Biosynthesis of a Novel Transformation-sensitive Heat-shock Protein That Binds to Collagen

REGULATION BY mRNA LEVELS AND IN VITRO SYNTHESIS OF A FUNCTIONAL PRECURSOR*

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The synthesis of a major collagen-binding glycoprotein of molecular weight 47,000 was previously shown to be altered by malignant transformation as well as by heat shock in chick embryo fibroblasts (Nagata, K., and Yamada, K. M. (1986) J. Biol. Chem. 261, 7531–7536 and Nagata, K., Saga, S., and Yamada, K. M. (1986) J. Cell Biol. 103, 223–229). In this paper, we examined the synthesis of this heat shock protein (hsp47) in terms of possible functional precursors and its regulation after heat shock and transformation by Rous sarcoma virus. Actinomycin D inhibited the induction of hsp47 after heat shock. Messenger RNAs purified from chick embryo fibroblasts (CEF), heat-treated CEF, and transformed CEF were analyzed in an in vitro translation system. In vitro translated products readily bound to gelatin-Sepharose, and levels were increased after heat shock and decreased after transformation. The increase in mRNA after heat shock was shown more directly by Northern assay using a synthetic oligonucleotide probe. We identified two putative precursors of hsp47 using an in vitro translation/processing system and tunicamycin: one is a 42-kDa primary translation product and the second is a 41-kDa polypeptide lacking signal peptide and carbohydrate moieties. Both of these precursors are biologically active as determined by gelatin-binding activity, in contrast to the lack of binding activity of precursors in several other membrane-associated receptor systems.

A collagen-binding membrane glycoprotein of M, = 47,000 has been found to be a novel transformation-sensitive heat-shock protein (1). The synthesis of this heat-shock protein (hsp47) is characteristically decreased in chick embryo fibroblasts (CEF)1 transformed by Rous sarcoma virus (RSV), although the degree of phosphorylation of hsp47 is increased after transformation (2). Hsp47 is also reported to be increased markedly during the differentiation of mouse F9 teratocarcinoma cells after the addition of retinoic acid and dibutyryl cyclic AMP (3).

Hsp47 is the only heat-shock protein described to date with the ability to bind to extracellular matrix proteins. This protein binds to gelatin (denatured types I and III) and to native collagen types I and IV (2, 3). Hsp47 has been found to be localized in the endoplasmic reticulum of fibroblasts by immunolocalization studies (4), a conclusion that is supported by the distinctive structure of its carbohydrate moieties (5).

Regulation of the induction of heat-shock proteins (hsp) has been examined in a variety of cells, particularly from Drosophila (6). Considerable evidence suggests that the induction of hsp is regulated at the transcriptional level in Drosophila cells and in many eukaryotic cells, although translational control or altered splicing of pre-mRNA into mature mRNA is also reportedly involved in heat-shock regulation (7, 8).

In this paper, we report that the induction of hsp47 by heat shock is regulated by control of mRNA levels and that the decrease of hsp47 synthesis in transformed CEF is probably also transcriptionally regulated. In addition, it was found that hsp47 is initially synthesized as a molecule of M, = 42,000, which is decreased to 41,000 after processing and then rises to 47,000 after glycosylation in the mature molecule. Both putative precursors and the final product display collagen-binding activity, in contrast to a variety of other membrane-associated binding molecules that appear to require biosynthetic processing to display functional activity.

EXPERIMENTAL PROCEDURES

Chemicals and Reagents—Gelatin-Sepharose 4B was purchased from Pharmacia LKB Biotechnology Inc. Amphoteric carrier ampholytes of pH range 3.5–10 were obtained from LKB. [35S]Methionine (specific activity 1100 Ci/mol) and in vitro translation/processing kit NEK-019 were from Du Point-New England Nuclear. Nonidet P-40 was obtained from Gellard/Schlesinger or Bethesda Research Laboratories and dithiothreitol from Behring Diagnostics or Wako Junyaku (Tokyo, Japan). Tunicamycin was purchased from Sigma and actinomycin D was from Pharmacia LKB Biotechnology Inc. Other reagents were obtained from Sigma, Bethesda Research Laboratories, Behring Diagnostics, Bio-Rad, or Wako Junyaku and were of the highest purity available from each company.

