was a strong linear relation between the increase in APP-751/APP-695 mRNA ratio in pyramidal neurons and the density of senile plaques within the hippocampus and entorhinal cortex. Thus, the increase in APP-751/APP-695 mRNA provides a molecular marker for regional variations in plaque density between individuals diagnosed with Alzheimer's disease by the commonly used composite criteria.

ACCUMULATIONS OF β amyloid in neuritic plaques and cerebral blood vessels are characteristic of Alzheimer's disease (AD). One hypothesis concerns the relation between altered proteolytic activity and β-amyloid deposition in plaques. The mRNA for amyloid protein precursor (APP) in aged human brain consists of two major alternatively spliced forms, APP-695 and APP-751; the latter has an exon encoding a Kunitz-type serine protease inhibitor (KPI) (*1–3*). Several other alternatively spliced or truncated KPI-containing APP mRNA forms (APP-770 and APP-543) are known (*3*), but are rare in aged human brain. APP-751 may be identical to the serine protease inhibitor protease nexin-II, on the basis of the available NH_2-terminal sequence (*4, 5*). Extracts of cells transfected with APP-770 have trypsin inhibitory activity (*6*). The APP is axonally transported and localized to synaptic membranes (*7–10*). An increase in the ratio of KPI-APP mRNA/APP-695 mRNA during AD could alter local membrane proteolytic activity and play a role in β-amyloid deposition and plaque formation. A major question is the distribution of each APP mRNA form with respect to local accumulations of amyloid.

Using probes specific for each APP RNA, we (*11, 12*) and others (*2, 13*) showed that the APP-751/APP-695 mRNA ratio increased in hippocampal and neocortical tissues during AD. In contrast, an indirect assay by in situ hybridization (*14*) indicated that APP-695 mRNA was increased in the nucleus basalis and locus ceruleus of individuals with AD. However, present data do not indicate whether there is a selective loss of neurons with high prevalence of a particular APP mRNA. It is therefore crucial to measure the neuronal prevalence of each APP transcript and to establish if both APP mRNA forms are present in the same neurons to understand the possible relation between changes in APP mRNAs and AD.

In situ hybridization with APP-751– and APP-695–specific probes (*15*) was performed on adjacent serial hippocampal sections from seven clinically and pathologically confirmed AD and four non-AD individuals. Neuronal grain density was determined by computer-assisted image analysis, and a comparative bridge microscope was used to colocalize the presence of each APP transcript in the same neuron from adjacent tissue sections (*16, 17*).

The APP-751/APP-695 grain density ratio was twofold higher in each subfield of AD hippocampus compared to non-AD hippocampus (Fig. 1 and Tables 1 and 2). The majority (five out of seven) of AD individuals had APP-751/APP-695 ratios that were higher than the four controls. Data presented show that specific regions of an AD brain may not show increased APP-751/APP-695 mRNA ratios if that region has low plaque density. We also found that individual pyramidal neurons contain both APP-695 and APP-751 mRNAs, by comparing identical cellular fields from adjacent sections (Fig. 2). Most (97%; $n = 104$) neurons common to both adjacent sections show cytoplasmic hybridization in the AD and non-AD specimens examined.

Neuronal loss and atrophy is common in AD, especially in hippocampal pyramidal fields (*18*). Because Northern (RNA) blot data have shown an increased APP-751/APP-695 mRNA ratio in hippocampus (*12*), it was important to determine whether this resulted from a loss of neurons that selectively contain APP-695 mRNA. Thus, we counted all neurons that hybridized to either APP-751– or APP-695–specific probes in adjacent tissue sections. The data are expressed as a ratio of neurons that contain 751 mRNA or 695 mRNA and show that the ratio of hippocampal pyramidal neurons containing each APP mRNA did not differ significantly between AD or non-AD individuals (Fig. 3).

Analysis across individuals showed a strong relation between the APP-751/APP-695 mRNA ratio and plaque density by Bielschowsky staining in the hippocampus

S. A. Johnson, T. McNeill, C. E. Finch, Andrus Gerontology Center, University of Southern California, Los Angeles, CA 90089.
B. Cordell, California Biotechnology, 2450 Bayshore Parkway, Mountain View, CA 94043.