Journal of Cell Science 104, 629-638 (1993)
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A stress-inducible 40 kDa protein (hsp40): purification by modified two-dimensional gel electrophoresis and co-localization with hsc70(p73) in heat-shocked HeLa cells

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SUMMARY

We have previously reported that a novel 40 kDa protein is induced by heat shock and several environmental stresses in mammalian and avian cells and that the N-terminal amino acid sequence of this 40 kDa protein has homology with the bacterial DnaJ heat-shock protein. We have purified this protein (40 kDa heat-shock protein, hsp40) from HeLa cells by modified two-dimensional gel electrophoresis and generated a polyclonal antibody against hsp40. This antibody was highly specific for human hsp40 and cross-reacted weakly with rat and Chinese hamster hsp40. Indirect immunofluorescence revealed that the hsp40 in HeLa cells accumulates in the nucleus, especially in the nucleolus, during heat shock and returns to the cytoplasm during the recovery period. The kinetics of the accumulation in the nucleoli and subsequent return to the cytoplasm of hsp40 was similar to that of hsp70. In addition, hsp40 was co-localized with hsc70(p73) in heat-shocked HeLa cells as demonstrated by double immunofluorescence staining. These results suggest that hsp40 (a DnaJ homologue) and hsp70 (a DnaK homologue) may act in concert to repair (refold) denatured proteins and protein aggregates in the nuclei and nucleoli of heat-shocked HeLa cells.

Key words: hsp40 (DnaJ homologue), hsp70 (DnaK homologue), co-localization

INTRODUCTION

Several polypeptides are known as heat- or stress-inducible proteins in both prokaryotes and eukaryotes. They are commonly referred to as heat-shock proteins (hsps) and are highly conserved throughout evolution (Schlesinger et al., 1982; Atkinson and Walden, 1985; Pardue et al., 1989). In the last several years it has become clear that significant amounts of these hsps are present in cells even in the absence of stress. The current notion is that these hsps perform basic and indispensable cellular functions at normal growth temperature in addition to protecting cells from stress-related deleterious effects. For example, the members of the hsp70 family and hsp60 have been shown to have a molecular chaperoning activity, involving them in the folding and assembly of nascent proteins, and in their translocation across the membranes of the endoplasmic reticulum and mitochondria (Chirico et al., 1988; Deshaies et al., 1988; Cheng et al., 1989; Beckman et al., 1990). It has also been suggested that hsp70 and its bacterial homologue, DnaK, associate with partially denatured (unfolded) proteins induced by heat shock and repair (refold) them using ATP as an energy source (Pelham, 1984, 1986; Skowyra et al., 1990).

Ohtsuka et al. (1990) have recently identified a novel hsp, hsp40, a 40 kDa protein induced by heat shock, sodium arsenite, cadmium chloride or azetidine carboxylic acid in mammalian and avian cells, in addition to the classical hsps. The N-terminal amino acid sequence of the human hsp40

has been shown most recently to share a limited homology with the bacterial DnaJ heat-shock protein; 24 of 48 amino acid residues are identical (50% identity, 69% similarity) (Hattori et al., 1992). The human hsp40 is also homologous to DnaJ-related proteins in yeast such as SCJ1, SEC63, YDJ1 and SIS1 (Hattori et al., 1992). Therefore, we conclude that the hsp40 is a mammalian homologue of DnaJ protein (Hattori et al., 1992), joining hsp90, hsp70 and hsp60, which are the eukaryotic homologoues of the bacterial hsps, HtpG, DnaK and GroEL, respectively (for review, see Georgopoulos et al., 1990). We have also shown that under certain conditions, hsp40 in HeLa cells is translocated into the nuclei and nucleoli by a brief heat shock in a manner analogous to hsp70 (Ohtsuka et al., 1986a), by using indirect immunofluorescence (Hattori et al., 1992).

In mammalian cells, the hsp70 family is known to consist of at least four major members, grp78 (localized in endoplasmic reticulum), grp75 (mitochondria), constitutive hsc70 (p73, cytoplasm) and inducible hsp70 (p72, cytoplasm) (for reviews, see Pelham, 1986; Welch, 1990). In this report, we refer to the constitutive form as hsc70 or p73 and to the inducible form as p72; the general term hsp70 implies both hsc70(p73) and p72.

In this report, we describe the characterization of a polyclonal antibody against hsp40, the comparison between the kinetics of translocation of hsp40 and hsp70 into the nucleoli and the subsequent return to the cytoplasm, and their co-localization in the nucleoli of heat-shocked HeLa cells. The method of the purification of hsp40 by modified two-dimensional gel electrophoresis is also described in detail.

MATERIALS AND METHODS

Cell culture, heating and pulse labeling

HeLa cells, NRK (normal rat kidney) cells, heat-resistant variants of HA-1 Chinese hamster fibroblasts (3021; Laszlo and Li, 1985), mouse mastocytoma cells (FMA1), chick embryo fibroblasts (CEF) were grown in Dulbecco's modified Eagle's minimal essential medium (Nissui, Tokyo, Japan) supplemented with 10% fetal bovine serum (Hazleton Res. Pro. Inc., Lenexa, KS). *Drosophila* Kc cells (Echalier and Ohanessian, 1970, a kind gift from Dr. F. Hirose of Aichi Cancer Center Research Institute) were grown in M3 (BF) medium (Cross and Sang, 1978) supplemented with 2% fetal bovine serum.

Cells at subconfluence in 100 mm or 35 mm culture dishes (Corning Glass Works, Corning, NY) were heated by immersing the dishes sealed with Parafilm (Amerian Can Co., Greenwich, CT) into a water bath, the temperature of which was controlled within ± 0.1 deg. C (Hayashi et al., 1991). After various treatments, cells were lysed in sodium dodecyl sulfate (SDS)-sample buffer (Laemmli, 1970) and boiled for 5 min, for one-dimensional SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

For pulse labeling, HeLa cells were heated at 45°C for 15 min and incubated at 37°C for the indicated period, then labeled with 0.74 MBq (20 μCi) of [^{35}S]methionine per plate (Tran^{35}S-label, 40.81 TBq/m mole L-methionine, ICN Biomedical Inc., Irvine, CA) for 1 h in methionine-free culture medium supplemented with 10% dialyzed fetal bovine serum. Control, non-heated cells were labeled at 37°C for 1 h in a similar manner. Cells were lysed by sonication in O'Farrell's lysis buffer (O'Farrell, 1975) (9.5 M urea, 2% Nonidet P-40, 5% 2-mercaptoethanol, 2% Pharmalyte (pH 3-10) (Pharmacia LKB Biotech., Sweden)) containing 1 μg/ml leupeptin, 1 μg/ml pepstatin and 1 mM phenylmethylsulphonyl fluoride (PMSF) and stored frozen at −70°C until use. Protein levels were quantitated by the Pierce protein assay kit (Pierce Chemical Co., Rockford, IL).

Gel electrophoresis

One-dimensional SDS-PAGE was done by Laemmli's method (1970) on a slab gel containing 10% acrylamide. Two-dimensional gel electrophoresis using non-equilibrium pH gradient electrophoresis (NEPHGE) (O'Farrell et al., 1977) as the first dimension was performed at 400 V for 3.5 h. The second dimension was run according to the methods of Laemmli (1970). Equal amounts of protein were analyzed in each sample by both methods. Following electrophoresis, the gels were stained with either Coomassie brilliant blue R-250 (CBB) or using a silver staining kit (Daiichi Pure Chemical Co., Tokyo, Japan), and photographed. The stained gels were then processed for fluorography as described (Ohtsuka et al., 1986b).

One-dimensional peptide mapping using V8 protease (Sigma Chemical Co., St. Louis, MO) was performed according to the methods of Cleveland et al. (1977).

