Cell Cycle–dependent Association of HSP70 with Specific Cellular Proteins

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Abstract. In asynchronous populations of HeLa cells maintained at control or heat shock temperatures, HSP70 levels and its subcellular distribution exhibit substantial heterogeneity as demonstrated by indirect immunofluorescence with HSP70-specific monoclonal antibodies. Of particular interest is a subpopulation of cells in which the characteristic nuclear accumulation and nucleolar association of HSP70 is not detected after heat shock treatment. This apparent variation in the heat shock response is not observed when synchronized cells are examined. In this study, we demonstrate that three monoclonal antibodies to HSP70, in particular, do not detect nucleolar-localized HSP70 in heat-shocked G_2 cells. This is not due to an inability of G_2 cells to respond to heat shock as measured by increased HSP70 mRNA and protein synthesis, or due to a lack of accumulation of HSP70 after heat shock in G_2. Rather the epitopes recognized by the various antibodies appear to be inaccessible, perhaps due to the association of HSP70 with other proteins. Nondenaturing immunoprecipitations with these HSP70-specific antibodies suggest that HSP70 may interact with other cellular proteins in a cell cycle–dependent manner.

THE highly conserved response of virtually all cell types and organisms to heat and other forms of physiological stress has been well characterized (reviewed in references 8 and 19). The key feature of this response is the rapid induction of a select group of proteins, collectively called the heat shock or stress-induced proteins. The exact number and size of induced proteins varies somewhat depending on the organism and the particular stress used, with the most highly conserved being a family of HSP70-related proteins (2, 13). Human cells contain at least four members in the 70-kD stress-responsive gene family; although the proteins are related by sequence and share a common antigenic determinant, their genes are differentially regulated (20, 34, 39). Two members of the human HSP70 gene family are: (*a*) P72, an abundant cellular protein synthesized constitutively at normal physiological temperatures and only slightly induced by heat shock (34, 36) which is reported in in vitro experiments to have an ATP-dependent clathrin-uncoating activity (6, 30), and (*b*) HSP70, the major stress-induced protein, which is also expressed at physiological temperatures in human cells, but in a cell cycle–dependent manner (20, 39). In previous publications from our laboratories HSP70 and P72 have also been referred to as HSP72 and P73, respectively. In HeLa cells expression of the HSP70 protein is restricted to the G_1/S boundary. Indirect immunofluorescence with HSP70-specific monoclonal antibodies reveals that the protein becomes concentrated in the nucleus of early S phase cells, but is diffusely distributed throughout the cytoplasm and nucleus during the remainder of the cell cycle (20). The abundance of the constitutively expressed P72 in the absence of physiological stress, and the cell cycle regulation of HSP70 indicate that these two proteins participate in normal processes in the unstressed cell. Indeed, recent data has implicated a role for these proteins in the translocation of polypeptides from their site of synthesis in the cytoplasm across the membrane of either the endoplasmic reticulum or the mitochondria (7, 9).

After heat shock, HSP70 accumulates within the nucleus, with much of the protein associated with nucleoli (1, 16, 25, 31, 36, 38). The localization of HSP70 within the region of the nucleolus involved in ribosomal assembly is correlated with visible, stress-induced alterations in nucleolar architecture. As the nucleoli recover their normal morphology, there is a corresponding exit of HSP70 from the organelle. Therefore, HSP70 has been suggested to participate in the repair of heat-induced nucleolar damage (18, 24, 25, 36, 38).

In studying the subcellular distribution of HSP70 in asynchronous cell populations we have observed variability in the nuclear association of HSP70 after heat shock (20, 36). Specifically, after heat shock many, but not all of the cells exhibit HSP70 within the nucleolus. In this study we demonstrate that this apparent variation is a consequence of the cell cycle. For example, we find that three HSP70-specific monoclonal antibodies are capable of detecting HSP70 within the nucleolus after heat shock treatment of only those cells in either G_1 or S phase. In contrast, G_2 cells do not exhibit nucleolar staining after heat shock when examined using these same monoclonal antibodies. Yet, we show that the G_2 cells were fully capable of inducing *HSP70* gene expression and elevated synthesis of the protein in response to heat shock treatment. Therefore, the inability to detect HSP70 within the nucleoli of the heat shock–treated G_2 cells ap-

pears to result from the inaccessibility of certain HSP70 epitopes, presumably due to association with other macromolecular components. We have identified HSP70-associated proteins by nondenaturing immunoprecipitation, and have found that these associations change throughout the cell cycle.

Materials and Methods

Cell Culture and Synchronization

HeLa cells were grown in DME supplemented with 10% FBS in 75 cm^2 tissue culture flasks or on glass coverslips. Synchronized populations of cells were obtained by mitotic detachment as described previously (20). Cells were heat shock-treated by incubation at 43°C for 90 min.

Characterization of Monoclonal Antibodies N6, N15, and N21

Monoclonal antibodies were produced from mice immunized with purified HeLa 72- and 70-kD stress proteins (35) and were characterized by immunoprecipitation analysis as described previously (36). The specificity of these antibodies, under denaturing conditions, for HSP70 is shown in Fig. 5.

Indirect Immunofluorescence

The intracellular distribution of HSP70 was determined using four different HSP70-specific mouse monoclonal antibodies. Cells growing on 12-mm glass coverslips were fixed and permeabilized by exposure to absolute methanol at −20°C for 2 min. The monoclonal antibodies were diluted as follows: C92 (characterized previously in reference 37) 1:100; N15, 1:200; N6, 1:400; and N21, 1:500. Fluorescein-conjugated goat anti-mouse antibody diluted 1:75 was used to detect the primary antibodies.

HSP70 mRNA Levels

HSP70 mRNA levels were determined by S_1-nuclease protection assays (3, 29, 40). Total cytoplasmic RNA was hybridized to a ^{32}P-labeled *HSP70* gene-specific probe and the RNA-DNA hybrids were digested with S_1 nuclease (Boehringer Mannheim Biochemicals, Indianapolis, IN). The protected fragments were analyzed by electrophoresis in 4% polyacrylamide/8 M urea gels.

HSP70 Synthesis and Accumulation

Cell cultures maintained at 37°C or subjected to a 90-min heat shock treatment at 43°C were labeled for 15 min at 37°C with [^{35}S]methionine (50 μCi/ml) in DME lacking methionine. After the labeling period, the medium was removed, the cells were washed with PBS, lysed in Laemmli sample buffer, and analyzed by one-dimensional SDS-PAGE (15).

The accumulation of HSP70 was determined by immunoblot analysis (5). Unlabeled cell lysates were electrophoretically separated on 12.5% SDS-polyacrylamide gels and transferred to nitrocellulose. Nitrocellulose filters were probed with the HSP70-specific monoclonal antibody C92 (diluted 1:100) followed by ^{125}I-labeled goat anti-mouse antibody.

