The Constitutive and Stress Inducible Forms of hsp 70 Exhibit Functional Similarities and Interact with One Another in an ATP-dependent Fashion

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Abstract. Mammalian cells constitutively express a cytosolic and nuclear form of heat shock protein (hsp) 70, referred to here as hsp 73. In response to heat shock or other metabolic insults, increased expression of another cytosolic and nuclear form of hsp 70, hsp 72, is observed. The constitutively expressed hsp 73, and stress-inducible hsp 72, are highly related proteins. Still unclear, however, is exactly why most eukaryotic cells, in contrast to prokaryotic cells, express a novel form of hsp 70 (i.e., hsp 72) after experiencing stress. To address this question, we prepared antibodies specific to either hsp 72 or hsp 73 and have compared a number of biological properties of the two proteins, both in vivo and in vitro. Using metabolic pulse-chase labeling and immunoprecipitation analysis, both the hsp 72 and hsp 73 specific antibodies were found to coprecipitate a significant number of newly synthesized proteins. Such interactions appeared transient and sensitive to ATP. Consequently, we suspect that both hsp 72 and hsp 73 function as molecular chaperones, interacting transiently with nascent polypeptides. During the course of these studies, we routinely observed that antibodies specific to hsp 73 resulted in the coprecipitation of hsp 72. Similarly, antibodies specific to hsp 72 were capable of coprecipitating hsp 73. Using a number of different approaches, we show that the constitutively expressed, pre-existing hsp 73 rapidly forms a stable complex with the newly synthesized stress inducible hsp 72. As is demonstrated by double-label indirect immunofluorescence, both proteins exhibit a coincident locale within the cell. Moreover, injection of antibodies specific to hsp 73 into living cells effectively blocks the ability of both hsp 73 and hsp 72 to redistribute from the cytoplasm into the nucleus and nucleolus after heat shock. These results are discussed as they relate to the possible structure and function of the constitutive (hsp 73) and highly stress inducible (hsp 72) forms of hsp 70, both within the normal cell as well as in the cell experiencing stress.

Members of the heat shock (stress) protein (hsp) family are being implicated as essential components of protein maturation. So far, two major classes of stress proteins have been shown to participate as so-called “molecular chaperones,” facilitating the early events of protein synthesis, protein folding, and perhaps higher ordered protein assembly (Ellis and Hemmingsen, 1989; Ellis and van der Vies, 1991). Members of the hsp 70 family, distributed throughout various intracellular compartments, interact with nascent polypeptides either undergoing synthesis on the ribosome, or being translocated into subcellular organelles (Haas and Wabl, 1983; Bole et al., 1986; Gething et al., 1986; Chirico et al., 1988; Deshaies et al., 1988; Kang et al., 1990; Beckmann et al., 1990; Mizzen et al., 1991; Welch, 1992). These results have prompted the suggestion that this interaction with a member of the hsp 70 family acts to slow or retard the folding of the nascent polypeptide until its synthesis or translocation event has been completed. Members of the hsp 60 (or groEL/ES) family also have been shown to interact with newly synthesized proteins (Bochkanova et al., 1988; Hemmingsen et al., 1988; Osterman et al., 1989; Martin et al., 1991; Trent et al., 1991; Mendoza et al., 1991). In contrast to the hsp 70 family however, members of the hsp 60 family are believed to function by actually catalyzing the early events of protein folding and perhaps protein assembly. It is likely that members of these two families of stress proteins participate in protein maturation events in a step-wise fashion. For example, newly synthesized and unfolded proteins complexed with a member of the hsp 70 family may be “handed off” to a member of the hsp 60 family where folding and/or assembly of the target polypeptide commences (Kang et al., 1990; Mizzen et al., 1991; Langer et al., 1992).

Most members of the hsp 70 family are constitutively expressed and reside within different intracellular compartments (for a review see Welch, 1990). These include: (a) the cytosolic and nuclear hsp 73; (b) grp 78 (or Bip), present...
within the lumen of the endoplasmic reticulum; and (c) grp 75, a component of the mitochondrial (or chloroplast) matrix. Whenever cells are subjected to metabolic stress (e.g., heat shock) another member of the hsp 70 family, hsp 72, is expressed. hsp 72 is highly related to hsp 73 (>90% sequence identity), and both proteins are observed to co-purify with one another (Welch and Feramisco, 1982). An unresolved question concerns why most eukaryotic cells, unlike prokaryotic cells, express a novel form of hsp 70 (i.e., hsp 72) after experiencing metabolic stress. Some investigators have suggested that the high level expression and accumulation of hsp 72 is a necessary requirement for the cell acquiring increased protection from a particular metabolic insult (Li and Werb, 1982; Li et al., 1982). In the present study, we have begun to address this key question using a number of approaches in vivo and in vitro. In addition to their transient interactions with nascent polypeptides, we find that constitutively expressed hsp 73 rapidly forms a stable complex with the highly inducible hsp 72 in cells following heat shock. Interestingly, similar to their interactions with newly synthesized proteins, the interaction of hsp 73 with hsp 72 appears to be regulated by ATP. Using both double-label indirect immunofluorescence and microinjection methods, we show that hsp 73 and hsp 72 move together in the cell experiencing stress.