Purification of hsp40

hsp40 was purified by a modified two-dimensional gel electrophoresis technique. HeLa cells growing at subconfluence in 100 mm dishes (a total of 500 dishes; 5×10^9 cells) were heated at 45°C for 15 min and allowed to recover for 16 h at 37°C, then lysed in O'Farrell's (1975) lysis buffer. NEPHGE in the first dimension was performed using a slab gel (135 mm × 145 mm × 2 mm) instead of a rod gel (130 mm × 2.5 mm). The position of hsp40 was determined using coloured pI markers (Oriental Yeast Co., Osaka, Japan). hsp40 migrated between pI values of 9.7 and 8.3. The portion of the gel containing hsp40 was cut out, applied to the second dimension, and hsp40 was detected as a band in the slab gel by CBB staining. This method, which we call Slab-NEPHGE/SDS-PAGE two-dimensional gel electrophoresis, allowed us to apply to the first-dimension gel 30-40 times the amount of protein (approximately 10 mg from 10 dishes) that can be applied under ordinary two-dimensional gel electrophoretic conditions. The band containing hsp40 was cut out, and the protein was eluted electrophoretically from the gel slice using an Amicon Centrilutor (Amicon Corp., Lexington, MA). Approximately 500 μg of purified hsp40 was obtained from 50 Slab-NEPHGE/SDS-PAGE gels.

Preparation of antibodies

An anti-hsp40 polyclonal antibody was obtained by immunizing a rabbit with purified hsp40 as described previously (Hattori et al., 1992). The anti-hsp70 polyclonal antibody used in this study was obtained as described (Ohtsuka et al., 1986b). This antibody is specific for both constitutive p73 and inducible p72.

Rat monoclonal antibodies against constitutive p73 were obtained as described in detail elsewhere (Ohtsuka, Laszlo et al. unpublished data). In brief, p73 was purified by standard methods (Welch and Feramisco, 1985) from heat-resistant variants of HA-1 Chinese hamster fibroblasts (Laszlo and Li, 1985) which had been exposed to sodium arsenite. Analysis of the purified materials with polyclonal antibody against hsp70 indicated the presence of p73 only. This material was used to generate monoclonal antibodies by fusion of spleen cells of immunized rat with the Y3-Ag1.2.3 rat myeloma cells using standard methods (Harlow and Lane, 1988). For double immunofluorescence studies, we used 1B5, one of the two monoclonal antibodies that we have isolated.

Immunological methods

For immunoblotting, the proteins of total cellular lysates were separated by SDS-PAGE and transferred to a nitrocellulose membrane (Schleicher & Schuell, Inc., Keene, NH) using the methods of Towbin et al. (1979). The membrane was blocked with 5% skim milk in TBS (50 mM Tris-HCl, pH 8.0, 154 mM NaCl) for 2 h. Anti-hsp40 polyclonal antibody (1/1,000 dilution), anti-hsp70 polyclonal antibody (1/400 dilution) or anti-p73 monoclonal antibody (1/200 dilution) were used as the first antibodies. Horseradish peroxidase-conjugated goat anti-rabbit IgG (Zymed Laboratories, Inc., So. San Francisco, CA) or goat anti-rat IgG (Zymed) both at 1/1,000 dilution were used as the second antibodies. Peroxidase activity was detected using 4-chloro-1-naphthol as a substrate.

For immunoprecipitation, HeLa cells were grown in 100 mm dishes. Cells were heated at 45°C for 15 min and incubated at 37°C for 6 h; for the last 5 h of recovery, cells were labeled with 0.74 MBq (20 μCi) of [^{35}S]methionine per plate in methionine-free medium. Control cells were labeled with the same medium at 37°C for 5 h. After labeling, cells were washed with cold PBS and lysed in RIPA buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS), containing 1 μg/ml leupeptin, 1 μg/ml pepstatin and 1 mM PMSF. The cell lysates were clarified by centrifugation for 5 min at 10,000 revs/min. Approximately the same amounts of TCA-precipitable radioactivity from control and heated cells were preabsorbed with 20 ml of insoluble Protein A (Zysorbin, non-viable *Staphylococcus aureus*, Zymed), which was previously boiled for 15 min in SDS-sample buffer and then washed three times with RIPA buffer. After 30 min of incubation, the lysates were clarified again. The supernatants were then incubated with 20 μl of anti-hsp40 antibody for 30 min at room temperature. Zysorbin (20 μl) was then added and the incubation continued for 10 min. The immunoprecipitates were collected by centrifugation and washed five times with RIPA buffer. The precipitated proteins were released from the Zysorbin by the addition of SDS-sample buffer followed by boiling for 5 min. The immunoprecipitates were then analyzed by SDS-PAGE followed by fluorography.

For the analysis of the intracellular localization of hsp40 and hsp70, HeLa cells were grown on glass coverslips (9 mm × 9 mm) in 35 mm dishes. After various treatments, cells were washed with cold PBS, and fixed in 100% methanol at −20°C for 10 min. The cells were then treated with 10% normal goat serum (MBL, Nagoya, Japan) in PBS for 1 h to inhibit nonspecific binding, and processed for immunofluorescence staining using the anti-hsp40 polyclonal antibody (1/100 dilution) or the anti-hsp70 polyclonal antibody (1/60 dilution) as the first antibody, and FITC-conjugated goat anti-rabbit IgG (H+L) (Zymed) as the second antibody (1/50 dilution). For double immunofluorescence staining, the first antibody was a mixture of rabbit anti-hsp40 polyclonal antibody (1/100 dilution) and rat anti-p73 monoclonal antibody (1/60 dilution), while the second antibody was a mixture of rhodamine-conjugated anti-rabbit IgG (1/20 dilution, Cappel, West Chester, PA) and FITC-conjugated anti-rat IgG (1/50 dilution, Zymed). Cells were photographed through a Fluorophoto microscope (Nikon, Tokyo, Japan), using Tri-X Pan (ASA 400, Kodak) for black-and-white prints and Kodacolor (ASA 400, Kodak) for colour prints. Exposure time was exactly the same for each photograph in one series of experiments. Cells displaying brightly stained nucleoli were counted as positively staining cells as described previously (Ohtsuka et al., 1986a; Hayashi et al., 1991).

Densitometry

The relative rate of hsp40 synthesis was measured by densitometry of fluorographs of two-dimensional gels using a VIDAS image analyzer (Zeiss, Germany), and relative amounts of hsp40, p73 and p72 detected by immunoblotting were measured using a CS-930 chromatoscanner (Shimadzu, Kyoto, Japan).

RESULTS

Purification of hsp40 by modified two-dimensional gel electrophoresis

Attempts to purify human hsp40 using conventional column chromatographic methods have not been successful so far. In order to prepare an antibody against hsp40, we purified hsp40 from HeLa cells using a modified two-dimensional gel electrophoretic technique (Slab-NEPHGE/SDS-PAGE), as described in Materials and Methods. In contol, non-heated HeLa cells, hsp40 was barely detectable (Fig. 1A). In contrast, the levels of hsp40 were markedly increased in cells exposed to a 45°C, 15 min heat-shock followed by 16 h recovery at 37°C (Fig. 1B). hsp40 migrated between pI values of 9.7 and 8.3 on Slab-NEPHGE, determined by using coloured pI markers, consistent with our previous observation that the pI value of hsp40 was approximately 9.2 in HeLa cells (Ohtsuka et al., 1990). When the portion of the gel containing hsp40 was cut out and applied to the second-dimension SDS-PAGE, the band corresponding to hsp40 was barely detectable in control cells (arrowhead in Fig. 1C), while it migrated as a major band in the lysate from heat-shocked cells (arrowhead in Fig. 1D). This band was cut out and hsp40 was eluted electrophoretically. The hsp40 purified in this manner migrated as a single band in one-dimensional SDS-PAGE (Fig. 1E). The protein contained in the hsp40-associated spot in Fig. 1B was identical with the protein contained in the hsp40-associated band in Fig. 1D as demonstrated by one-dimensional peptide mapping (Fig. 1F). The purified hsp40 migrated as two spots by two-dimensional gel electrophoresis (Fig. 1G). The left (basic) spot in Fig. 1G corresponded to the original hsp40 in Fig. 1B, since it co-migrated with hsp40 contained in control or heat-shocked HeLa cell lysates (unpublished observation). The right (acidic) spot in Fig. 1G with slightly higher molecular size might represent a modified form of hsp40 generated during purification, since both spots showed essentially the same pattern in one-dimensional peptide mapping (unpublished results). Therefore, since these two spots contain peptides associated with hsp40 and no other material was detected in this gel by silver staining, we concluded that the hsp40 isolated in this manner was essentially pure. We have found the acidic form of hsp40 in whole cell lysates at 24-36 hours after the initial heat shock. This form was detected by both pulse-chase experiments and CBB staining (unpublished data). The exact nature of the modification associated with the acidic form is not clear at this time.