Nondenaturing Immunoprecipitations

The proteins associated with HSP70 were identified by nondenaturing immunoprecipitation using HSP70-specific monoclonal antibodies. HeLa cells were continuously labeled with [^{35}S]methionine (40 μCi/ml) in DME lacking methionine containing 10% FBS and 10% normal DME. Heat shock samples were incubated at 43°C for the last hour of the labeling period. After labeling, the cells were washed with PBS and lysed in 10 mM Tris pH 7.4, 150 mM NaCl, 1% Triton X-100, 1% deoxycholate, 0.1% SDS (RIPA buffer). The cell lysates were sonicated, preabsorbed with protein A-agarose, and clarified by centrifugation. The cleared lysates were incubated with the HSP70-specific monoclonal antibodies at 4°C with gentle rocking for 1–2 h. Protein A-agarose beads were added and the incubation continued for 30 min. The immunoprecipitates were washed five times with RIPA buffer and the precipitated proteins released from the beads by addition of SDS gel electrophoresis sample buffer followed by boiling for 5 min. The immunoprecipitates were analyzed by SDS-PAGE on 12.5% gels.

Results

Heterogeneity in the Levels and Subcellular Distribution of HSP70 in Asynchronous Cell Populations

Immediately after heat shock treatment of mammalian cells, HSP70 accumulates in the nucleus, with a portion of the protein specifically associated with nucleoli (16, 25, 36). While 85–90% of the cells in an asynchronous population display this characteristic nuclear and nucleolar localization of HSP70, there exists a subpopulation of heat-shocked cells that exhibits a different pattern of HSP70 staining. This is illustrated in Fig. 1, in which asynchronous populations of HeLa cells growing on glass coverslips were incubated for 90 min at 43°C. The cells were fixed and HSP70 distribution examined using three HSP70-specific monoclonal antibodies. (C92 is described in reference 38, for specificity of N6 and N21 see Fig. 5.)

When monoclonal antibody C92 was used (Fig. 1 *A*) we observed two very different patterns of HSP70 distribution. The majority of cells exhibited staining in the nucleus, most of which was primarily coincident with nucleoli. A subpopulation of cells, however, exhibited very little nuclear fluorescence, and no nucleolar staining (Fig. 1 *A*, *arrows*). Heat-shocked cells stained with antibodies N6 and N21 (Fig. 1 *B* and *C*, respectively) similarly exhibited two classes of HSP70 distribution. The majority of cells showed intense HSP70 fluorescence coincident with nucleoli; antibodies N6 and N21, in contrast to C92, also revealed increased nuclear staining of other nonnucleolar structures. Again, in a subpopulation of the cells (indicated by arrows in Fig. 1) HSP70 was not detected within the nucleoli, despite a considerable amount of nuclear fluorescence. Thus, using three different HSP70-specific monoclonal antibodies we consistently observed heterogeneity with respect to the distribution of HSP70 after heat shock treatment of asynchronous populations of cells.

Subcellular Localization of HSP70 in Synchronized Cells

Our previous studies examining the distribution of HSP70 in HeLa cells grown at 37°C indicated differential staining patterns as a function of the cell cycle. During the G_1 and G_2 phases of the cell cycle HSP70 is diffusely distributed throughout the entire cell, whereas, during S phase HSP70 becomes concentrated in the nucleus (20). Therefore, we investigated whether the heterogeneity in nuclear and nucleolar staining patterns in heat-stressed cells (shown in Fig. 1) might also be cell cycle related.

HeLa cells grown on glass coverslips were synchronized by removal of the serum from the media for 36 h as described previously (39). Fresh serum was added to the cells, and 6 h later the cells were incubated at 43°C for 90 min, then fixed and analyzed for the distribution of HSP70 using the C92 antibody. In contrast to the variation observed with asynchronous cells, cells synchronized by serum stimulation exhibited a uniform nuclear and nucleolar distribution of HSP70 after heat shock (Fig. 2). In virtually every cell examined, intense nucleolar staining of HSP70 was observed. These results indicate that the variation in the nuclear and nucleolar distribution of HSP70 in heat-treated cells is most likely dependent upon the growth state of the cells. The apparent lack

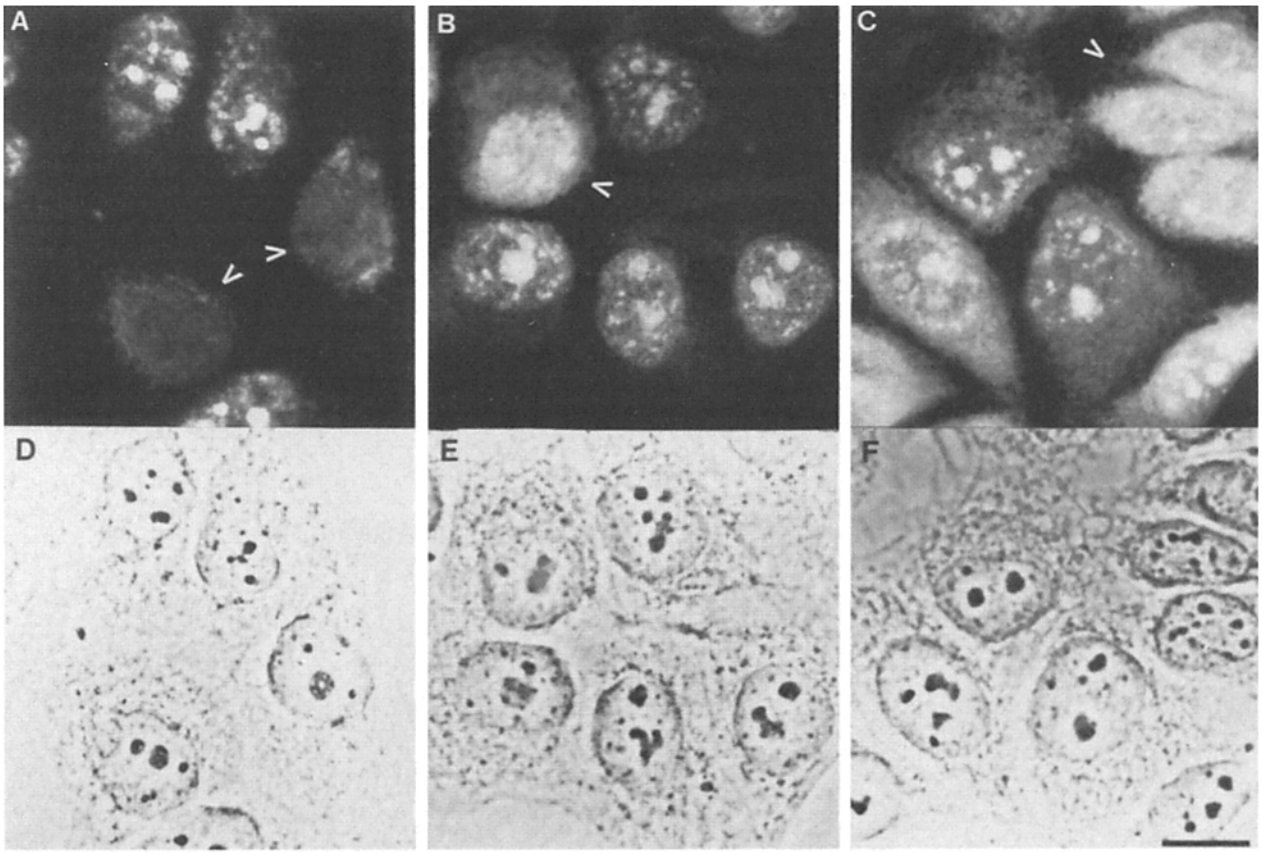


Figure 1. Distribution of HSP70 in asynchronous cells after heat shock, as determined by indirect immunofluorescence using monoclonal antibodies C92, N6, and N21. Asynchronously growing HeLa cells on glass coverslips were incubated at 43°C for 90 min. After heat shock the coverslips were fixed and analyzed by indirect immunofluorescence as described in Materials and Methods. (*A–C*) Fluorescent micrographs of cells stained with C92 (*A*), N6 (*B*), and N21 (*C*). (*D–F*) The corresponding phase-contrast micrographs. Bar, 10 μm.

of nuclear or nucleolar staining in some cells within an asynchronous population could be due to an inability to induce HSP70 synthesis in a particular phase of the cell cycle, or alternatively may reflect cell cycle–dependent changes in the availability of the HSP70 epitope recognized by the C92 antibody.