Cell Culture and Metabolic Labeling—CEF were maintained in Vogt’s GM medium (9), passed using 0.05% trypsin, 0.02% EDTA (GIBCO), and used for experiments between passages 3 and 6. Subconfluent cultures of approximately 6–10 X 10^6 cells/35-mm dish were labeled for 1 h with 0.1 mCi/ml [35S]methionine, and cell lysates were prepared as described previously (2).

Preparation of mRNA—RNA was prepared from CEF, heat-shocked CEF, and CEF transformed with Rous sarcoma virus (Schmidt-Ruppin strain) by the protocol of Adams et al. (10). After washing once with cold PBS, cells were harvested by trypsinization,

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‡ The abbreviations used are: CEF, chick embryo fibroblasts; SDS, sodium dodecyl sulfate; RSV, rous sarcoma virus; hsp, heat-shock protein; grp, glucose-related proteins; SDS, sodium dodecyl sulfate.
was collected by centrifugation for 25 min at 5,000 rpm. The pellet was reprecipitated twice. The RNA was dissolved in water and poly(A)+ RNA was separated from total RNA by two cycles of oligo(dT) cellulose column chromatography.

In Vitro Translation and Processing—Equal amounts of mRNA determined spectrophotometrically at 260 nm were added to the reaction mixture for in vitro translation using rabbit reticulocyte lysates following the manufacturer's recommendations (Du Pont-New England Nuclear). The translated products, which were labeled with [35S]methionine, were applied to gelatin-Sepharose to affinity-purify hsp47 as described previously (2). The gelatin-bound proteins were assayed by SDS-polyacrylamide gel electrophoresis. Where indicated, a canine pancreatic microsomal membrane fraction was added to the in vitro translation reaction mixture as recommended by the company to analyze in vitro processing.

Northern Blotting Assay—Oligonucleotides were labeled by the method of Wallace et al. (11). Labeled oligonucleotides were separated from unreacted [γ-32P]ATP by chromatography on a Whatman DE52 cellulose column. Unbound fractions were pooled and used directly in hybridization experiments.

Poly(A)+ RNAs were separated on 1% agarose gels containing formaldehyde and transferred to nylon membrane filters (GeneScreen Plus, Du Pont-New England Nuclear) as described in the commercial manual. Blotted filters were prehybridized overnight at 42 °C in 5 × SSC (1 × SSC = 0.15 M NaCl, 15 mM sodium citrate, pH 7.0) containing 50% formamide, 0.04% polyvinylpyrrolidone, 0.04% bovine serum albumin, 0.04% Ficoll, 1% SDS, and 100 μg/ml denatured salmon sperm DNA.

An oligonucleotide probe for hsp47 was synthesized based on data from amino-terminal amino acid sequencing (further sequences to be published elsewhere) using a Model 120A gas-phase protein synthesizer (Applied Biosystems, Foster City, CA). The amino acid sequence used for the probe was Met-Ala-Lys-Asp-Lys-Asn-Met, using β-cyanoethyl diisopropyl phosphoramidites (American Bionetics).

Filters were then hybridized with 5'-32P-end-labeled oligonucleotide (2 × 10⁶ cpm/ml) at 42 °C for 16–18 h in 6 × NET, 1 × Denhardt's solution (1 × NET is 0.15 M NaCl, 1 mM EDTA, 0.05 M Tris, pH 8.0, and 1 × Denhardt's solution is 0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.02% bovine serum albumin). The specific activity of the probe was >10⁵ cpm/pmol. The filters were washed several times with 5 × SSC at room temperature and then once with 6 × SSC at 65 °C for 5 min. The filters were exposed at −80°C for 24 h using Fuji RX-O film with Grenex 8 intensifying screens.

Gel Electrophoresis—One-dimensional SDS-polyacrylamide gel electrophoresis was performed according to the methods of Laemmli using 10% resolving gels or 8–13% gradient gels (12). Prestained molecular weight markers (Bethesda Research Laboratories) consisted of myosin heavy chain (Mr = 200,000), phosphorylase b (Mr = 97,400), bovine serum albumin (Mr = 68,000), ovalbumin (Mr = 43,000), α-chymotrypsinogen (Mr = 25,700), and β-lactoglobulin (Mr = 18,400). The slab gel was treated with Autofluor (National Diagnostics) before drying, and then fluorography of the dried gel was performed with Kodak X-OMat film or Fuji RX-O-H film.