Antibody characterization

The purified hsp40 described in the previous section was used as antigen for the generation of an anti-hsp40 polyclonal antibody. A newly obtained anti-p73 monoclonal antibody (unpublished data) was also used in this study. We characterized these antibodies by immunoblotting and/or

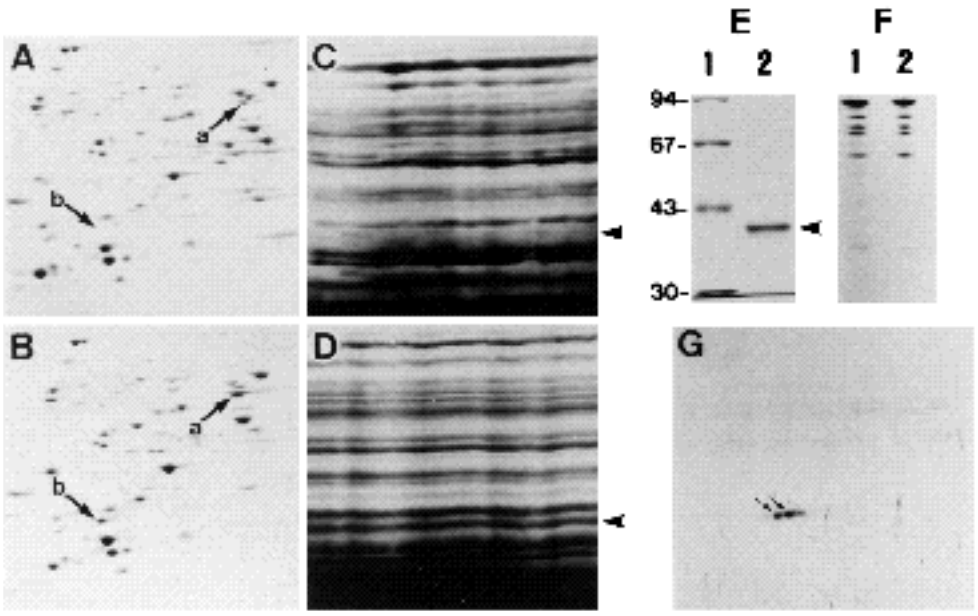

Fig. 1. Purification of hsp40 from HeLa cells by Slab-NEPHGE/SDS-PAGE two-dimensional gel electrophoresis. Lysates of control (A and C) and heat-shocked (45°C for 15 min followed by recovery at 37°C for 16 h) (B and D) cells were subjected to ordinary two-dimensional gel electrophoresis (A and B) or Slab-NEPHGE/SDS-PAGE (C and D) as described in Materials and Methods. Gels were stained with CBB. The acidic end of the first dimension is on the right-hand side of (A) and (B). a and b in (A) and (B) indicate inducible hsp70(p72) and hsp40, respectively. The arrowhead in (C) and (D) indicates hsp40. (E) One-dimensional SDS-PAGE analysis of purified hsp40. Lane 1, molecular mass markers, phosphorylase *b* (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), and carbonic anhydrase (30 kDa); lane 2, purified hsp40 (0.3 μg). The gel was stained with CBB. (F) V8 protease partial peptide mapping analysis of hsp40. Lane 1, the spot of hsp40 indicated as b in (B); lane 2, the band of hsp40 indicated by the arrowhead in (D). The gel was silver stained. (G) Two-dimensional gel (NEPHGE/SDS-PAGE) analysis of purified hsp40 (0.5 μg). The purified hsp40 showed two spots (double arrow). See the text for more details. The acidic end of the first dimension is to the right. The gel was silver stained. Please note the absence of any other material on the gel.

immunoprecipitation. On immunoblots, the anti-hsp40 antibody specifically recognized a 40 kDa band in lysates from heat-shocked cells (Fig. 2A, lanes 1 and 2), as well as the purified hsp40 (Fig. 2A, lane 3). The antibody reacted with only one spot in two-dimensional immunoblots (Fig. 2B and C); this spot corresponded exactly to the spot associated with hsp40 identified by CBB staining (Fig. 1B). The high specificity of the anti-hsp40 serum for hsp40 was also demonstrated by non-denaturing immunoprecipitation (Fig. 2D, lanes 7 and 8). Several bands ranging from 43 to 70 kDa appear to coprecipitate with hsp40. These bands, however, were considered to be nonspecific, since they were also precipitated by the preimmune serum (Fig. 2D, lane 6). Similar results were obtained when immunoprecipitation was performed in RIPA buffer without SDS (data not shown). Thus, the anti-hsp40 antibody appears to specifically recognize both the native and denatured forms of hsp40. Specific association of hsp40 with other proteins was not detected under our experimental conditions. The results of the foregoing experiments also indicated that hsp40, while barely detectable, if at all, in control, non-heated HeLa cells, is remarkably induced by heat shock, consistent with the results in Fig. 1A and B and with our previous reports (Ohtsuka et al., 1990; Hattori et al., 1992).

Next, we examined the species specificity of the polyclonal anti-hsp40 and anti-hsp70 antibodies and the monoclonal anti-p73 antibody. As shown in Fig. 3A, the anti-hsp40 antibody was highly specific for hsp40 in HeLa (human) and NRK (rat) cells (lanes 1-4). The antibody also reacted faintly with hsp40 from 3012 (Chinese hamster) cells (Fig. 3A, lanes 7 and 8). hsp40 from FMA1 (mouse) and CEF (chick) cells was not detected under these experimental conditions, which are known to induce elevated levels of hsp40 (Ohtsuka et al., 1990). However, a slight cross-reaction was observed at a higher concentration of the antibody (1/100 dilution) in these cells (data not shown). No cross-reacting material was detected in lysates of Kc (*Drosophila*) cells even at high concentrations of the antibody (Fig. 3A, lanes 11 and 12). The same samples were also probed with an anti-hsp70 antibody (Fig. 3B) (Ohtsuka et al., 1986b), which recognized both p73 and p72. In HeLa cells, both p73 and p72 were expressed constitutively at 37°C, and p72 was increased approximately fivefold by heat shock (Fig. 3B, lanes 1 and 2). In other cells, only p73 was detected at normal growth temperature, while both p73 and p72 were detected under heat-shock conditions except in CEF (Fig. 3B, lanes 3-12). The anti-p73 monoclonal antibody was specific for p73 in all cells examined; it did not react with Kc cells (Fig. 3C). Overall these results indicate that the anti-hsp40 antibody appears to be more species specific than the anti-hsp70 antibodies.

Induction of hsp40 in HeLa cells

We have previously reported that the extent of the induction of hsp40 was dependent both on the specific cell lines studied and on the types of stress (Ohtsuka et al., 1990). In HeLa cells, a heat shock of 15 minutes at 45°C was most effective at inducing hsp40. We examined the kinetics of induction and accumulation of hsp40 in HeLa cells after such a heat shock by pulse-labeling with [^{35}S]methionine and immunoblotting with anti-hsp40 antibody (Fig. 4). The elevated synthesis of hsp40 was detected 1-2 h after the heat shock, reached a maximum at 5-6 h; this was followed by a gradual decrease until its synthesis was not detected by 12 h after the heat shock (Fig. 4A). The relative rate of hsp40 synthesis was determined by densitometric scanning