Materials and Methods

Cell Culturing and Metabolic Labeling

Cells used in all experiments were maintained at 37°C in DME supplemented with 10% bovine calf serum. For steady state labeling, the cells were incubated with a mixture of [35S]methionine and [35S]cysteine (ICN, “ translating” specific activity 1,120 Ci/mMol) in medium consisting of 95% methionine-free DME, 5% complete DME, and 5% bovine calf serum. For pulse-chase experiments, cells were labeled as described both previously and in the figure legends (Beckmann et al., 1992).

Antibody Preparation

A mAb (C92) specific for the inducible form of hsp 70 (i.e., hsp 72), as well as a mAb (N27) which recognizes both hsp 72 and hsp 73, have been characterized and described previously (Welch and Suhlan, 1986; Riabowol et al., 1988). A polyclonal antibody specific for the constitutive form of hsp 70, (hsp 73) was produced in rabbits immunized with a 20- amino acid synthetic peptide derived from the carboxy-terminal region of hsp 73 (see Fig. 1) and covalently linked to ovalbumin.

Protein Purification, Immunoprecipitation Analysis, and Gel Electrophoresis

For the isolation of individual forms of hsp 70: (a) bovine brains were used for the purification of hsp 73; (b) human 293 cells were subjected to heat shock and further incubated at 37°C for 12 h and used to isolate hsp 72 and hsp 73. In each case, the proteins were purified by ion-exchange chromatography followed by affinity chromatography on ATP agarose columns (Welch and Feramisco, 1985). After their purification, the proteins were concentrated by NH4SO4 precipitation and extensively dialyzed to remove residual ATP.

Immunoprecipitation using the hsp 72 or hsp 73 antibodies was performed using the monoclonal C92 antibody (specific for hsp 72, diluted 1/1,000) and the mouse monoclonal N27 antibody (specific for hsp 72, diluted 1/800) was done as described previously (Riabowol et al., 1988). For gel filtration, Hela cells, steady state labeled with [35S]methionine, were lysed by the addition of 0.1% Triton X-100 in PBS supplemented with 5 mM MgCl2 and clarified by centrifugation at 16,000 g for 15 min at 4°C. The resultant supernatant was applied to a sepharose 6B column (100 × 1.2 cm) and the column developed in Buffer B (20 mM Tris, pH 7.4, 20 mM NaCl, 0.1 mM EDTA, 5 mM β-mercapto ethanol [BME]). Proteins eluting off the column were analyzed by SDS-PAGE and by Western blotting using the N27 antibodies. Analysis of purified proteins by HPLC was performed using a Zorbax SE-250 column (9.4 × 250 mm; Dionex Corp., Sunnyvale, CA). Purified proteins, solubilized in PBS (1 mg/ml), were injected into the column and the column developed in PBS, either in the absence or presence of 5 mM MgATP. The position of the proteins eluting off the column were monitored by absorbance at 280 nm. For in vitro transcription, capped messenger RNA encoding human hsp 72 was synthesized via transcription of a plasmid, pSP64T (Krieg and Melton, 1984), containing a bacteriophage SP6 promoter, the 5' untranslated region of X. laevis β-globin, the cDNA for human HSP72 (Hunt and Morimoto, 1983) and the 3' untranslated region of β-globin under conditions previously described (Hansen et al., 1986). Rabbit reticulocyte lysate treated with micrococcal nuclease (Jackson and Hunt, 1983) was programmed with hsp 72 mRNA and incubated at 24°C for 60 min. Initiation of further protein synthesis was blocked by the addition of 7-methyl-guanosine monophosphate and edeine (final concentrations of 4 mM and 10 μM, respectively). Polypeptide elongation was allowed to proceed for an additional 15 min to ensure that all of the hsp 72 polypeptide chains had been completed. To 10 μl of in vitro translation were added either 50 μl PBS Mg containing 12 mM MgATP, 48 mM creatine phosphate and 50 μg/ml creatine phosphokinase (ATP supplemented) or 60 μl of aseptic ATP (ATP depleted). After 10 min at 24°C, native immunoprecipitations were performed using either pre-immune rabbit serum or the rabbit hsp 73 antibody, as described above.

Results

Characterization of Antibodies Specific for Hsp 73 and Hsp 72

As a first step toward characterizing similarities and/or differences between the constitutive (hsp 73) and stress-
inducible (hsp 72) forms of cytosolic/nuclear hsp 70, antibodies specific to each protein were prepared. As we have described previously, a mouse mAb (C92) was prepared and shown to be specific for hsp 72 (Welch and Suh, 1986). For the preparation of the hsp 73 specific antibody, a synthetic peptide corresponding to amino acids 650–670 of hsp 73 (near the carboxy terminus) was prepared. As is shown in Fig. 1, this region of hsp 73 exhibits a number of sequence differences as compared with that of hsp 72. A rabbit polyclonal antibody was prepared and characterized using two different cell lines: human Hela cells and the rodent line, BHK cells. Previous studies have shown that Hela cells (like those of most primate cell lines) constitutively express hsp 73 and, to a lesser degree, hsp 72 when grown under normal conditions (Welch et al., 1983; Welch and Feramisco, 1984). BHK cells, (like most rodent cell lines), express hsp 73, but not hsp 72 when grown at 37°C. After heat shock treatment, both cell lines synthesize the stress inducible hsp 72 at relatively high levels for many hours, even after their return to 37°C. As is shown in Fig. 2, when cells are lysed under denaturing conditions, the mouse C92 antibody immunoprecipitated only the faster migrating hsp 72 species, while the rabbit peptide polyclonal antibody appeared specific for the relatively slower migrating hsp 73. To more definitively identify the antigens being recognized, the immunoprecipitation products obtained from the Hela cells were analyzed by two-dimensional gel electrophoresis (Fig. 2, C and D). The rabbit peptide antibody recognized the more acidic hsp 73 isoforms while the C92 antibody specifically immunoprecipitated most all of the more basic isoforms of hsp 72.