Two-dimensional gel electrophoresis was performed with non-equilibrium pH gradient electrophoresis as the first dimension, using pH 3.5–10 ampholytes, with the second dimension consisting of a 10% polyacrylamide SDS slab gel (13).

RESULTS

Effect of Actinomycin D on the Induction of hsp47—CEF cultures were heat-treated at 45 °C for 3 h and then labeled with [35S]methionine for 1 h. Besides three major heat-shock proteins (hsp90, hsp70, and hsp25), a band with a molecular size of 47,000 daltons (hsp47) was also clearly induced by heat shock as shown by one-dimensional gradient gel electrophoresis (compare Fig. 1, lanes 1 and 3). Fig. 2 shows the incorporation of [35S]methionine into trichloroacetic acid-insoluble fractions of cell lysates. Although total protein synthesis was drastically inhibited after 3 h at 45 °C, the induction of heat-shock proteins compensated for the reduction in protein synthesis (Fig. 1, lane 3), resulting in only a slight decrease in the incorporation of total radioactivity into the trichloroacetic acid-insoluble fraction (Fig. 2).

![Fig. 1. Effect of actinomycin D on the induction of hsp47. Actinomycin D (2 μg/ml) was added 30 min before and during heat treatment of CEF at 45 °C for 3 h. After heat shock, cultures were labeled with 0.1 mCi/ml [35S]methionine-free Dulbecco's modified Eagle's minimal essential medium at 37 °C for 1 h. Aliquots of cell extracts containing equal amounts of trichloroacetic acid-precipitable radioactivity were analyzed by SDS-gel electrophoresis (8–15% polyacrylamide). Lanes 1 and 2, nontreated CEF with (lane 2) or without (lane 1) actinomycin D and lanes 3 and 4, heat-treated CEF with (lane 4) or without (lane 3) actinomycin D. The positions of prestained molecular weight markers (Bethesda Research Laboratories) are shown in the right-hand margin.](image1)

![Fig. 2. Effect of actinomycin D on total incorporation of [35S]methionine into the trichloroacetic acid-precipitable fraction of cell extracts. CEF cultures were heat-treated with or without actinomycin D as described in the legend for Fig. 1. Radioactivity in trichloroacetic acid-precipitable material collected on glass microfiber filters (GF/C, Whatman) was determined in a liquid scintillation counter (LKB Rack-Beta 1215 scintillation spectrometer); values are the average of closely agreeing duplicate determinations.](image2)
Actinomycin D was added to the CEF 30 min prior to and during heat shock in order to examine whether the induction of hsp47 required newly synthesized mRNA. Although actinomycin D at a concentration of 2 μg/ml (and even at 20 μg/ml; data not shown) inhibited total protein synthesis only slightly at 37°C, an equal concentration of actinomycin D inhibited total protein synthesis of the cells treated at 45°C to one-third (Fig. 2). Actinomycin D (2 μg/ml) added to cells prior to heat shock inhibited the induction of all three major heat-shock proteins almost completely (Fig. 1, lane 4). The induction of hsp47 by heat shock was also inhibited to basal levels (Fig. 1, lane 4). This result suggests transcriptional regulation of the expression of hsp47, as reported for other major heat-shock proteins (22).

In Vitro Translation of mRNA—We prepared RNAs from CEF, heat-treated CEF, and RSV-transformed CEF, and purified poly(A)+ RNAs by two cycles of oligo(dT)-cellulose chromatography. Equal amounts of poly(A)+ RNA determined spectrophotometrically were translated in vitro into polypeptides labeled with [35S]methionine using a rabbit reticulocyte translation system. The products of in vitro translation were applied directly to gelatin-Sepharose columns to affinity-purify functional hsp47 precursor(s).

The molecular size of the in vitro translation product purified by gelatin-Sepharose appeared smaller than that of native hsp47, with a size of approximately 42,000 daltons. Fig. 3 shows that quantities of this gelatin-binding protein precursor translated in vitro were increased after heat shock at 42°C for 4 h and that a further increase was observed when the mRNA was isolated from cells treated at 45°C for 4 h. Conversely, the 42-kDa band translated from mRNA from cells transformed by RSV was significantly fainter than that of control normal counterparts (compare lane 4 with lane 1 in Fig. 3).