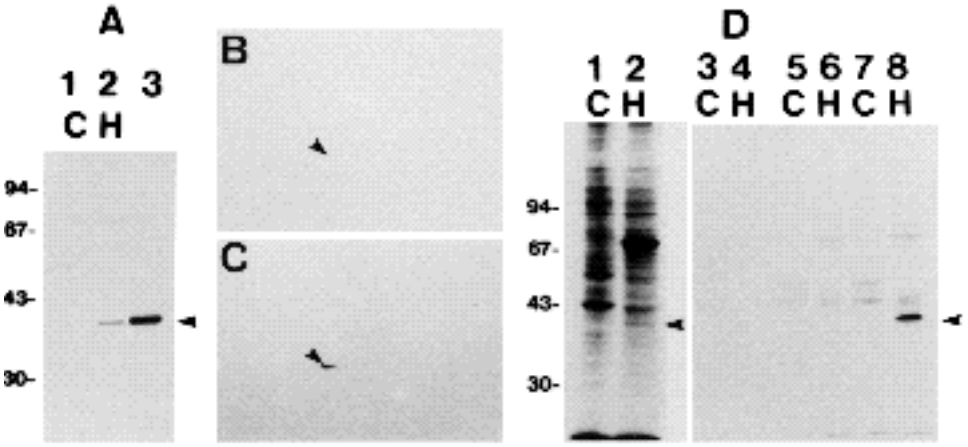

Fig. 2. Characterization of a polyclonal antibody against hsp40. (A) One-dimensional immunoblot analysis with the antibody against hsp40. Lane 1, whole cell lysate of control HeLa cells; lane 2, whole cell lysates of heat-shocked HeLa cells (45°C for 15 min then incubated for 16 h at 37°C); lane 3, purified hsp40 (0.3 μg). (B and C) Two-dimensional immunoblot analysis with the same antibody. Lysates from control (B) or heat-shocked (C) HeLa cells were subjected to NEPHGE/SDS-PAGE and processed for immunoblotting. (D) Immunoprecipitation analysis of the antibody. Lanes 1, 3, 5 and 7, HeLa cells labeled with [^{35}S]methionine for 5 h at 37°C; lanes 2, 4, 6 and 8, HeLa cells heated at 45°C for 15 min then incubated at 37°C for 6 h and labeled with [^{35}S]methionine for the last 5 h. Lanes 1 and 2, whole cell lysate; lanes 3 and 4, immunoprecipitates obtained with Zysorbin alone; lanes 5 and 6, immunoprecipitates obtained with the preimmune serum; lanes 7 and 8, immunoprecipitates obtained with the anti-hsp40 antibody. After electrophoresis, the gel was processed for fluorography. The arrowheads indicate the location of hsp40.

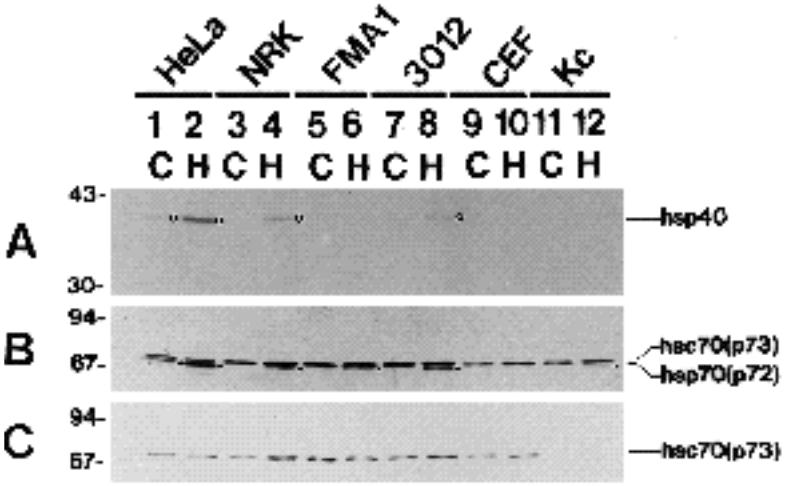

Fig. 3. Immunoblot analysis of the species specificity of antibodies used in this study. (A) Immunoblot of lysates from HeLa (human, lanes 1 and 2), NRK (rat, lanes 3 and 4), FMA1 (mouse, lanes 5 and 6), 3012 (Chinese hamster, lanes 7 and 8), CEF (chick, lanes 9 and 10) and Kc (*Drosophila*, lanes 11 and 12) cells probed with the anti-hsp40 antibody. Odd numbers, non-heat-shocked control cells; even numbers, heat-shocked cells. HeLa, NRK and 3012 cells were heated at 45°C for 15 min then incubated at 37°C for 16 h. FMA1 cells were heated at 42°C for 4 h and incubated at 37°C for 2 h in suspension culture with gentle shaking. CEF cells were heated at 46°C for 30 min and incubated at 37°C for 16 h. Kc cells (normal growth temperature is 25°C) were heated at 36°C for 1 h then incubated at 25°C for 6 h. Small circles on the right of some lanes indicate the location of hsp40. (B) The samples shown in (A) were probed with a polyclonal antibody against hsp70. The upper band indicates the constitutive p73 and the lower band the inducible p72. Small dots on the right of some lanes indicate the location of p72. (C) The samples shown in (A) were probed with an anti-p73 monoclonal antibody (1B5). Note that this antibody is specific for p73.

of the gels illustrated in Fig. 4A, the results of which are plotted in Fig. 4D (filled circles). The amount of hsp40 detected by immunoblotting increased gradually after the heat shock and reached near maximum at 8 h, and it remained relatively constant thereafter up to 24 h (Fig. 4C and D, open circles). The relative amounts of p73 and p72 were also measured by immunoblotting (Fig. 4B), and quantitated by densitometry (Fig. 4D, open squares and open triangles, respectively). These data indicate that the kinetics of accumulation of hsp40 in HeLa cells after a 15 min, 45°C heat shock were similar to the kinetics of accumulation of p72.

Heat-shock-induced alterations in the localization of hsp40

In mammalian cells, both major forms of hsp70, p73 and p72, are localized mostly in the cytoplasm at normal growth temperatures and are translocated rapidly into the nuclei and the nucleoli by heat shock, followed by a gradual return to the cytoplasm during the recovery period after heat shock (Pelham, 1984; Welch and Feramisco, 1984; Ohtsuka et al., 1986a; Welch and Mizzen, 1988). In a previous report, we presented some preliminary evidence that hsp40 is also translocated into nuclei and nucleoli by heat shock (Hattori et al., 1992). We compared the kinetics of the translocation of hsp40 into the nucleoli and its subsequent return to the cytoplasm during and after a 42°C, 2 h heat shock to the kinetics of translocation of hsp70 under the same conditions in HeLa cells. As shown in Fig. 5A, no apparent fluorescence signal was observed in control cells with the anti-hsp40 polyclonal antibody. In contrast, when cells were heated at 42°C for 2 h, large and small globular structures in the nuclei, corresponding to phase-dense nucleoli (see Fig. 7), were stained brightly (Fig. 5B). In other experiments, we found an increased amount of hsp40 in whole cells, especially in the nuclear fraction, after a 42°C, 2 h heat shock by immunoblotting with the same antibody (Ohtsuka and Hattori, unpublished results). These results indi-