Using metabolic pulse-chase radiolabeling and immunoprecipitation under native-like conditions, we previously reported a transient interaction of hsp72/hsp73 with newly synthesized proteins. Now having antibodies specific to either hsp 73 or hsp 72 we investigated which of the two proteins (or both) were interacting with newly synthesized proteins. Three cell lines were chosen for the analysis owing to their differences in the relative expression of hsp 72 and hsp 73 at 37°C: Hela cells (which express high levels of hsp 73 and slightly lower levels of hsp 72); BHK cells (which express only hsp 73); and human 293 cells (which express appreciable amounts of both hsp 73 and hsp 72) (Fig. 3 A).

Each of the three different cell lines were labeled with [35S]methionine for 15 min and then harvested immediately (i.e., pulsed) or alternatively, were labeled for 15 min and then further incubated in the absence of radiolabel for an additional 60 min (i.e., pulse chased). The cell lysates then were examined by immunoprecipitation using: (a) the hsp 72 specific antibody; (b) the hsp 73 specific antibody; or (c) a mixture of mAbs which recognize both hsp 73 and hsp 72. As was observed previously, when using the mAb mixture a significant amount of newly synthesized proteins were found to coprecipitate with hsp 73 and hsp 72 in either the Hela (Fig. 3 B), BHK (Fig. 3 C) or 293 (Fig. 3 D) cells (Fig. 3, lanes 4). After a 60-min chase period however, significantly fewer proteins were observed to coprecipitate with hsp 73 and hsp 72 (Fig. 3, lanes 8). Similarly, when using

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\text{NH}_2\text{CGPGMPGGFPGGAPPGGA}SK\text{S-COOH (hsp 72)} \\
\text{GPGGFAGQPKGSQXXXS-COOH (hsp 73)}
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**Figure 1.** Sequence of the hsp 73 synthetic peptide used to raise the hsp 73 specific rabbit polyclonal antibody. Shown below is the sequence of hsp 73 used to generate a synthetic peptide for subsequent conjugation to ovalbumin and immunization. Also shown is the corresponding region of hsp 72. The hsp 73 sequence shown represents amino acids 650–670. Amino acids underlined at the NH2 and COOH termini represent residues that were included in the synthetic peptide for water solubility and cross-linking purposes.

**Figure 2.** Characterization of antibodies specific for hsp 72 or hsp 73. Hela cells growing at 37°C, or subjected to a 43°C/90 min heat shock treatment and then returned to 37°C, were labeled with [35S]methionine for 6 h. Cells were harvested in Laemmli sample buffer containing 1% SDS, heated at 100°C, and the cell lysates used for denaturing immunoprecipitation analysis using antibodies raised against the hsp 73 peptide, or a mouse mAb (C92) specific for hsp 72. Shown are the immunoprecipitation products from the Hela cells (A) and BHK cells (B). Lanes 1 and 2 represent the proteins present in the lysates of cells grown at 37°C (lane 1) or after heat shock (lane 2). Lanes 3–5 and 6–8 represent the immunoprecipitation products from the 37°C and the heat-shock treated cell lysates, respectively. Lanes 3 and 6, no first antibody; lanes 4 and 7, hsp 72 antibody; and lanes 5 and 8, hsp 73 antibody. To further confirm the specificities of these antibodies, some of the immunoprecipitation products were analyzed by two-dimensional gel electrophoresis. Shown in C are the proteins present in the 37°C Hela cell lysate (upper panel), and the corresponding hsp 73 immunoprecipitation product (lower panel). Shown in D are the proteins present in the heat-shock treated Hela cell lysate (upper panel), and the corresponding hsp 72 immunoprecipitation product (lower panel). The positions of hsp 73 isoforms are indicated by a downward pointing arrow, and the positions of the hsp 72 isoforms are indicated by an upward pointing arrow.
Hsp 72 and hsp 73 interact with newly synthesized proteins. Hela, BHK, and 293 cells growing on 35-mm dishes at 37°C were labeled with [35S]methionine for 15 min. Some of the pulse-labeled cells were harvested immediately, while to the others, the label was removed and the cells further incubated for 1 h at 37°C. As described in Materials and Methods section, immunoprecipitations were performed from the pulsed and pulse-chased labeled cell lysates using the: (a) hsp 72 antibody, (b) hsp 73 antibody, and (c) a mixture of mAbs specific for both hsp 72 and hsp 73. In A is shown the relative amounts of hsp 72 and hsp 73 present in each of the three cell lysates as determined by Western blot analysis using the mAb mixture. Lane 1, Hela cells; lane 2, BHK cells; and lane 3, 293 cells. In B (Hela), C (BHK), and D (293) are shown the resultant immunoprecipitation products from the pulse and pulse-chased cells. Lane designations are the same in each panel. Lanes 1-4 show the immunoprecipitation products from the 15-min pulse-labeled cell lysates, while lanes 5-8 represent the immunoprecipitation products from the pulse and pulse-chased cells. Lane designations are the same in each panel. Lanes 1-4 show the immunoprecipitation products from the 15-min pulse-labeled cell lysates, while lanes 5-8 represent the immunoprecipitation products from the pulse and pulse-chased cells. Lane 1, no antibody; lane 2, hsp 72 antibody; lane 3, hsp 73 antibody; and lane 4, mAb mixture. The positions of hsp 72 and hsp 73 are indicated by arrowheads.