To distinguish between these two possibilities we examined the expression and the distribution of HSP70 through-

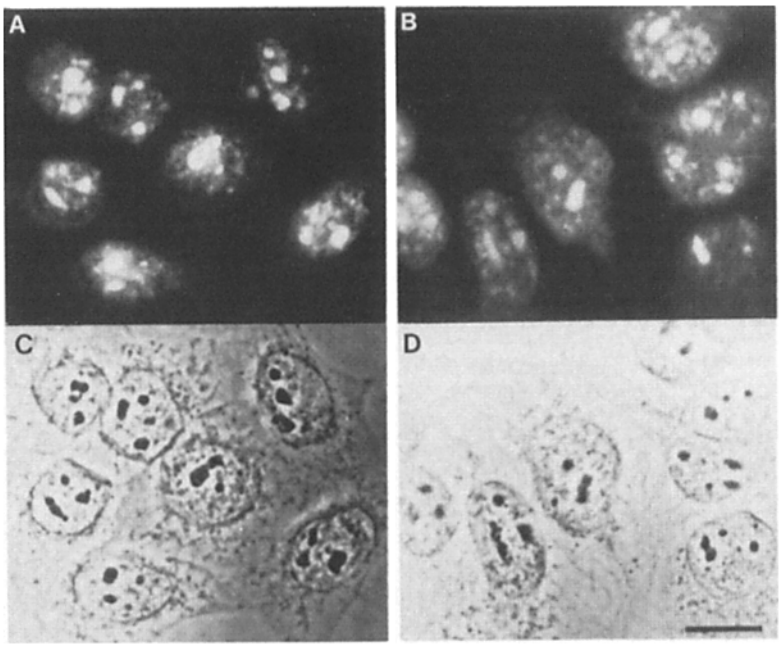


Figure 2. Distribution of HSP70 after heat shock of synchronized cells as determined by indirect immunofluorescence using monoclonal antibody C92. HeLa cells growing on glass coverslips were synchronized by serum starvation for 36 h in DME lacking serum followed by stimulation with DME containing 10% FBS. 6 h poststimulation the cells were incubated at 43°C for 90 min. After heat shock the coverslips were fixed and analyzed by indirect immunofluorescence using monoclonal antibody C92 as described in Materials and Methods. (*A* and *B*) Fluorescent micrographs of two different fields of cells. (*C* and *D*) the corresponding phase-contrast micrographs. Bar, 10 μm.

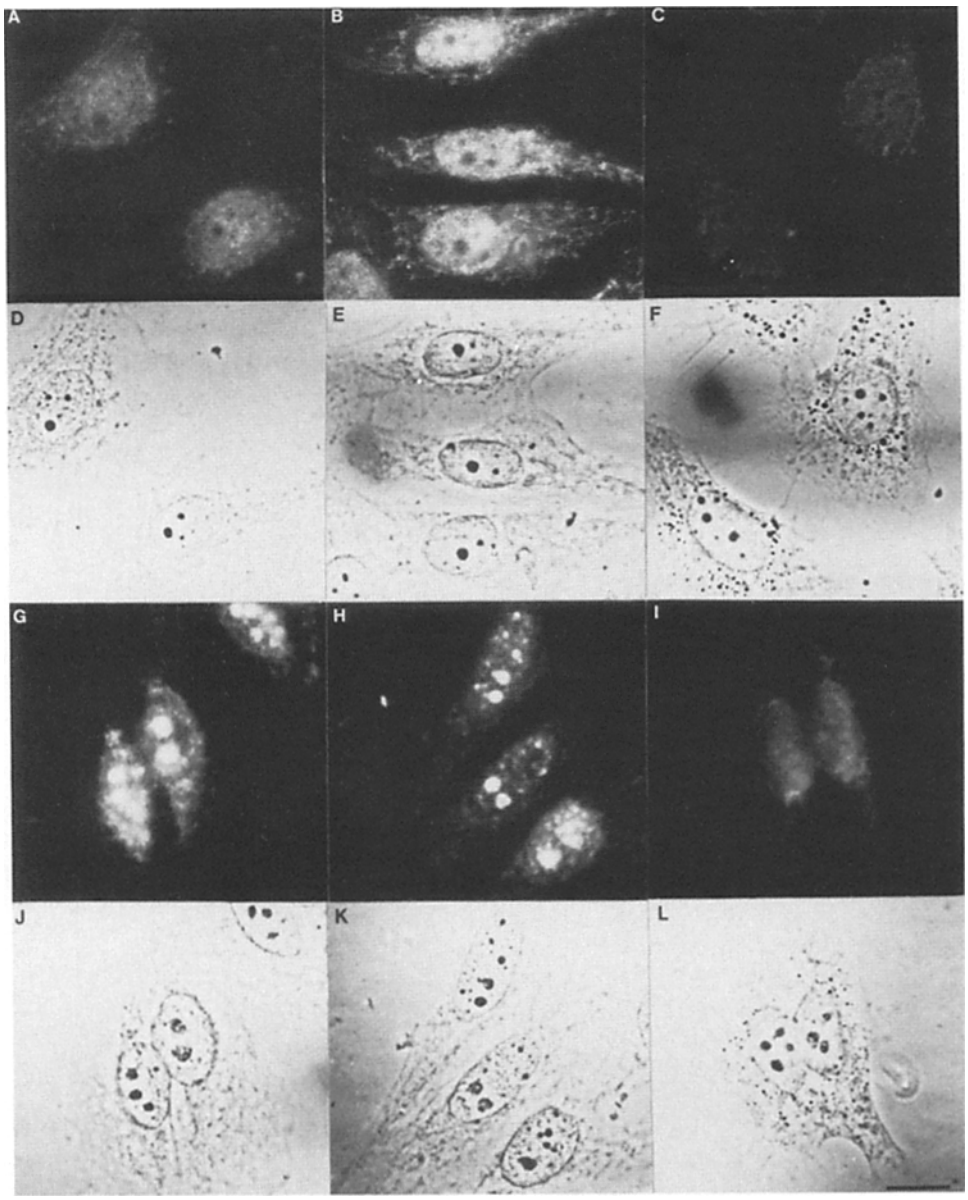


Figure 3. Intracellular distribution of HSP70 throughout the cell cycle before and after heat shock treatment of synchronized cells. HeLa cells were synchronized by mitotic detachment as described in Materials and Methods. In G_1, S, and G_2 (10-, 12- and 19-h postplating) one coverslip was immediately fixed while another was further incubated for 90 min at 43°C before fixation. The fixed cells were then analyzed for the distribution of HSP70 by indirect immunofluorescence using monoclonal antibody C92 as described in Materials and Methods. (*A–C*) Fluorescent micrographs of control 37°C cells in G_1 (*A*), S (*B*), and G_2 (*C*). (*D–F*) Corresponding phase-contrast micrographs. (*G–I*) Fluorescent micrographs of cells heat shock treated in either the G_1 (*G*), S (*H*), or G_2 (*I*) phase of the cell cycle. (*J–L*) Corresponding phase-contrast micrographs. Bar, 10 μm.