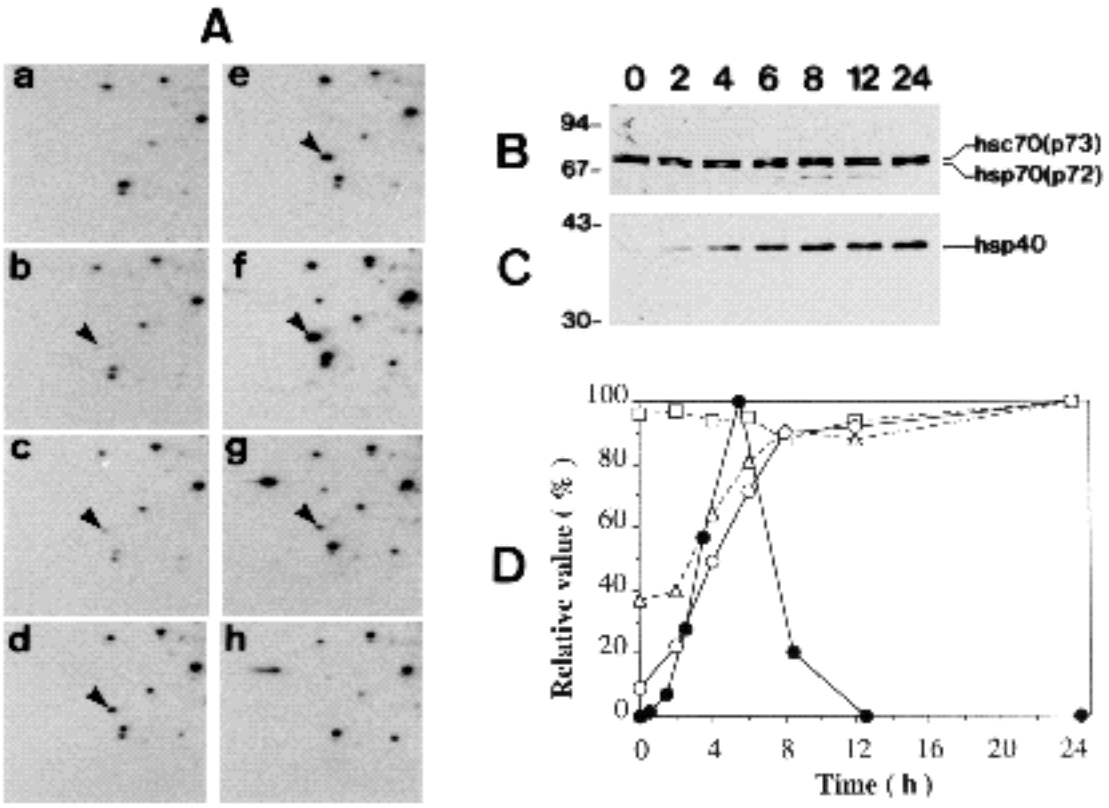

Fig. 4. Induction and accumulation of hsp40 in HeLa cells. (A) Cells growing at 37°C were pulse-labeled with [^{35}S]methionine for 1 h at 37°C (a); cells exposed to 45°C for 15 min were allowed to recover at 37°C, and then pulse-labeled with [^{35}S]methionine for 1 h at 0-1 (b), 1-2 (c), 2-3 (d), 3-4 (e), 5-6 (f), 8-9 (g) and 12-13 h (h) of recovery. After labeling, cells were lysed and subjected to NEPHGE/SDS-PAGE followed by fluorography. Approximately equal amounts of proteins were loaded in the first dimension. Fluorographs of gels around hsp40 are shown with the acidic end to the right. Arrowheads indicate the location of hsp40. (B) Accumulation of p73 and p72 in cells after heat shock as determined by immunoblot analysis with the polyclonal antibody against hsp70. Cells were heated at 45°C for 15 min and incubated at 37°C for a time period (hours) indicated above each lane, then washed with PBS, lysed in SDS-sample buffer and subjected to one-dimensional SDS-PAGE followed by immunoblotting. Lane 0, control non-heat-shocked cells. The upper band corresponds to p73 and the lower band to p72. (C) Accumulation of hsp40 in cells after heat shock. The samples shown in (B) were probed with the polyclonal antibody against hsp40. A small amount of hsp40 was detected in the control cells in this preparation (lane 0). (D) Relative rate of hsp40 synthesis (●) quantitated by densitometry of each fluorograph in (A) using a VIDAS image analyzer. Maximum value of the relative synthesis (5-6 h after the heat shock) was taken to be 100%. The relative amounts of hsp40 (○), p73 (□) and p72 (△) in cells at various times of recovery from the 15 min at 45°C heat shock were quantitated by densitometry of each immunoblot in (B) and (C) using a CS-930 chromatoscanner. The maximum values observed in the 24 h samples were taken to be 100%. The abscissa indicates incubation time (h) at 37°C after the heat shock.

cate that hsp40 was induced during heating to 42°C and accumulated in the nuclei and the nucleoli. During recovery at 37°C from the 42°C, 2 h heat shock, the nucleolar staining with the anti-hsp40 antibody gradually diminished, and by 9 h recovery no appreciable nucleolar staining was observed (Fig. 5C-F). This result indicates that hsp40 returned to the cytoplasm, since the cytoplasm was stained brightly at 6-9 h, and no appreciable degradation products were observed during the recovery period as determined by immunoblotting (see Fig. 2A, lane 2, or Fig. 3A, lane 2). HeLa cells were also stained with an anti-hsp70 polyclonal antibody under the same heating conditions. The cells with brightly stained nucleoli were counted as positively stained cells and the percentage of the stained cells was plotted against time during and after heating (Fig. 6). The kinetics of the heat-induced translocation of hsp40 were very similar to that of hsp70 during and after heating at 42°C for 2 h (Fig. 6A). We obtained similar results when cells were heated at 45°C for 15 min, allowed to recover at 37°C for 16 h to increase the amount of hsp40 present, and then heated again at 43°C for 30 min and allowed to recover at 37°C for up to 4 h (Fig. 6B).

Co-localization of hsp40 with hsp70 (p73)

As described in the previous section, during the recovery period after heat shock, hsp40 and hsp70 were localized in the nucleoli in some cells but not in others. Therefore, we examined whether or not the nucleolar localization of hsp40 and hsp70 occurred in the same cells under these conditions, by double immunofluorescence staining using the anti-hsp40 polyclonal antibody and the p73-specific monoclonal antibody. HeLa cells were heated at 45°C for 15 min, incubated at 37°C for 16 h, and then heated again at 43°C for 30 min. Immediately after the second heat shock, both hsp40 and p73 were localized in the nucleoli in the majority of the *same* cells (Fig. 7E and F). During the recovery

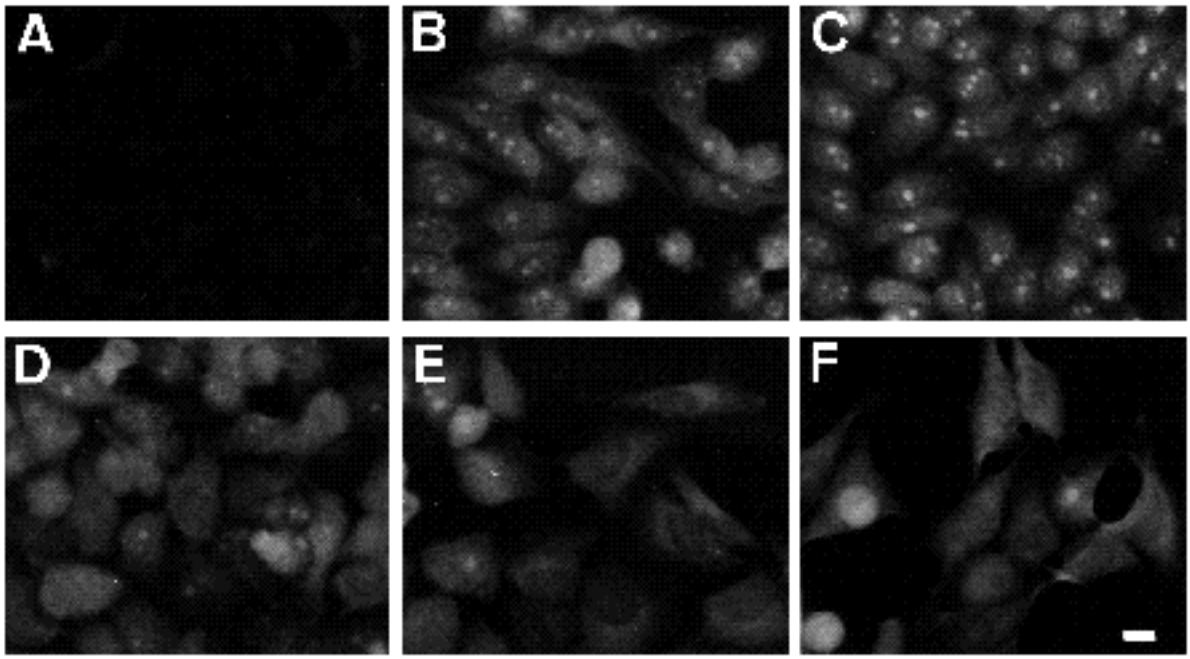

Fig. 5. Intracellular localization of hsp40 in HeLa cells during and after heat shock. (A) Control, non-heated cells. Cells were heated at 42°C for 2 h (B), followed by recovery at 37°C for 1 (C), 3 (D), 6 (E) and 9 h (F). Cells were then fixed and processed for indirect immunofluorescence staining with the polyclonal antibody against hsp40 as described in Materials and Methods. Exposure time for each photograph was 30 s. Bar, 10 μm.