Figure 3. Hsp 72 and hsp 73 interact with newly synthesized proteins. Hela, BHK, and 293 cells growing on 35-mm dishes at 37°C were labeled with [35S]methionine for 15 min. Some of the pulse-labeled cells were harvested immediately, while to the others, the label was removed and the cells further incubated for 1 h at 37°C. As described in Materials and Methods section, immunoprecipitations were performed from the pulsed and pulse-chased labeled cell lysates using the: (a) hsp 72 antibody, (b) hsp 73 antibody, and (c) a mixture of mAbs specific for both hsp 72 and hsp 73. In A is shown the relative amounts of hsp 72 and hsp 73 present in each of the three cell lysates as determined by Western blot analysis using the mAb mixture. Lane 1, Hela cells; lane 2, BHK cells; and lane 3, 293 cells. In B (Hela), C (BHK), and D (293) are shown the resultant immunoprecipitation products from the pulse and pulse-chased cells. Lane designations are the same in each panel. Lanes 1-4 show the immunoprecipitation products from the 15-min pulse-labeled cell lysates, while lanes 5-8 represent the immunoprecipitation products from the pulse and pulse-chased cells. Lane 1, no antibody; lane 2, hsp 72 antibody; lane 3, hsp 73 antibody; and lane 4, mAb mixture. The positions of hsp 72 and hsp 73 are indicated by arrowheads.

The hsp 73 specific antibody, appreciable amounts of newly synthesized proteins were observed to transiently interact with hsp 73 (Fig. 3, pulse, lane 3, chase, lane 7 in each panel). Interestingly, in only the 293 cell lysates, which express relatively high levels of hsp 72 at 37°C, did a significant amount of newly synthesized proteins co-precipitate with hsp 72 (Fig. 3 D, lane 2).

Hsp 73 and Hsp 72 Stably Interact with One Another

Upon examination of the data presented in Fig. 3, it can be seen that regardless of the antibody used, not all of the newly synthesized proteins were released from hsp 72 or hsp 73 after the 1-h chase period. In addition to a number of high molecular mass proteins, a doublet at ~70 kD was evident in the immunoprecipitates after the chase period (e.g., see Fig. 3, B and D, lanes 6-8). We suspected that this doublet represented hsp 72 and hsp 73. To test this, Hela cells and BHK cells were subjected to heat shock treatment, returned to 37°C and then labeled for 6 h with [35S]methionine. After the 6-h labeling period, the label was removed and the cells further incubated (in the absence of radiolabel) for 2 h. This labeling protocol should insure a significant amount of radiolabeled hsp 72 and hsp 73, and provide suitable time for other newly synthesized (and radiolabeled) polypeptides to be released from their hsp 72 and hsp 73 chaperones. The radiolabeled cells were harvested, ATP levels depleted, and native immunoprecipitations performed using the hsp 72 or hsp 73 specific antibodies (Fig. 4). From the Hela cell lysates, immunoprecipitation using the rabbit hsp 73 antibody resulted in the coprecipitation of hsp 73 and hsp 72 (Fig. 4 B, upper panel). Conversely, immunoprecipitation with the...
We next turned our attention to determining the absolute amounts of hsp 72 and hsp 73 that exist together in an apparent complex. Hela cells and BHK cells were grown at 37°C, or alternatively, the cells were subjected to a 43°C heat shock treatment and then returned to 37°C for 6 h. The cells were harvested, ATP levels depleted via addition of apyrase, and the lysates used for native immunoprecipitation analysis. Proteins present in the resultant immunoprecipitates were resolved on an SDS-gel, the proteins transferred to nitrocellulose, and then examined by Western blot analysis using the monoclonal antibody N27 (which recognizes both hsp 73 and hsp 72). From the 37°C Hela cells (Fig. 5 A) the hsp 72 antibody precipitated hsp 72 along with a small amount of hsp 73. Conversely, using the hsp 73 antibody resulted in the capture of hsp 73 and modest amounts of hsp 72. After heat shock treatment of the Hela cells, the relative amounts of hsp 73 and hsp 72 coprecipitating with one another increased significantly. Very similar results were observed for the heat-shock-treated BHK cells. Thus, after heat shock, significant amounts of hsp 73 forms a stable interaction with the highly stress-inducible hsp 72.