out the cell cycle, both before and after heat shock treatment. A highly synchronous population of HeLa cells was obtained by selective mitotic detachment (20). At various times throughout the cell cycle the distribution of HSP70, both before and after a 90-min 43°C heat shock treatment, was monitored by immunofluorescence using the C92 antibody (Fig. 3). Progression through the cell cycle was monitored by [^{3}H]thymidine incorporation into TCA-precipitable material (data not shown). In all of the following cell cycle experiments, the incorporation of [^{3}H]thymidine was maximal ~12 h after plating the mitotic cells, and was 10- to 15-fold higher than the incorporation at 4 or 8 h after plating, corresponding to a mitotic index of >90%.

Similar to the cells synchronized by serum deprivation, the population synchronized by mitotic detachment appeared homogeneous with respect to both HSP70 levels and its intracellular localization. The change in the levels and distribution of HSP70 throughout the cell cycle in unstressed cells has been described previously (20), and is shown here (Fig. 3, *A–C*) for comparison to the heat-shocked samples (Fig. 3, *G–I*). 10 h after plating, the population had progressed to late G_1. The unstressed cells (Fig. 3 *A*) showed low levels of HSP70 diffusely distributed throughout the entire cell. After heat shock, the cells in G_1 (Fig. 3 *G*) exhibited intense nucleolar staining; HSP70 is also detected within other areas of the nucleus. A similar pattern of HSP70 distribution was observed in serum-deprived cells that were heat shock treated 6 h after serum stimulation (Fig. 2). As the cells entered S phase (12 h after plating) there was an increased accumulation of HSP70 in the nuclei of unstressed cells (Fig. 3 *B*), consistent with increased synthesis of the protein in S phase cells (20). After heat shock, the S phase cells (Fig. 3 *H*) exhibited HSP70 in the nucleoli similar to that observed for G_1 cells. By 19 h after plating, DNA synthesis was

completed and the cells had entered G_2. At this point in the cell cycle HSP70 was diffusely distributed throughout the cell before the heat shock (Fig. 3 *C*). The relative level of staining in G_2 cells was somewhat less than that observed in either G_1 or S phase cells. In addition, the localization of HSP70 in the G_2 cells after heat shock treatment was different than that in other phases of the cell cycle when using the C92 antibody (Fig. 3 *I*). Specifically, there appeared to be only a slight increase in the amount of HSP70 staining after heat shock treatment, as compared with that seen in either G_1 or S phase cells after heat shock. In addition, little or no nucleolar accumulation of HSP70 was evident in the heat-treated G_2 cells, even though the nucleoli were clearly visible by phase-contrast microscopy (Fig. 3 *L*). These results indicate that the variation observed in the distribution of HSP70 in an asynchronous population of cells after heat shock reflects the position within the cell cycle that each cell is in at the time of heat shock treatment. Specifically, the C92 antibody reveals nucleolar staining of HSP70 in cells heat shock treated while in the G_1 or S phases of the cell cycle, but not in those cells heat treated during G_2.

Does the Cell Cycle Influence the Heat Shock Response?

To determine the basis of this apparent lack of nucleolar staining in heat-shocked G_2 cells, we examined *HSP70* mRNA levels and protein synthesis after heat shock treatment throughout the cell cycle. HeLa cells were synchronized by mitotic detachment and total cytoplasmic RNA was isolated before and after a 90-min, 43°C heat shock administered during G_1 (4 h after plating), early S phase (12 h), late S/G_2 (19 h), G_2 (21 h), and G_2/M (23 h). Equivalent amounts of RNA (5 μg) from each sample were annealed to a ^{32}P-end-labeled *HSP70* gene-specific template and digested with S_1 nuclease. The protected fragments of 150 nucleotides, detected by electrophoresis in polyacrylamide/urea gels, correspond to the 5′-untranslated region of the HSP70 mRNA (29). After heat shock, *HSP70* mRNA levels were induced 10- to 20-fold at all points throughout the cell cycle (Fig. 4 *A*). In this particular experiment the level of *HSP70* mRNA after heat shock was slightly lower at the 19- and 21-h timepoints. However, this minor variation was not significant enough to account for the lack of response as seen by immunofluorescence.

We next examined whether the levels of HSP70 synthesis after heat shock varied during the cell cycle. Synchronized HeLa cells were pulse-labeled with [^{35}S]methionine at 37°C for 15 min, either before or after a 90-min, 43°C heat shock treatment administered at either 4, 12, 19, 21, or 23 h after plating mitotic cells. Equal numbers of pulse-labeled cells were then analyzed by SDS-PAGE and the proteins visualized by fluorography. Fig. 4 *B* demonstrates that the increased synthesis of HSP70 after heat shock was approximately the same at all points in the cell cycle. Also note the S phase–specific expression of the protein (at 12 h) in the unstressed cells as previously described (20).

To this point we had demonstrated that *HSP70* mRNA levels and the overall patterns of stress protein synthesis appeared similar at all points within the cell cycle after heat shock. Nevertheless, after heat shock the immunofluorescent patterns of HSP70 in cells within the G_2 phase of the cell cycle appeared markedly different from that of the cells