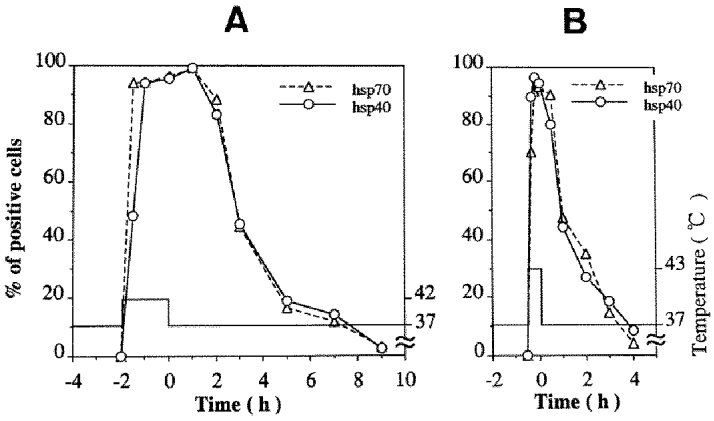

Fig. 6. Comparison of the kinetics of the accumulation of hsp40 (○) and hsp70 (△) in the nucleoli during heat shock and their subsequent return to the cytoplasm during the recovery period at 37°C after the heat shock in HeLa cells. (A) Cells heated at 42°C for 2 h, and allowed to recover at 37°C for up to 9 h. The abscissa indicates incubation time (h) at 42°C and 37°C. (B) Cells heated at 45°C for 15 min and incubated at 37°C for 16 h, then heated again at 43°C for 30 min and allowed to recover at 37°C for up to 4 h. The abscissa indicates incubation time (h) at 43°C and 37°C. At the indicated times, cells were fixed and processed for indirect immunofluorescence. Cells were stained separately with the polyclonal antibodies against hsp40 and hsp70, which were visualized with a FITC-conjugated goat anti-rabbit IgG, and photographed. The number of cells with brightly stained nucleoli were counted as positively stained cells. The ordinate indicates percentage of positively stained cells. Each data point represents at least 400 cells, the total of three independent experiments.

period at 37°C after the second heat shock, hsp40 and p73 were also localized in the nucleoli of the *same* cells (Fig. 7H, I, K and L), although fluorescence of hsp40 in the nucleoli of a few cells was slightly brighter than that of p73 (e.g. a cell in the bottom right-hand lower corner of Fig. 7K). Similar co-localization of hsp40 and p73 was observed during the recovery period after heat-shock at 42°C for 2 h (data not shown). These results suggest the co-translocation and co-localization of hsp40 and p73 under heat-shock conditions, although no appreciable association of hsp40 with p73 was observed in the immunoprecipitation experiments (Fig. 2D). We did not examine whether or not hsp40 co-localized with p72. However, they may also be co-localized in heat-shocked cells, since p72 itself as well as p73 is translocated into the nuclei and nucleoli during heat shock and returns to the cytoplasm during the recovery period in mammalian cells (Pelham, 1984; Welch and Feramisco, 1984; Ohtsuka et al., 1986a; Welch and Mizzen, 1988; Hayashi et al., 1991).

DISCUSSION

We have isolated hsp40 from HeLa cells using a modified two-dimensional gel electrophoretic technique, Slab-NEPHGE/SDS-PAGE. We obtained 10-15 μg of purified hsp40 from ten 100 mm culture dishes of HeLa cells using one Slab-NEPHGE/SDS-PAGE gel. The hsp40 purified in this manner was used to obtain the partial amino acid sequence of the N-terminal (48 amino acid residues of

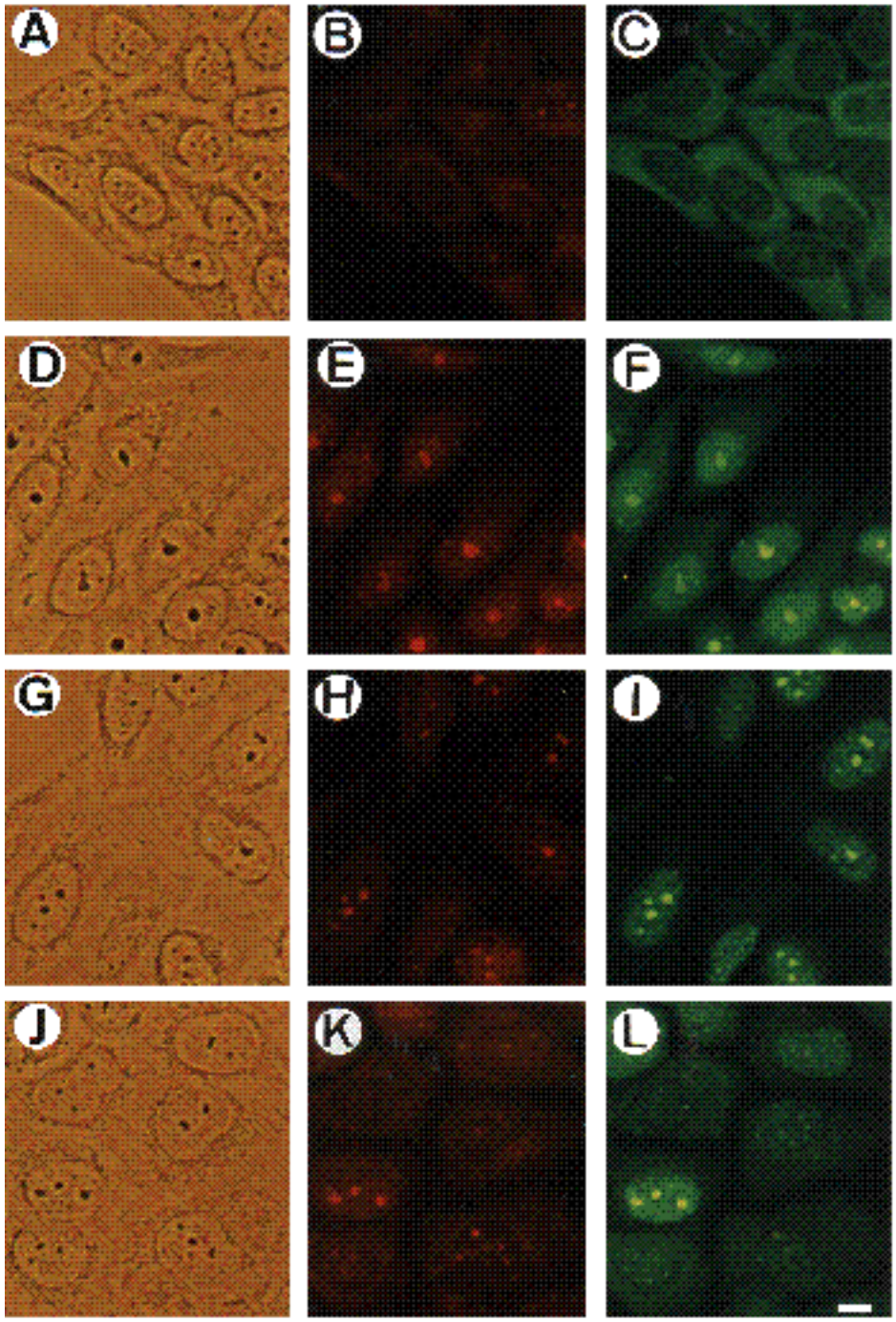

Fig. 7. Co-localization of hsp40 and hsc70(p73) in heated HeLa cells. Control, non-heated cells (A, B and C). Cells were heated at 45°C for 15 min and incubated at 37°C for 16 h, then heated again at 43°C for 30 min (D, E and F) and allowed to recover at 37°C for 1 h (G, H and I) and for 3 h (J, K and L). Cells were fixed and processed for double immunofluorescence staining with a mixture of the anti-hsp40 polyclonal, and the anti-p73 monoclonal (1B5) antibodies, which were then visualized with a mixture of rhodamine-conjugated anti-rabbit IgG and FITC-conjugated anti-rat IgG. The phase-contrast micrographs are shown in (A), (D), (G) and (J); the hsp40-specific fluorescence micrographs (rhodamine) of each corresponding field are shown in (B), (E), (H) and (K); the hsc70(p73)-specific fluorescence micrographs (FITC) of each corresponding field and shown in (C), (F), (I) and (L). Exposure times were 1.5 s for (A), (D), (G) and (J), 150 s for (B), (E), (H) and (K) and 50 s for (C), (F), (I) and (L), respectively. Bar, 10 μm.