Tunicamycin is known to inhibit N-linked glycosylation. The hsp47 of cells treated with tunicamycin followed by labeling with [35S]methionine migrated more rapidly than that of nontreated cells according to SDS-polyacrylamide gel electrophoresis (Fig. 3, lanes 6 and 7). The apparent molecular size of tunicamycin-treated hsp47 was about 41,000 daltons. To verify that the 42-kDa band purified by gelatin-Sepharose from the product translated in vitro was the precursor of hsp47, we analyzed the translation products by two-dimensional gel electrophoresis. The 42,000-dalton product migrated with the same isoelectric point as hsp47, but was slightly smaller (Fig. 4A). Isolated 42-kDa protein purified from the in vitro translation mixture by gelatin-Sepharose copurified with hsp47 precursor(s).

Quantities of the translated 42-kDa precursor of hsp47 were substantially increased after heat shock (Fig. 4B). Moreover, this polypeptide was decreased in cells transformed by RSV (Fig. 4C). These results indicate alterations in levels of translatable mRNA for hsp47 after heat shock and transformation.

Northern Analysis of mRNA—The expression of hsp47 by heat shock was also shown to be regulated by levels of mRNA by performing Northern blot analysis. Poly(A)+ RNA, the amounts of which were adjusted by determining absorbance at 260 nm, was subjected to agarose gel electrophoresis, blotted onto nylon membrane filter, and hybridized with a synthetic oligonucleotide probe. After the filter was washed at 65°C for 5 min as described under "Experimental Procedures," only one band was found to hybridize with the probe. The amount of mRNA was clearly increased after heat shock at 45°C for 4 h (Fig. 5), which is again consistent with transcriptional regulation of hsp47. The estimated size of the message was approximately 4.5 kilobases. Since the 5′-untranslated region of this message is 600 bases in length according to primer extension experiments (data not shown), the 3′ non-coding region of this mRNA appears to be about 2.5 kilobases in length.

Analysis of the Precursors of hsp47—To clarify the differences in apparent molecular size between the tunicamycin-treated hsp47 and its in vitro translated precursor, we used 8–15% gradient polyacrylamide SDS slab gel electrophoresis (in place of 10% gels). A clear difference was still observed between the two bands (Fig. 6, lanes 2 and 3). Lane 5 in Fig. 6 consisted of a mixture of three proteins: mature hsp47 which was purified from cells labeled in culture with [35S]methionine, hsp47 from cells treated with tunicamycin, and in vitro translated precursor; the size differences between these species were still found in this mixing experiment.

The approximate molecular sizes of the tunicamycin-treated and in vitro translated hsp47 precursor were 41,000 and 42,000 daltons, respectively. Fibronectin was the other protein copurified with hsp47 by gelatin-Sepharose chromatography. The molecular size of fibronectin purified from tunicamycin-treated cells was also less than that of nontreated cells (Fig. 6, lanes 1 and 2).

We added a microsomal membrane fraction to the in vitro translation system to obtain in vitro processing of the translated products. As shown in Fig. 6, lane 4, the band of the in vitro translated product after in vitro processing coincided exactly with that obtained after tunicamycin treatment. The difference in molecular size caused by processing was also apparent after mixing the two species before and after proc.
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FIG. 4. Two-dimensional nonequilibrium pH gradient gel electrophoresis (NEPHGE) of in vitro translated products. Labeled products translated in vitro from equal amounts of mRNAs were analyzed by two-dimensional gel electrophoresis consisting of nonequilibrium pH gradient electrophoresis as the first dimension (right to left) and SDS-10% polyacrylamide slab gel electrophoresis as the second dimension (top to bottom). A, nontreated CEF; (B) CEF treated at 45°C for 4 h; (C) RSV-transformed CEF. Arrowheads indicate the in vitro translated precursors of hsp47. Open circle in A indicates the position where mature hsp47 molecules are detected (see Refs. 1 and 2).

Fig. 5. Northern blot analysis. Total RNA was isolated from CEF (1) and from CEF-treated at 45°C for 4 h (2), and mRNA was purified by two cycles of oligo(dT)-cellulose column chromatography. mRNA was fractionated in a 1% agarose-formaldehyde gel, transferred to a nylon membrane filter, and hybridized with the synthetic oligonucleotide probe labeled with [γ-32P]ATP.