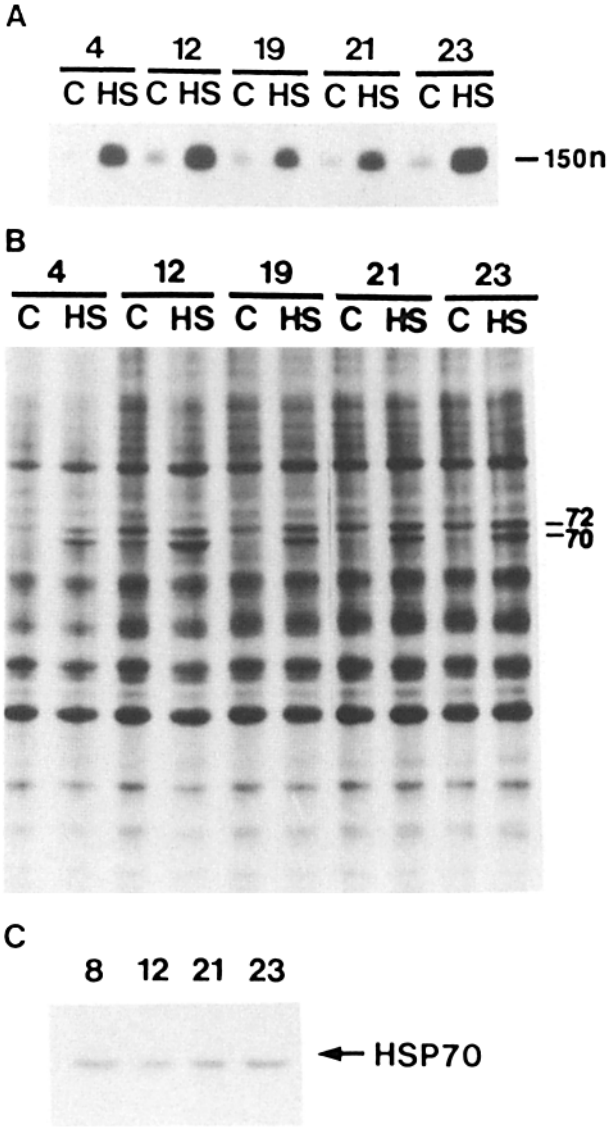


Figure 4. HSP70 mRNA levels, protein synthesis, and HSP70 accumulation before and after heat shock treatment at various points in the cell cycle. HeLa cells were synchronized by mitotic detachment as described in Materials and Methods. At the indicated hour after plating (in each case presented at the top of the panel), the cells were either directly harvested (control) or heat shock treated at 43°C for 90 min and then harvested. These samples were then used to probe the relative content of either *HSP70* mRNA (*A*) or protein (*C*). In parallel the cells, at the indicated hours after plating, were analyzed by pulse-labeling with [^{35}S]methionine for 15 min at 37°C either before (control) or after a 90-min, 43°C heat shock treatment (*B*). (*A*) Cytoplasmic RNA was prepared and 5 μg from each sample was hybridized to a ^{32}P-labeled *HSP70* gene-specific probe and digested with S_1 nuclease. The nuclease-resistant fragments were analyzed by electrophoresis on a 4% polyacrylamide/8 M urea gel. (*B*) Protein synthesis was analyzed by pulse-labeling cells with 50 μCi/ml [^{35}S]methionine for 15 min at 37°C. Equal numbers of cells were analyzed by SDS-PAGE on a 12.5% polyacrylamide gel. Shown is a fluorograph of the gel with the positions of the HSP70 and P72 proteins indicated on the right. (*C*) HSP70 accumulation in heat-shocked cells was determined by Western blotting. Lysates from equal numbers of heat-shocked cells were fractionated by SDS-PAGE and the proteins transferred to nitrocellulose. The filter was then probed with monoclonal antibody C92 followed by ^{125}I-labeled goat anti-mouse antibody. The autoradiograph of the filter is shown.

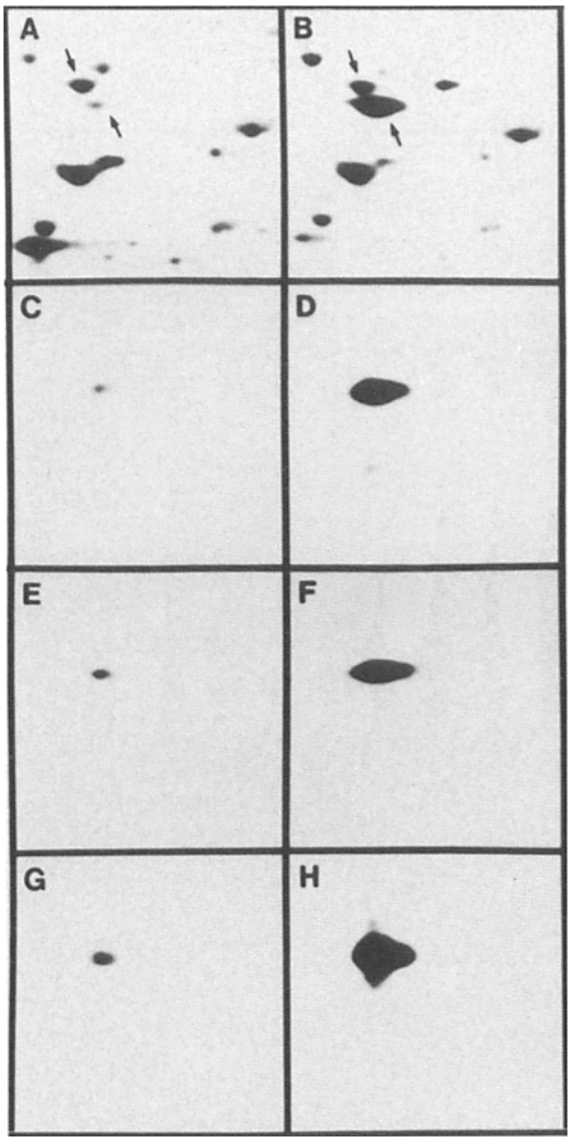


Figure 5. Specificity of monoclonal antibodies N6, N15, and N21 for HSP70. HeLa cells growing on 10-cm plastic dishes were labeled with 10 μCi/ml [^{35}S]methionine for 1 h at 37°C (control), or were incubated for 90 min at 43°C and then labeled at 37°C for 1 h (heat shock). The labeled cells were solubilized in Laemmli sample buffer, boiled 10 min, and used in immunoprecipitation studies with monoclonal antibodies N6, N15, and N21 as previously described (37). The control and heat shock cell lysates and the resultant immunoprecipitates were analyzed by two-dimensional gel electrophoresis (pH 5–7 isoelectric focussing in the first dimension, followed by 12.5% SDS-PAGE in the second dimension). (*A* and *B*) Control and heat shock cell lysates, respectively. (*C* and *D*) Control and heat shock immunoprecipitates using N6. (*E* and *F*) Control and heat shock immunoprecipates using N15. (*G* and *H*) Control and heat shock immunoprecipitates using N21. Downward pointing arrow in *A* and *B* indicates P72, while the upward pointing arrow indicates the highly stress-inducible HSP70 protein.

within either G_1 or S phase. We therefore examined the absolute levels of HSP70 protein after heat shock by immunoblotting. Mitotically synchronized HeLa cells were given a 90-min, 43°C heat shock treatment at either 8, 12, 21, or 23 h after plating. After heat treatment the cells were lysed in Laemmli sample buffer and equal numbers of cells were fractionated by SDS-PAGE. After transferring the proteins to nitrocellulose, the filter was probed with C92 followed by ^{125}I-labeled goat anti-mouse IgG. The amount of HSP70 that accumulated after heat shock was nearly identical in cells from all phases of the cell cycle (Fig. 4 *C*). Thus, we conclude that G_2 cells respond to heat shock with increased expression of HSP70 similar to that found for the G_1 and S phase cells, and that the inability to detect HSP70 by immunofluorescence using the C92 antibody is not due to the absence of the HSP70 protein.