hsp40) (Hattori et al., 1992) and to generate an antibody against hsp40. This novel purification method consists of only three steps, electrophoresis in the first dimension and in the second dimension, and electroelution. It may be applicable for the purification of other cellular proteins present in small amounts. If a protein of interest can be detected with Coomassie brilliant blue staining in a regular two-dimensional gel, microgram amounts of protein can be purified using this method. Such quantities are sufficient for the determination of a partial amino acid sequence (Matsudaira, 1987). Using several Slab-NEPHGE/SDS-PAGE gels, quantities of pure protein sufficient for the preparation of an antibody can also be obtained. In order to avoid the contamination of other proteins with similar molecular size and pI to the protein of interest, carrier Ampholines of different pH range may have to be used for the first dimension.

It has been reported that two bacterial heat-shock proteins, DnaK (the homologue of hsp70) and DnaJ, form an operon located at 0.3 min on the *Escherichia coli* map and their expression is regulated by the same promoter (for review, see Georgopoulos et al., 1990). It has been suggested that these two proteins function together in the dissociation of a protein complex involved in the replication

of bacteriophage λ, in both the presence and absence of another heat-shock protein, GrpE (Georgopoulos et al., 1990; Zylicz et al., 1989). They are also thought to be necessary for the monomerization of the RepA dimer in an ATP-dependent reaction, which leads to the binding of RepA monomer with RepA binding sites and to the replication of P1 plasmid (Wickner et al., 1991). Moreover, it has been shown that the DnaK, DnaJ and GrpE proteins are required in vivo for the renaturation (refolding) of denatured λ repressor (Gaitanaris et al., 1990) and that DnaK protein itself can protect RNA polymerase from heat inactivation and reactivate heat-inactivated RNA polymerase in an ATP hydrolysis-dependent manner in vitro (Skowyra et al., 1990). However, direct evidence for the association of DnaK with DnaJ has not been reported. In eukaryotic cells, the members of the hsp70 family have been shown to have a molecular chaperoning activity involved in the translocation of nascent protein from the cytoplasm across the membrane of the endoplasmic reticulum or mitochondria (Chirico et al., 1988; Deshaies et al., 1988) and in the folding and assembly of nascent proteins (Beckman et al., 1990). It has also been suggested that hsp70 associates with abnormal or unfolded proteins induced by heat shock or other stresses and promotes their repair (refolding and reassembly) in the nuclei and nucleoli in an ATP-dependent manner (Pelham, 1984, 1986; Lewis and Pelham, 1985).

In a previous report (Hattori et al., 1992), we have shown that the hsp40 found in HeLa (human) cells is homologous to the bacterial heat-shock protein DnaJ (Bardwell et al., 1986; Ohki et al., 1986; Lathigra et al., 1988) and DnaJ-related proteins in yeast, such as SCJ1, SEC63, YDJ1 and SIS1, and that hsp40 is localized in the nuclei, and especially in the nucleoli in heat-shocked HeLa cells. In this report, we have demonstrated that the kinetics of the heat-induced translocation of hsp40 to the nucleoli and its subsequent return to the cytoplasm are essentially similar to those of hsp70 (Fig. 6). Furthermore, both hsp40 and p73 are co-localized in the nucleoli in the same cells as a consequence of heat-induced translocation and also during the recovery period (Fig. 7). On the basis of the function of bacterial DnaK and DnaJ proteins, these results suggest that hsp70 (DnaK homologue) and hsp40 (DnaJ homologue) may act together to repair heat-denatured proteins in the nuclei and nucleoli of heat-shocked mammalian cells. Mammalian hsp70 has been shown to have a nucleolar localization sequence (Dang and Lee, 1989; Milarski and Morimoto, 1989). It remains to be elucidated whether or not hsp40 is translocated in the nuclei and nucleoli in association with p73/p72, and/or whether hsp40 itself contains a nuclear or nucleolar localization sequence.

Recently, four different DnaJ-homologous genes have been isolated from yeast. The SCJ1 protein is localized in the interior of mitochondria and appears to be involved in protein import into mitochondria (Blumberg and Silver, 1991). The product of the *NPL1/SEC63* gene has been shown to be important for protein assembly into the endoplasmic reticulum and the nucleus (Sadler et al., 1989). The YDJ1 protein is concentrated in a perinuclear ring as well as in the cytoplasm, as demonstrated by indirect immunofluorescence, and it cofractionates with nuclei and microsomes (Caplan and Douglas, 1991). The *MAS5* gene was isolated by complimentation of a mutant phenotype that has a defect in mitochondrial protein import at the nonpermissive temperature (Atencio and Yaffe, 1992). DNA-sequence comparison has revealed that YDJ1 is identical to MAS5 protein. The function of MAS5 (YDJ1) protein is likely to be involved in protein import into mitochondria in the cytoplasm or on the mitochondrial surface (Atencio and Yaffe, 1992). Curiously, the MAS5 protein is heat-inducible but YDJ1 protein is not (Caplan and Douglas, 1991; Atencio and Yaffe, 1992). Finally, the SIS1 protein is localized throughout the cell but is more concentrated at the nucleus as determined by indirect immunofluorescence (Luke et al., 1991). The SIS1 protein is heat-inducible but its exact function(s) are not known at present. Whether or not SCJ1 and NPL1/SEC63 proteins are induced by heat shock has not yet been examined. The intracellular localization of these proteins in heat-shocked yeast cells also remains to be investigated.

More recently, two human homologues of the DnaJ protein, HSJ1 and HDJ1, were identified by sequencing techniques (Raabe and Manley, 1991; Cheetham et al., 1992). HSJ1 protein is expressed preferentially in neurons and biochemical fractionation experiments suggest that the HDJ1 protein is localized in, or is associated with, the nucleus. The function(s) and heat-inducibility of HSJ1 and HDJ1 proteins are not known. Comparison of amino acid sequences has shown that both HSJ1 and HDJ1 proteins are homologous but not identical to our hsp40 (24 and 26 of 48 amino acid residues of the N-terminal are identical, respectively) (Raabe and Manley, 1991; Cheetham et al., 1992; Hattori et al., 1992). This indicates that there are several human DnaJ-homologous proteins. Thus, the HSJ1 protein, HDJ1 protein and hsp40 may represent a family of DnaJ-homologous proteins in mammalian cells. We are in the process of cloning the human hsp40 gene; comparison of the complete DNA sequences of hsp40, HDJ1 and HSJ1 will test the validity of this hypothesis.

This work was supported by Grants-in-Aid for Cancer Reserch (02151033 and 03151037) and for General Scientific Research (03304051 and 03680186) from the Japanese Ministry of Education, Science and Culture. The monoclonal hybridoma clones, 1B5 and 4C2, were prepared by K.O. at Section of Cancer Biology, Department of Radiology, Washington University School of Medicine, St. Louis, MO, in collaboration with Drs. B. Lokeshwar and A. Laszlo, and the work was supported by NIH grants CA-42591 and CA-49018 (A.L.). We thank Dr. K. Utsumi (Aichi Cancer Center Research Institute) for the densitometry using an image analyzer VIDAS and Mrs. S. Tokumasu for assistance in the preparation of this manuscript.

REFERENCES

Atencio, D. P. and Yaffe, M. P. (1992). MAS5, a yeast homolog of DnaJ involved in mitochondrial protein import. *Mol. Cell. Biol.* **12**, 283-291.

Atkinson, B. G. and Walden, D. B., eds (1985). *Changes in Eukaryotic Gene Expression in Response to Environmental Stress*. Academic Press, New York.