**ATP Dependence of Hsp 73 and Hsp 72 Interaction**

To determine whether the interaction between hsp 72 and hsp 73 might be ATP dependent, Hela cells and BHK cells growing at 37°C, or after a 43°C/90-min heat shock treatment were steady state labeled for 6 h with [35S]methionine. After a 2-h chase period in the absence of radiolabel, the cells were solubilized in nonionic detergents. 5 mM ATP was added to one half of the lysate, while endogenous ATP levels were depleted from the other half of the cell lysate via the addition of apyrase, and native immunoprecipitations were performed (Fig. 6). In the 37°C Hela lysates first depleted of ATP, immunoprecipitations using either the hsp 72 specific antibody (Fig. 6, lane 2) or hsp 73 specific antibody (Fig. 6, lane 3) resulted in the coprecipitation of hsp 73 and hsp 72 (Fig. 6 A, Hela 37). However, if ATP was added to the cell lysate before the immunoprecipitation analysis, hsp 72 and hsp 73 no longer were observed to

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**Figure 6.** The interaction of hsp 72 with hsp 73 appears sensitive to ATP. Hela and BHK cells maintained at 37°C, or subjected to heat shock treatment (43°C/90 min) and returned to 37°C, were labeled with [35S]methionine for 6 h. Cell lysates were prepared and apyrase was added to one half of the lysate to deplete endogenous ATP levels. To the other half of the lysate, ATP was added (final, 5 mM). After a 30-min incubation, the cell lysates were used for native immunoprecipitation analysis using antibodies specific to hsp 72 or hsp 73. A shows the results using the Hela cell lysates, which B shows the results using the BHK cell lysates. Lane designations are the same in each panel: lane C, cells labeled at 37°C; lane HS, cells labeled after heat shock; lane 1, preimmune serum (ATP depleted lysate); lane 2, hsp 72 antibody (ATP depleted lysate); lane 3, hsp 73 antibody (ATP depleted lysate); lane 4, preimmune serum (ATP supplemented lysate); lane 5, hsp 72 antibody (ATP supplemented lysate); and lane 6, hsp 73 antibody (ATP supplemented lysate).
coprecipitate, regardless of the antibody used in the analysis (Fig. 6 A, lanes 5 and 6). After heat shock, significant amounts of hsp 72 and hsp 73 were observed to coprecipitate, but again only in those cell lysates first depleted of ATP (Fig. 6 A, Hela HS). Essentially identical results were observed for the BHK cells labeled after the heat shock treatment (Fig. 6 B). Note, however, that the intensity of radiolabeled hsp 73 which coprecipitated when using the hsp 72 antibody was significantly less than that observed when using the hsp 73 antibody (e.g., compare Fig. 6 B, BHK HS, lanes 2 and 3). Most likely, in the BHK cells after heat shock the newly synthesized (and therefore highly radiolabeled) hsp 72 rapidly becomes complexed with the pre-existing (and therefore relatively low unlabeled) hsp 73.

To examine further the interaction of hsp 72 with hsp 73, a number of other biochemical studies were performed both in vitro and in vivo. First, we examined the properties of purified hsp 72 and hsp 73, and in particular determined their native molecular mass (e.g., monomer, dimer, etc.). For such studies we purified: (a) hsp 73 from bovine brains; (b) hsp 72 from 293 cells which were heat shock treated and then recovered overnight at 37°C; and (c) a mixture of hsp 72 and hsp 73 from Hela cells. The material purified from the Hela cells represents an approximate equal molar mixture of hsp 72 and hsp 73, while the material purified from bovine brain represents >95% pure hsp 73, and the material purified from 293 cells represents >95% pure hsp 72 (Fig. 7 A, insets). Analysis of the various purified proteins by HPLC gel filtration revealed two major elution peaks (Fig. 7 A, solid lines). The two different peaks, as determined by calibration of the HPLC columns with appropriate standards, represent apparent dimers (earlier peak) and monomers (later peak) of the purified proteins. Interestingly, the relative proportions of these two major peaks was observed to vary amongst the different preparations. Upon repeating the gel filtration analysis, this time with 5 mM ATP present in the column developing buffer, the gel filtration profiles were observed to change (Fig. 7 A, dotted lines). In each case, the earlier migrating material (peak 1) disappeared concomitant with an increase in the amount of material present within the second peak. Using "native" polyacrylamide gel electrophoresis, very similar results to those observed using gel filtration were observed. While most of the Hela hsp 72 and hsp 73, as well as the 293 hsp 72, migrated as a single species, the bovine brain hsp 73 material was resolved into two populations (Fig. 7 B). Although not shown, prior addition of ATP to the bovine brain hsp 73 material resulted in the disappearance of the slower migrating species concomitant with an increase in the amount of the faster migrating species. In an attempt to reconstitute the apparent in vivo interaction of hsp 72 with hsp 73, purified hsp 73 was added to the purified hsp 72 and the mixture analyzed by both gel filtration and native polyacrylamide gel electrophoresis. Regardless of whether the analysis was performed in the presence of ATP or in the presence of apyrase (to remove any residual ATP left over from the purification of the proteins), we were unable to reconstitute the apparent interaction of hsp 72 with hsp 73 (data not shown).