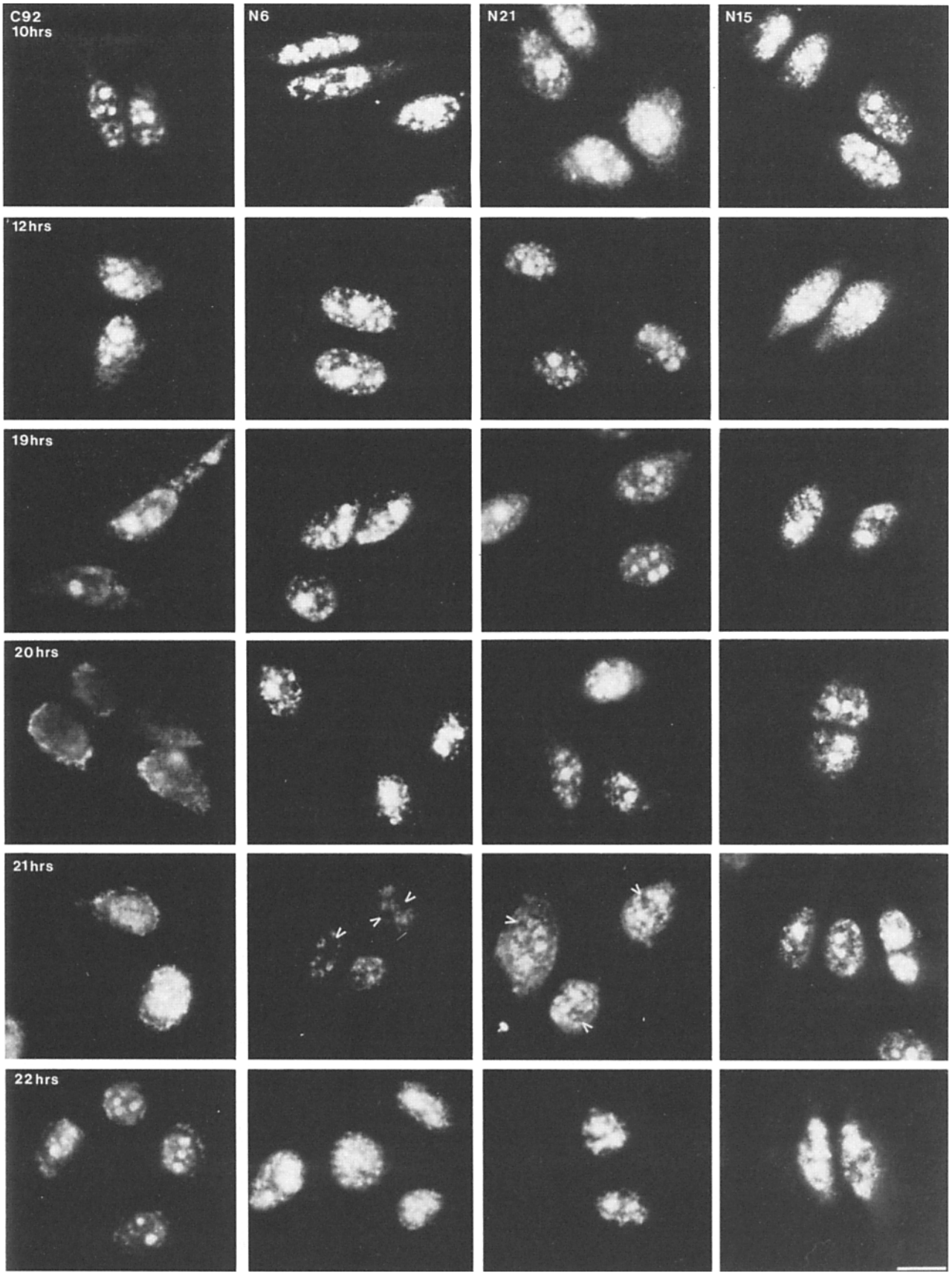
Cell Cycle–dependent Variation in the Accessibility of HSP70 Epitopes

Based on the results of the previous section, we suspected that the observed lack of immunofluorescence in the heat-shocked G_2 cells was due to an inability of the C92 antibody to bind its epitope. To address this possibility, we repeated the cell cycle experiments using other HSP70-specific monoclonal antibodies. The specificity of each antibody was first demonstrated by two-dimensional gel analysis of immunoprecipitates from [^{35}S]methionine-labeled lysates of asynchronous control and heat-shocked HeLa cells. As shown in Fig. 5, the monoclonal antibodies N6, N15, and N21 immunoprecipitated only HSP70, the highly inducible member of the 70-kD stress protein family.

These HSP70-specific monoclonal antibodies were then used to assess the distribution of HSP70 after heat shock at various points throughout the cell cycle similar to that described earlier using the C92 antibody. HeLa cells were synchronized by mitotic detachment and the harvested mitotic cells were plated on glass coverslips. At various times throughout the cell cycle, the coverslips were transferred to 43°C for 90 min. The cells were then fixed and stained with each of the four monoclonal antibodies (C92, N6, N15, N21). These results are presented in Fig. 6. Each vertical column represents a progression through the cell cycle with a single monoclonal antibody, whereas each horizontal row depicts the timepoint examined. Since all the coverslips in this experiment were generated from the same pool of mitotic cells, the results with the four different antibodies can be directly compared at each timepoint.

In late G_1 cells (10-h postplating, Fig. 6, row 1), the distribution of HSP70 after heat shock appeared very similar using the four different antibodies. Accumulation of HSP70 in the nucleus and its association with phase-dense nucleoli was observed. However, there were some minor differences with respect to the nonnucleolar, nuclear distribution of the protein. For instance, antibodies N21 and N15 revealed

Figure 6. Intracellular localization of HSP70 in heat-shocked cells throughout the cell cycle as determined by indirect immunofluorescence using monoclonal antibodies C92, N6, N21, and N15. HeLa cells were synchronized by mitotic detachment as described in Materials and Methods. At various times throughout the cell cycle, four coverslips were incubated at 43°C for 90 min and then fixed and processed for immunofluorescence as described in Materials and Methods. The timepoints shown are: row 1, late G_1 (10-h postplating); row 2, early S phase (12-h); row 3, late S/G_2 (19-h); row 4, G_2 (20-h); row 5, G_2 (21-h); row 6, G_2/M (22-h). Each vertical column represents the staining pattern as determined by incubation with C92, N6, N21, and N15, left to right, respectively. Bar, 10 μm.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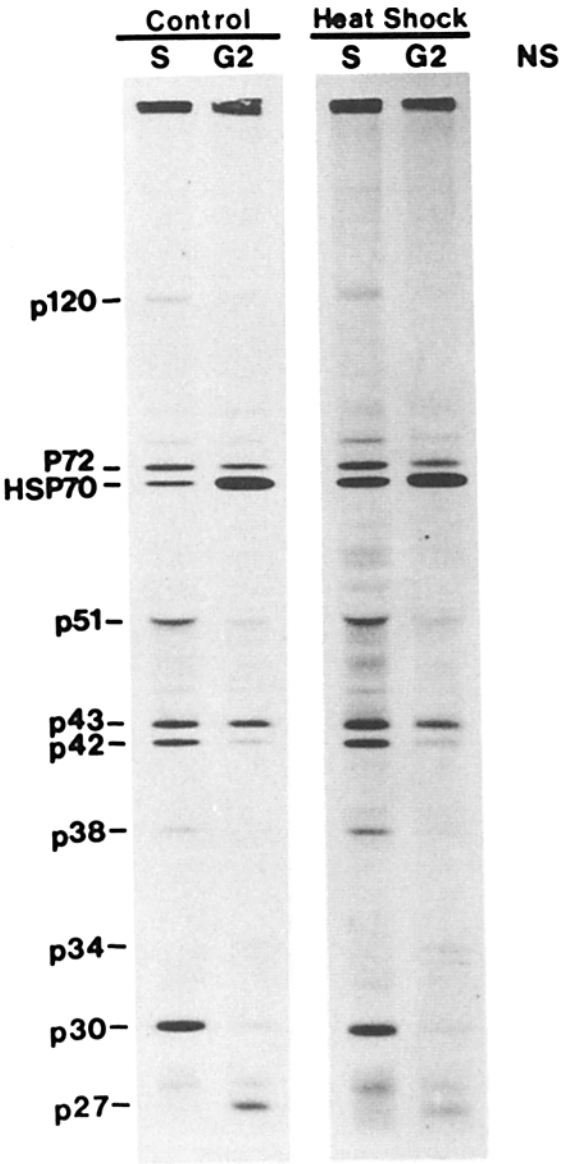