DISCUSSION

In a previous study (1), we reported that a membrane glycoprotein of Mr = 47,000 daltons is a novel heat shock protein in CEF. This heat-shock protein (hsp47) has unique characteristics in that (a) it is one of only two major collagen-binding proteins in detergent extracts of CEF, the other of which is fibronectin, (b) the synthesis of hsp47 is decreased after RSV transformation of fibroblasts, and (c) the phosphorylation of hsp47 is increased after transformation (2). HSP47 binds to type I and type IV collagen and also to gelatin (denatured types I and III collagen) (2, 3).

The major new findings in this study are (a) in vitro translation of CEF mRNA produces a functionally active collagen-binding hsp47 precursor; (b) both microsomal processing of the primary translation product and tunicamycin

Fig. 6. Analysis for functional hsp47. CEF cultures were labeled with 0.1 mCi/ml [35S]methionine for 1 h in the presence (lane 2) or absence (lane 1) of 2 μg/ml tunicamycin. Aliquots of cell extracts containing equal amounts of trichloroacetic acid-precipitable radioactivity were analyzed on an SDS-gradient polyacrylamide slab gel (8-15%). In vitro translation was performed for mRNAs isolated and purified from CEF in the presence (lane 4) or absence (lane 3) of a microsomal membrane fraction. Lane 5 is the mixture of samples applied to lanes 1-3, and lane 6 is the mixture of samples applied to lanes 3 and 4.
inhibition of N-linked glycosylation produce a slightly smaller product that is also functional; (c) actinomycin D blocks the induction of hsp47 after heat shock; (d) levels of translatable mRNA for hsp47 are increased after heat shock and are also decreased after transformation, and (e) Northern blot analysis further indicates that regulation occurs at the level of mRNA accumulation after heat treatment.

In this system, precursor molecules appear to be functionally active without a requirement for processing, at least as determined by their ability to bind to collagen. This finding contrasts with such a requirement for several other membrane-associated receptors or binding proteins. For example, the mammalian fibronectin receptor requires a lengthy maturation period spanning hours that appears to involve processing of an asparagine-linked oligosaccharide moiety (14). The receptors for insulin and epidermal growth factor also require processing, which for the former receptor was shown to involve a conformational alteration (15–17). Tunicamycin treatment blocks the function of a variety of other receptors, including for low density lipoprotein, IgM, IgE, and acetylcholine (reviewed in Ref. 18). Thus, the fact that hsp47 can bind readily to gelatin after in vitro translation or after inhibition of N-linked glycosylation by tunicamycin indicates that binding activity resides in its primary structure, without any requirement for the processing needed for function of a number of other membrane-associated binding proteins.

In many prokaryotic and eukaryotic cells, transcriptional regulation is proposed to be the major control mechanisms for the expression of hsps (6, 19, 20), although translational control and other mechanisms have also been reported (7, 8, 21). The induction of expression of hsp47 by heat shock is probably also regulated at the level of transcription, as suggested by three findings in this paper: the inhibition of hsp47 expression by the addition of actinomycin D, the increase in hsp47 precursor translated in vitro from mRNA purified from heat-treated cells, and finally the Northern blot demonstration of an increase in hsp47 mRNA after heat shock. Kelley et al. (22) reported that mRNA from chick cells treated at 45 °C produced proteins of ~15, 18, 33, 45, and 55 kDa in addition to the three major heat-shock proteins in an in vitro translation system. It is not clear whether the 41-kDa precursor of hsp47 we find corresponds to Kelley’s 45-kDa band because of the lack of other information such as PI values and binding characteristics, as well as differences in cell type (passage number).

In this study, the precursor of hsp47 translated in vitro was also shown to be decreased in transformed cells when equal amounts of mRNA from normal and transformed cells were added to the translation system, again indicating a change in translatable mRNA levels responsible for altered biosynthesis. Kurkinen et al. (3) reported that hsp47, which they term “colligin,” was induced in the F9 teratocarcinoma cell line after the cells differentiated into parietal endoderm cells following the addition of retinoic acid and dibutyryl cyclic AMP. The expression of hsp47 is thus regulated in three independent events, that is, heat shock, transformation, and differentiation. It will eventually be interesting to compare regulatory mechanisms, for example, whether the heat-shock transcription factor discovered for hsp70 (23, 24), is involved in these three events. Differences in time courses of induction of hsp47 and other hsps suggest the possibility of independent regulation (2). It will also be useful to examine whether the hsp47 gene has the same consensus sequences as other hsps (25, 26), since Schlesinger has defined the hps by two criteria: the presence of characteristic consensus sequences in the promoter region of each hsp gene as well as the ability to be induced by heat shock (27). We are presently attempting to clone cDNAs for hsp47.