Our failure to reconstitute an interaction of hsp 72 with hsp 73 in vitro, prompted our examining the relative distribution of the two proteins in whole cell lysates via gel filtration. Hela cells were subjected to heat shock treatment and allowed a subsequent recovery period at 37°C to insure maximum synthesis and accumulation of hsp 72 and hsp 73. The cells were lysed in nonionic detergents, the material applied to a Sepharose 6B gel filtration column, and analysis of the resultant fractions eluting off the column performed via Western blot analysis using the N27 antibody (which recognizes both hsp 72 and hsp 73). Note that the vast majority of the two proteins migrated together through the column with a relative constant stoichiometry.

We examined whether the apparent ATP-dependent interaction of hsp 73 and hsp 72 was a reversible phenomenon in the whole cell lysates. Hela cells were heat shock treated, returned to 37°C, and steady state labeled with [35S]methionine. After labeling, the cells were lysed with nonionic de-
mM ATP followed by the addition of apyrase (to deplete the added ATP). After the appropriate treatments, the lysates were used for native immunoprecipitation analysis employing the hsp 72 specific mAb (C92). Shown in A are the immunoprecipitation products. Lane 1, ATP-depleted lysate; Lane 2, ATP-supplemented lysate; and Lane 3, lysate to which ATP was added and subsequently depleted.

In a second experiment, a rabbit reticulocyte lysate (which contains only hsp 73) was programmed with a mRNA coding for hsp 72. After a 1 h translation in the presence of [35S]methionine, the lysate was split into three equal portions: (a) one portion was depleted of ATP; (b) another portion supplemented with 5 mM ATP; and (c) one portion treated with 5 mg/ml puromycin. After a 30-min incubation, the added ATP was depleted via the addition of apyrase, and then subsequently depleted.

Native immunoprecipitations using the hsp 72 specific antibody (C92) were then performed from the three different treated cell lysates (Fig. 8 A). As was observed before, prior depletion of ATP in the cell lysate resulted in the hsp 72 specific antibody coprecipitating both hsp 72 and hsp 73. In contrast, added ATP resulted in the dissociation of hsp 73 and hsp 72. In those lysates treated first with ATP, and then subsequently depleted of ATP via apyrase, immunoprecipitation with the hsp 72 specific antibody now resulted in the coprecipitation of both hsp 72 and hsp 73 (Fig. 8 A, lane 3). Thus, we suspect that in vivo the interaction between hsp 72 and hsp 73 is a reversible phenomenon, likely dependent on ATP and perhaps facilitated by other cellular components.

To further address the possibility that pre-existing hsp 73 rapidly forms a stable complex with newly synthesized hsp 72, we turned to an in vitro translation system. As determined by Western blot analysis, rabbit reticulocyte lysates contain appreciable amounts of hsp 73, but little or no hsp 72 (W. J. Hanson and W. J. Welch, manuscript in preparation). After a coupled transcription and translation of human hsp 72 in the presence of [35S]methionine, we examined for the possible presence of a hsp 73-hsp 72 complex via immunoprecipitation analysis using our hsp 73 specific antibody (Fig. 8 B). In those lysates first depleted of ATP, the hsp 73 specific antibody was observed to co-precipitate the radiolabeled, newly synthesized hsp 72.

### Hsp 73 and Hsp 72 Exist as a Complex In Vivo

Previous studies have shown that the highly stress-inducible form of hsp 70 (hsp 72) localizes within the nucleus and nucleolus of cells after heat shock (Welch and Feramisco, 1984; Pelham, 1984). Now, with the availability of an antibody specific to hsp 73, similar studies examining the intracellular locale of hsp 73 were performed. Hela cells and BHK cells growing on glass coverslips were subjected to heat shock treatment and then returned to 37°C for 2 h. The cells were fixed and processed for double-label indirect immunofluorescence using both the mouse anti hsp 72 and the rabbit anti hsp 73 antibodies. As shown in Fig. 9, hsp 72 and hsp 73 exhibited a coincident locale, primarily within the nucleus and nucleolus in either the Hela (Fig. 9, A–C) or BHK (Fig. 9, D–F) cells after heat shock. Note that while not all of the BHK cells exhibited a nuclear/nucleolar locale of the proteins, those that did always showed a similar, if not identical, deposition of the two proteins (Fig. 9, D–F).