Figure 7. Nondenaturing immunoprecipitations from control and heat-shocked cell lysates of S phase and G_2 cells. HeLa cells were synchronized by mitotic detachment as described in Materials and Methods. The synchronized cells were labeled with [^{35}S]methionine at 37°C for a total of 6 h before harvesting in S phase or G_2. In parallel, cells labeled at 37°C for 4.5 h were incubated, still in the presence of radiolabel, at 43°C for 1.5 h. The labeled cell lysates were prepared for nondenaturing immunoprecipitation and incubated with monoclonal antibody C92 as described in Materials and Methods. The immunoprecipitated proteins were analyzed by SDS-PAGE. Shown is a fluorograph of the gel. The estimated molecular weights of precipitated proteins are indicated to the left. *NS*, proteins immunoprecipitated with a nonspecific antiserum.

more background nuclear staining than C92. As the cells progressed into S phase (12-h postplating, Fig. 6, row 2) the distribution of HSP70 after heat shock appeared similar to that observed for the heat-treated G_1 cells. However, as was shown in Fig. 3 *H*, cells heat shocked in early S phase exhibited a considerable amount of HSP70 in association with other nonnucleolar components, a result which is best illustrated with antibodies C92, N6, and N15 (Fig. 6, columns 1, 2, and 4).

As DNA synthesis subsided and the cells entered G_2 (19 and 20 h postplating, Fig. 6, rows 3 and 4) the four antibodies revealed distinct patterns of staining. Similar to the results described earlier, the C92 antibody revealed little detectable HSP70 associated with nucleoli after heat shock treatment of the G_2 cells (Fig. 6, column 1). In contrast, antibodies N6, N21, and N15 (Fig. 6, columns 2, 3, and 4, respectively) were able to detect HSP70 within the nucleoli of the heat-shocked G_2 cells. As the cells progressed through G_2 (21- and 22-h postplating, Fig. 6, rows 5 and 6) the nucleolar localization of HSP70 could again be detected by the C92 antibody (column 1). This disappearance and reappearance of the nucleolar staining of HSP70 was also observed with the N6 and N21 antibodies (Fig. 6, columns 2 and 3). At 21-h postplating (Fig. 6, row 5), the N6 antibody showed diminished overall fluorescence, with much of the staining restricted to nonnucleolar regions of the nucleus. The N21 antibody still revealed intense staining in areas of the nucleus, but these did not correspond to nucleoli. The nucleoli themselves appeared as dark regions in which HSP70 staining was excluded (Fig. 6, *arrowheads*).

Throughout the timecourse of the cell cycle, as well as in asynchronous heat-shocked HeLa cells, we observed minimal variation in the patterns of HSP70 staining when antibody N15 was used. In contrast, the ability to detect HSP70 within the nucleoli after heat shock using antibodies C92, N6, and N21 varied throughout the cell cycle.

Analysis of Proteins Associated with HSP70 by Nondenaturing Immunoprecipitation

When using the C92, N6, and N21 antibodies, the variability in immunostaining after heat shock at different points during the cell cycle was suggestive that the antigenic sites of HSP70 recognized by the antibodies were obscured; perhaps by cell cycle–specific interactions with other components. To address this possibility, we sought to identify proteins that may interact with HSP70 at different points in the cell cycle. HeLa cells were synchronized by mitotic detachment, and 6 h before harvesting the cells were labeled at 37°C with [^{35}S]methionine. In parallel, cells labeled at 37°C for 4.5 h were transferred to 43°C, still in the presence of label, for 90 min. Control and heat-shocked cells were then harvested in either G_1 (8-h postplating), S (13-h), or G_2 (21-h), and the cell lysates used in nondenaturing immunoprecipitations with the C92 antibody as described in Materials and Methods. Cellular proteins immunoprecipitated using HSP70-specific antibodies were analyzed by SDS-PAGE (Fig. 7).

When a nonspecific antiserum was used few, if any, proteins were found precipitated (Fig. 7, *NS*). However, when the C92 antibody was used, immunoprecipitations performed with lysates from control or heat-shocked cells resulted in the coprecipitation of a number of other proteins in addition to HSP70 (Fig. 7). Some of the coprecipitating proteins were present in both the control and heat shock samples at all points in the cell cycle. For example, p42, p43, and p72 were apparent in all of the immunoprecipitations. Analysis of these samples by two-dimensional gel electrophoresis revealed that the 72-kD protein present in the immunoprecipitates comigrated precisely with P72, the constitutive

member of the 70-kD stress protein family (data not shown). Under more severe (e.g., boiling in SDS sample buffer) conditions that disrupt protein–protein interactions, the C92 antibody does not immunoprecipitate P72 (38). Therefore, the presence of P72 in the immunoprecipitations performed under nondenaturing conditions suggests that HSP70 and P72 may interact with one another; a result consistent with previous studies showing these proteins to copurify over several steps of ion exchange and affinity chromatography (35).

Comparison of the immunoprecipitated proteins from control and heat-shocked cells, within the same point of the cell cycle, revealed that heat shock treatment did not significantly alter the pattern of coprecipitated proteins. In some experiments a few additional proteins were immunoprecipitated after the heat shock treatment. However, the overall pattern of proteins was very similar in control and heat-shocked cells, indicating that HSP70 may interact with some of the same proteins in stressed and unstressed cells.

While the patterns of coprecipitated proteins differed little when control and heat-shocked cells were compared, examination of immunoprecipitations performed at different points in the cell cycle revealed some differences. Specifically, proteins with molecular masses of ~120, 51, 38, and 30 kD were selectively enriched in the immunoprecipitates from S phase cells. Likewise, proteins of ~34 and 27 kD appeared to be enriched in the immunoprecipitates from G_2 cells (Fig. 7). We did not detect any G_1-specific proteins in the immunoprecipitations (data not shown). The changes in the patterns of coprecipitated proteins in S phase or G_2 cells indicate that different proteins may be in contact with HSP70 at different points in the cell cycle. Therefore, we suggest that the inability of certain monoclonal antibodies to detect HSP70 at specific points in the cell cycle may be due to cell cycle–specific interactions with other proteins, thereby masking the antigenic sites recognized by the antibodies.

Discussion

In the present study we have demonstrated that the ability of certain monoclonal antibodies to detect HSP70 within the nucleus and nucleolus of heat-shocked cells is a cell cycle–specific phenomenon. The cell cycle–dependent accessibility of HSP70 epitopes is suggestive of HSP70 interactions with other cellular components during the course of the cell cycle. Indeed, we have shown that HSP70-specific monoclonal antibodies will, under nondenaturing conditions, immunoprecipitate other cellular proteins distinct from HSP70, and that some of these proteins apparently associate with HSP70 within specific phases of the cell cycle.