We also established two putative precursors for hsp47 in this paper. In vitro translation produces a 42-kDa polypeptide that can bind to gelatin-Sepharose. The 42-kDa polypeptide appears to be the precursor of hsp47, because (a) the only two major collagen-binding molecules at physiological salt concentrations are hsp47 and fibronectins (250 kDa), (b) the isoelectric point of the 42-kDa polypeptide was identical to hsp47, (c) mRNA from cells heat-shocked at 42 or 45 °C synthesized much larger amounts of 42-kDa polypeptide than that from nontreated cells in the in vitro translation system, and (d) mRNA of cells transformed with RSV produced lower amounts of 42-kDa polypeptide. In contrast, Kurkinen et al. (3) reported that no proteins synthesized in vitro using parietal endoderm mRNA could be bound to gelatin-Sepharose. This discrepancy may be due to differences in methods or cell types.

The second putative precursor of hsp47 is the 41-kDa polypeptide, which is detected after processing of in vitro translated polypeptides, as well as cells treated with tunicamycin. The difference in apparent molecular size between the 42- and 41-kDa polypeptides suggests the presence of a signal peptide at the amino terminus of hsp47, because the addition of a microsomal membrane fraction containing signal peptidase to the reaction mixture clearly reduced the size of the in vitro translated gelatin-binding polypeptide to a species comigrating with nonglycosylated hsp47 precursor from tunicamycin-treated cells. Kurkinen et al. (3) reported that colligin has two glycosylation sites on the protein, as evidenced by their experiments using different concentrations of tunicamycin; these oligosaccharides are N-linked moieties characteristic of proteins of the endoplasmic reticulum (5). Fig. 7 summarizes a tentative scheme for the synthesis and processing of hsp47.

The function of hsp47 besides collagen-binding is uncertain, but it displays interesting characteristics. Polyclonal and monoclonal antibodies against hsp47 reveal localization of this protein to the endoplasmic reticulum (4). Moreover, binding of hsp47 to gelatin is disrupted by pH ≤ 6.3 (4). It is of interest that similar or even lower pH values are characteristic for certain vesicular secretory and endocytic compartments of cells involved in protein processing or translocation (reviewed in Ref. 28). Although the physiological function of hsp47 is unclear at present, this property of pH dependence could permit the regulated release of bound ligands from membrane-associated hsp47 by a simple local reduction in pH.
pH, as is thought to occur in some step(s) of protein and receptor sorting.

Glucose-regulated proteins (grps) have also been classified as members of the stress-protein family (29, 30); grp78 is also known to be localized to endoplasmic reticulum (29), and grp84 is in endoplasmic reticulum and on the cell surface (31). Recently, the tetrapeptide sequence Lys-Asp-Glu-Leu (KDEL), located at the carboxyl terminus, was shown to be important for protein localization in the endoplasmic reticulum; grp78 was also shown to have a KDEL sequence at the carboxyl terminus (32). It will thus be interesting to determine in the future whether hsp47 contains an identical tetrapeptide sequence.

Recently, binding functions have been discovered for other heat-shock proteins. Hsp70 enters the nucleus and is concentrated in nucleoli after heat shock, and it has been shown to bind to preribosomes (33). Heat-shock cognate protein binds to nascent heavy chains lacking light chain sequence.

Grp78, also a member of the hsp70 family, is identical with pp60c-src which is the src-gene product of Rous sarcoma virus (37, 38). Hsp90 also binds to receptors for glucocorticoids (39). Hsp90 and hsp100 bind to actin filaments to cause gelation of actin solutions, and the binding of hsp90, but not hsp100, to actin filaments is regulated by calcium and calmodulin (40, 41). Hsp47, therefore, adds a new category of binding properties to the heat-shock protein family with a function expressed even by its precursors.

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REFERENCES