We were curious to know whether hsp 73 could relocate into the nucleus and nucleolus under conditions where the stress inducible hsp 72 had not yet accumulated to any appreciable extent. Therefore, BHK cells growing at 37°C, or alternatively given a brief, but severe heat shock treatment (45°C for 20 min) were analyzed for the localization of the pre-existing, constitutively expressed hsp 73. Previous studies have shown that little or no hsp 72 is synthesized within the first few hours after such a severe heat shock treatment (Mizzen and Welch, 1988). In the BHK cells maintained at 37°C, a diffuse cytoplasmic and nuclear distribution of hsp 73 was observed (Fig. 10, A and B). Immediately after heat shock treatment, hsp 73 now was found concentrated within the nucleolus (Fig. 10, C and D). Thus, pre-existing hsp 73 is capable of relocating into the nucleolus even when hsp 72 has not yet been synthesized. Next we examined whether hsp 73 and hsp 72 would redistribute together as a complex in cells after heat shock. Therefore, BHK cells were first provided a 43°C/1 h heat shock and then allowed a recovery period at 37°C for 12 h. After 12 h of recovery, most all of the hsp 73 and hsp 72 have returned to the cytoplasm (Welch and Suhani, 1986). These cells then were subjected to a 45°C/20 min heat shock treatment and the distribution of hsp 72 and hsp 73 determined by double-label indirect immunofluorescence (Fig. 10, E and F). In almost every cell, hsp 72 and hsp 73 exhibited an identical locale.

Having shown that hsp 72 and hsp 73 interacted with one another, as well as colocalized in the cell after heat shock, we examined whether we could block their redistribution from the cytoplasm into the nucleus and nucleolus as a result of antibodies specific to either protein. In particular, would antibodies specific to hsp 72 block the ability of hsp 72 to redistribute into the nucleus and nucleolus after heat shock? Therefore, Hela cells growing at 37°C (and expressing both hsp 72 and hsp 73) were injected with the purified rabbit hsp 73 antibody. After their injection, the cells were subjected to a 43°C/90-min heat shock treatment, returned to 37°C for 1 h and then examined by indirect immunofluorescence. The un.injected cells displayed a typical
nuclear and nucleolar locale of hsp 72 (Fig. 11 C). Cells injected with the rabbit anti hsp 73 antibody (Fig. 11 B), however, showed a marked reduction in the amount of hsp 72 present within the nucleolus after heat shock (Fig. 11 C). In a complimentary experiment, we injected the mouse antibodies specific to hsp 72 and then ascertained the relative distribution of hsp 72 after heat shock. In those cells injected with the anti hsp 72 antibodies (Fig. 11 E), only a slight reduction in the amount of nuclear and nucleolar hsp 73 was observed (Fig. 11 F). The only obvious difference was the lack of well defined hsp 73 nucleolar staining, as compared with the staining of hsp 73 in the surrounding, un.injected cells. Because the relative levels of hsp 73 are greater than that of hsp 72 in Hela cells growing at 37°C, we repeated this latter experiment, this time in cells which had received a prior heat shock treatment and recovery period (to allow for increased expression and accumulation of hsp 72); the rationale being that with higher levels of hsp 72 now present, all of hsp 73 would exist in a complex with hsp 72. When these cells were injected with the anti hsp 72 antibodies (Fig. 11 H), and then subjected to a heat shock treatment, a significant reduction in the amount of hsp 73 present within the nucleus and nucleolus was observed (Fig. 11 I).

Discussion

Within the last few years considerable progress has been realized in our understanding of the structure and function of the hsp 70 family of stress proteins. Related forms of hsp 70 have been discovered within the cytoplasm and nucleus, the lumen of the ER, the mitochondrial and chloroplast matrix, and perhaps even a form near or on the cell membrane (reviewed by Welch, 1990). Each of the related forms of hsp 70 bind avidly to ATP (Welch and Feramisco, 1985), and each appear to function as mediators of protein maturation (Haas and Wabl, 1983; Boie et al., 1986; Gething et al., 1986; Chirico et al., 1988; Deshaies et al., 1988; Kang et al., 1990; Beckmann et al., 1990; Mizzen et al., 1991). For example, the different hsp 70 family members, within their own distinctive subcellular compartment, have been shown to interact transiently with other proteins which are in the course of maturation. We and others have suggested that the members of the hsp 70 family function to slow down or retard the folding proteins which are in the course of synthesis and/or translocation into organelles (Haas and Wabl, 1983; Beckmann et al., 1990; Kang et al., 1990; Ellis and van der Vies, 1991; Mizzen et al., 1991).