Despite the vast literature characterizing the stress response in many different systems, the function of the stress proteins remains unknown. The 70-kD heat shock proteins have been reported to associate with many different cellular components, including the cytoskeleton, microtubules, the nuclear scaffold, hnRNA, and a cell surface glycoprotein (12, 14, 17, 32, 33). Whether these reported associations are relevant or whether they may simply reflect the abundance and the effusive distribution of the proteins in the cell is still unclear. However, the use of well-characterized, specific immunological reagents rather than biochemical fractionation techniques should provide clarification. The diversity of the results may actually reflect the ability of the 70-kD stress proteins to perform similar biochemical functions in many areas of the cell. Recent studies in yeast have also implicated the 70-kD stress proteins in the translocation of polypeptides across the membranes of the endoplasmic reticulum and mitochondria (7, 9).

Studies on the intracellular localization of HSP70, by both biochemical fractionation and by indirect immunofluorescence with HSP70-specific antibodies, have demonstrated that HSP70 is distributed throughout the cytoplasm and nucleus in unstressed cells (1, 31, 36). After heat shock treatment, HSP70 redistributes becoming concentrated in the nucleus and specifically associated with nucleoli (1, 16, 25, 31, 36, 38). To understand why some cells in an asynchronous population do not exhibit nucleolar staining and why this apparent heterogeneity is not observed when synchronized cells are examined, we used a collection of monoclonal antibodies to study the localization of HSP70 by indirect immunofluorescence in synchronized cells heat shocked at various points in the cell cycle. The antibodies appear to fall into two classes with regard to their ability to detect HSP70 within the nucleolus; those whose epitopes remained accessible throughout the cell cycle, and those that were unable to bind their epitope at a specific point in the cell cycle. Monoclonal antibody N15, an example of the first class, displayed little heterogeneity in nucleolar staining in asynchronous populations of heat-shocked cells. Consistently, N15 was able to detect nucleolar-localized HSP70 after heat shock throughout all points of the cell cycle examined. In contrast, when antibodies C92, N6, and N21 were used, nucleolar staining was not detected when cells in G_2 were heat shocked. Since we have demonstrated by immunoblotting and by immunofluorescence with N15 that a portion of the HSP70 protein is present within the nucleolus in the G_2 heat-shocked cells, we conclude that the lack of detection of nucleolar-distributed HSP70 by C92, N6, and N21 in G_2 cells indicates that the epitopes recognized by these antibodies periodically become inaccessible, perhaps due to interactions with other components. Furthermore, the timing of epitope inaccessibility varies slightly (19-h postplating for C92 vs. 21-h for N6 and N21) indicating that the presumed interactions may be very dynamic.

Using the various monoclonal antibodies we have demonstrated that several proteins coprecipitate with HSP70 under the nondenaturing conditions used here. These proteins either share a common epitope with HSP70, or they are precipitated through an association with HSP70. Since the HSP70 antibodies used here have been shown to precipitate only HSP70 from total cell lysates under denaturing conditions (Fig. 5; reference 38) we conclude that the proteins coprecipitating most likely are specific components with which HSP70 interacts.

Furthermore, these interactions can be divided into two classes: more dynamic interactions that appear to change at certain points in the cell cycle, and more static interactions that remain constant throughout the entire cell cycle. HSP70 itself is also cell cycle regulated, being expressed only in S phase in HeLa cells (20). In addition, some of the proteins that are precipitated in association with HSP70 may themselves be cell cycle–regulated proteins. For example, coprecipitation of p38 and p30, which were enriched in the immunoprecipitates from S phase cells, was only observed if the

cells were labeled at the G_1/S boundary. Cells labeled at any other point of the cell cycle did not show an association of p30 and p38 with HSP70 (data not shown). The interaction of HSP70 with p38 and p30 also appeared short-lived. When synchronized cells were pulse-labeled for 1 h at the G_1/S boundary, then chased in the absence of label, no p30 or p38 was observed to coprecipitate with HSP70 indicating that either the interaction of HSP70 with p38 and p30 is transient or the proteins themselves have short half-lives. Identification of these cell cycle–regulated proteins should help us to understand the function of HSP70, with respect to its expression and distribution in the nucleus during S phase.

In contrast to p30 and p38, a number of other proteins were found in apparent association with HSP70 at all points during the cell cycle. We have identified the 72-kD protein present in all of the immunoprecipitations as P72, the constitutive member of the HSP70 family. Since the antibodies used do not precipitate P72 under conditions that disrupt protein–protein interactions, these results indicate that P72 and HSP70 may interact with one another, or with similar proteins, and may function together in the cell. The two proteins are highly related structurally, both bind ATP and are extemely difficult to separate chromatographically (35). In addition, the recent demonstration that P72 and HSP70 show the same intracellular distribution after heat shock treatment supports the idea that they may function similarly in the cell (37).

When comparing the proteins associated with HSP70 before and after heat shock treatment we found few changes in the pattern of coprecipitated proteins. This could be explained, in part, by the nature of the experimental protocols used. For example, by indirect immunofluorescence analyses a dramatic redistribution of HSP70 from the cytoplasm to the nucleus and nucleolus is observed after heat shock (36). In the immunoprecipitation studies, in which the cells were solubilized in nonionic detergents, some of the nuclei and nucleoli may have been removed from the lysate during clarification. Thus, the immunoprecipitations may only have revealed a subset of HSP70–substrate interactions. Also, the labeling conditions and the time of harvest may have influenced the ability to detect new associations that occur after heat shock treatment. What is noteworthy, however, is the fact that most of the proteins found associated with HSP70 before heat shock treatment remained associated with the protein after heat shock. One interpretation of these results is that the function of HSP70 in response to stress may just be an extension of its role in the unstressed cell. Models have been proposed in which the physical interaction of HSP70 with other proteins or protein complexes is involved in their repair after heat shock (18, 24). Our results suggest that HSP70 is interacting with some of these same substrates in the unstressed cell. Consequently, this may explain the finding that in thermotolerant cells the presence of the stress proteins allows for the accelerated return to normal functioning of cellular components and processes known to be disrupted by the heat shock treatment (21, 37).

Proteins that interact with other members of the HSP70 family have been identified. For example, GRP78, related to HSP70 both immunologically and by sequence homology, is a luminal protein of the endoplasmic reticulum (22, 34). GRP78 binds to immunoglobulin heavy chains and improperly glycosylated or misfolded proteins in the endoplasmic reticulum (4, 10, 11, 22, 28). The apparent *Escherichia coli* homologue of HSP70, the dnaK protein, is involved in the replication of bacteriophage lambda, and has recently been shown to participate in the replication of oriC (41, 27). dnaK interacts directly with both lambda P, a phage-encoded replication protein, and grpE another *E. coli* heat shock protein (41, 42). Finally, in mammalian cells both the constitutive P72 and the heat-inducible HSP70 have been reported to bind clathrin and to facilitate the uncoating of clathrin-coated vesicles in vitro (6, 30). The same proteins have also been demonstrated in complexes with the product of the cellular oncogene, p53 (26). A common feature of all these interactions, and also of that between HSP70 and nucleoli, is that they appear dependent upon ATP (6, 18, 22, 30, 41). Thus, the 70-kD family of stress proteins appear to be involved in ATP-dependent interactions with a number of different cellular components; and therefore are likely to be involved in a variety of different cellular processes. Identification of some of the cellular proteins that HSP70 interacts with in human cells should provide new information concerning its role in various cellular processes.

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