Germane to the present study is the fact that eukaryotic cells express both a constitutive (e.g., hsp 73) and a highly stress-inducible (e.g., hsp 72) form of hsp 70. Although encoded by distinct genes, both hsp 72 and hsp 73 exhibit an extremely high degree of sequence relatedness. One major difference however, is the fact that the gene encoding hsp 72 lacks intervening sequences, while the maturation of hsp 73...
mRNA, like most other cellular mRNAs, requires processing (reviewed by Craig, 1985). Since mRNA processing activities appear compromised in cells after heat shock treatment (Mayrand and Pederson, 1983; Yost and Lindquist, 1986), the high level expression of hsp 72 after heat shock simply may represent a way for the cell to rapidly increase the levels of cytosolic and nuclear hsp 70 by bypassing the requirement for mRNA processing activities. A number of studies, however, support the idea that hsp 72 and hsp 73 are truly distinct proteins as it relates to the physiology of the cell. First, cells which have been subjected to a mild or sublethal stress event, sufficient to induce the high level expression and accumulation of hsp 72, now exhibit increased protection upon their exposure to a subsequent stress event (Gerner and Schneider, 1975; Henle and Leeper, 1976; Mizzen and Welch, 1988). This phenomenon, referred to as acquired thermotolerance, appears to correlate closely with the overall amount of hsp 72 produced in the cell after the initial or “priming” stress event (Li and Werb, 1982). Yet other experiments also indicate differences between the constitutively and highly stress-inducible forms of hsp 70. For example, Lindquist and co-workers have succeeded in establishing a Drosophila cell line which constitutively expresses the highly stress-inducible form of hsp 70 (Feder et al., 1992). Early after transformation, and as the cells begin expressing the stress-inducible form of hsp 70, there occurs a substantial reduction in the rate of cell growth. During this period of relatively slow growth, the expressed protein is distributed in a diffuse manner throughout the cell. With time in culture, the transfected cells eventually regain their nor-
Figure 11. hsp 72 and hsp 73 redistribute into the nucleus and nucleolus as a complex in cells after heat shock. Hela cells growing on glass coverslips at 37°C were injected with antibodies specific to either hsp 72 or hsp 73. After a 30-min incubation at 37°C, the cells were subjected to a 43°C/90-min heat-shock treatment and then returned to 37°C for 2 h. The cells then were fixed and processed for indirect immunofluorescence using either the hsp 72- or hsp 73-specific antibodies. To identify those cells injected, the cells were stained with goat anti-rabbit antibodies (to detect the anti hsp 73 antibody) or rabbit anti-mouse antibodies (to detect the hsp 72 antibody); A–C: cells injected with rabbit anti hsp 73 (B) and then analyzed for the distribution of hsp 72 (C); D–F: cells injected with mouse anti hsp 72 (E) and then analyzed for the distribution of hsp 73 (F). In a second experiment, Hela cells were subjected to heat shock and returned to 37°C for 12 h (to allow for maximal synthesis and accumulation of hsp 72). The cells were injected with the mouse antibody specific for hsp 72, then subjected to a 45°C/20-min heat shock treatment and then analyzed for the distribution of hsp 73; G–I: cells injected with the anti hsp 72 antibody (H) and then analyzed for the distribution of hsp 73 (I). Arrowheads in C, F, and I indicate the antibody injected cells.
mal rate of growth, concomitant with the sequestration of hsp 70 within a cytoplasmic granule-like structure. Thus, the presence of the highly stress-inducible form of hsp 70 may, at least in some cells, serve to inhibit activities associated with normal growth. Moreover, the high level expression and accumulation of the stress inducible form of hsp 70 may be a necessary requirement for the cells acquisition of a thermotolerant phenotype.

Using metabolic pulse-chase labeling and immunoprecipitation with antibodies that recognize both hsp 72 and hsp 73, we previously concluded that the two proteins interacted transiently with a number of newly synthesized proteins in Hela cells. Herein, using antibodies which can discriminate between either hsp 73 or hsp 72, we find that either one of the proteins apparently are capable of transiently interacting with newly synthesized proteins. Throughout the course of these studies, however, we observed what appeared to be a relatively stable association of hsp 73 with hsp 72, especially in cells after heat shock. We suspect that the pre-existing hsp 73 forms a relatively stable complex with the heat-inducible, newly synthesized hsp 72. First, antibodies specific to hsp 73 coprecipitated appreciable amounts of newly synthesized and radiolabeled hsp 72. In contrast, antibodies to hsp 72 resulted in the coprecipitation of only modest amounts of radiolabeled hsp 73 (e.g., see Figs. 4 and 6). Yet, when the absolute amounts of the two proteins coprecipitating with one another was determined, they appeared to be present in approximately a 1:1 stoichiometry (e.g., see Fig. 5). These results could be explained if the pre-existing (and therefore unlabeled) hsp 73 was interacting with the newly synthesized (and therefore radiolabeled) hsp 72. Indeed, in a rabbit reticulocyte lysate programmed with mRNA encoding hsp 72, the pre-existing (and unlabeled) hsp 73 present within the reticulocyte lysate rapidly formed a stable complex with the newly synthesized and radiolabeled hsp 72.

Our biochemical studies were complimented by in vivo studies examining the subcellular distribution of hsp 72 and hsp 73. In rodent cells expressing only hsp 73, heat shock treatment resulted in an immediate redistribution of hsp 73 into the nucleus and nucleolus. As the highly stress inducible hsp 72 began to accumulate in cells after heat shock, it also was found to concentrate within the nucleus and nucleolus. Whenever a particular cell exhibited a nucleolar locale for hsp 72, that same cell also displayed a similar locale for hsp 73. Our microinjection experiments supported the idea that the two proteins, in many cases, move into the nucleus and nucleolus as a complex. For example, when antibodies specific to hsp 73 were injected into living cells, we observed a corresponding reduction in the amount of both hsp 73 and hsp 72 present within the nucleus and nucleolus after heat shock.

The significance of this interaction between constitutive hsp 73 and stress-inducible hsp 72 is not yet clear. We suspect that in many cell types, such as rodents, hsp 73 likely exists in an equilibrium between monomeric and oligomeric forms, the latter perhaps a dimer. This is suggested by our HPLC analysis demonstrating two major forms of hsp 73, with the oligomeric form (probably a dimer) dissociating upon addition of ATP. Our data showing that the hsp 73 homodimer, as well as the hsp 72-73 heterodimer, are disrupted by the addition of ATP may have important implica-

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