Bardwell, J. C. A., Tilly, K., Craig, E., King, J., Zylicz, M. and Georgopoulos, C. (1986). The nucleotide sequence of the *Escherichia*

coli K12 *dnaJ*$^{+}$ gene. A gene that encodes a heat shock protein. *J. Biol. Chem.* **261**, 1782-1785.

Beckman, R. P., Mizzen, L. A. and Welch, W. J. (1990). Interaction of hsp70 with newly synthesized proteins: Implications for protein folding and assembly. *Science* **248**, 850-854.

Blumberg, H. and Silver, P. A. (1991). A homologue of the bacterial heat-shock gene *DnaJ* that alters protein sorting in yeast. *Nature* **349**, 627-630.

Caplan, A. J. and Douglas, M. G. (1991). Characterization of YDJ1: A yeast homologue of the bacterial dnaJ protein. *J. Cell Biol.* **114**, 609-621.

Cheetham, M. E., Brion, J.-P. and Anderton, H. (1992). Human homologues of the bacterial heat-shock protein DnaJ are preferentially expressed in neurons. *Biochem. J.* **284**, 469-476.

Cheng, M. Y., Hartl, F.-U., Martin, J., Pollock, R. A., Kalousek, F., Neupert, W., Hallberg, E. M., Hallberg, R. L. and Horwich, A. L. (1989). Mitochondrial heat-shock protein hsp60 is essential for assembly of proteins imported into yeast mitochondria. *Nature* **337**, 620-625.

Chirico, W. J., Waters, M. G. and Blobel, G. (1988). 70K heat shock related proteins stimulate protein translocation into microsomes. *Nature* **332**, 805-810.

Cleveland, D. W., Fischer, S. G., Kirschner, M. W. and Laemmli, U. K. (1977). Peptide mapping by limited proteolysis in sodium dodecyl sulfate and analysis by gel electrophoresis. *J. Biol. Chem.* **252**, 1102-1106.

Cross, D. P. and Sang, J. H. (1978). Cell culture of individual *Drosophila* embryos. I. Development of wild type cultures. *J. Embryol. Exp. Morphol.* **45**, 161-172.

Dang, C. V. and Lee, W. M. F. (1989). Nuclear and nucleolar targeting sequences of c-*erb*-A, c-*myb*, N-*myc*, p53, HSP70 and HIV *tat* proteins. *J. Biol. Chem.* **264**, 18019-18023.

Deshaies, R. J., Koch, B. D., Werner-Washburne, M., Craig, E. A. and Schekman, R. (1988). A subfamily of stress proteins facilitates translocation of secretory and mitochondrial precursor polypeptide. *Nature* **332**, 800-805.

Echalier, G. and Ohanessian, A. (1970). In vivo culture of *Drosophila melanogaster* embryonic cells. *In Vitro* **6**, 162-172.

Gaitanaris, G. A., Papavassiliou, A. G., Rubock, P., Silverstein, S. J. and Gottesman, M. E. (1990). Renaturation of denatured λ repressor requires heat shock proteins. *Cell* **61**, 1013-1020.

Georgopoulos, C., Ang, D., Liberek, K. and Zylicz, M. (1990). Properties of the *Escherishia coli* heat shock proteins and their role in bacteriophage λ growth. In *Stress Proteins in Biology and Medicine* (ed. R. I. Morimoto, A. Tissières, and C. Georgopoulos), pp. 191-121. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Harlow, E. and Lane, D. (1988). *Antibodies: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Hattori, H., Liu, Y.-C., Tohnai, I., Ueda, M., Kaneda, T., Kobayashi, T., Tanabe, K. and Ohtsuka, K. (1992). Intracellular localization and partial amino acid sequence of a stress-inducible 40 kDa protein in HeLa cells. *Cell Struct. Funct.* **17**, 77-86.

Hayashi, Y., Tohnai, I., Kaneda, T., Kobayashi, T. and Ohtsuka, K. (1991). Translocation of hsp-70 and protein synthesis during continuous heating at mild temperatures in HeLa cells. *Radiat. Res.* **125**, 80-88.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-685.

Laszlo, A. (1988). Regulation of the synthesis of heat-shock proteins in heat-resistant variants of Chinese hamster fibroblasts. *Radiat. Res.* **116**, 427-441.

Laszlo, A. and Li, G. C. (1985). Heat-resistant variants of Chinese hamster fibroblasts altered in expression of heat shock protein. *Proc. Nat. Acad. Sci. USA* **82**, 8029-8033.

Lathigra, R. B., Young, D. B., Sweetser, D. and Young, R. A. (1988). A gene from *Mycobacterium tuberculosis* which is homologous to the DnaJ heat shock protein of *E. coli*.*Nucl. Acids Res.* **16**, 1636.

Lewis, M. J. and Pelham, H. R. B. (1985). Involvement of ATP in the nuclear and nucleolar functions of the 70 kd heat shock protein. *EMBO J.* **4**, 3137-3143.

Luke, M. M., Sutton, A. and Arndt, K. T. (1991). Characterization of SIS1, a *Saccharomyces cerevisiae* homologue of bacterial dnaJ protein. *J. Cell Biol.* **114**, 623-638.

Matsudaira, P. (1987). Sequence from picomole quantities of proteins electroblotted onto polyvinylidene difluoride membranes. *J. Biol. Chem.* **262**, 10035-10038.

Milarski, K. L. and Morimoto, R. I. (1989). Mutational analysis of the human hsp70 protein: Distinct domains for nucleolar localization and adenosine triphosphate binding. *J. Cell Biol.* **109**, 1947-1962.

O'Farrell, P. H. (1975). High resolution two-dimensional electrophoresis of proteins. *J. Biol. Chem.* **250**, 4007-4021.

O'Farrell, P. Z., Goodman, H. M. and O'Farrell, P. H. (1977). High resolution two-dimensional electrophoresis of basic as well as acidic proteins. *Cell* **12**, 1133-1142.

Ohki, M., Tamura, F., Nishimura, S. and Uchida, H. (1986.) Nucleotide sequence of the *Escherichia coli dnaJ* gene and purification of the gene product. *J. Biol. Chem.* **261**, 1778-1781.

Ohtsuka, K., Masuda, A. Nakai, A. and Nagata, K. (1990). A novel 40 kDa protein induced by heat shock and other stresses in mammalian and avian cells. *Biochem. Biophys. Res. Commun.* **166**, 642-647.

Ohtsuka, K., Nakamura, H. and Sato, C. (1986a). Intracellular distribution of 73000 and 72000 dalton heat shock proteins in HeLa cells. *Int. J. Hyperthermia* **2**, 267-275.

Ohtsuka, K., Tanabe, K., Nakamura, H. and Sato, C. (1986b). Possible cytoskeletal association of 69,000- and 68,000-dalton heat shock proteins and structural relations among heat shock proteins in murine mastocytoma cells. *Radiat. Res.* **108**, 34-42.

Pardue, M. L., Feramisco, J. R. and Lindquist, S., eds (1989). *Stress-Induced Proteins.* Alan R. Liss, Inc. New York.

Pelham, H. R. B. (1984). Hsp70 accelerate the recovery of nucleolar morphology after heat shock. *EMBO J.* **3**, 3095-3100.

Pelham, H. R. B. (1986). Speculations on the functions of the major heat shock and glucose-regulated proteins. *Cell* **46**, 959-961.

Raabe, T. and Manley, J. L. (1991). A human homologue of the *Escherichia coli* DnaJ heat-shock protein. *Nucl. Acids Res.* **19**, 6645.

Sadler, I., Chiang, A., Kurihara, T., Rothblatt, J., Way, J. and Silver, P. (1989). A yeast gene important for protein assembly into the endoplasmic reticulum and the nucleus has homology to DnaJ, an *Escherichia coli* heat shock protein. *J. Cell Biol.* **109**, 2665-2675.

Schlesinger, M. J., Ashburner, M. and Tissières, A., eds (1982). *Heat Shock from Bacteria to Man*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Skowyra, D., Georgopoulos, C. and Zylicz, M. (1990). The E. coli *dnaK* gene product, the hsp70 homolog, can reactivate heat-inactivated RNA polymerase in an ATP hydrolysis-dependent manner. *Cell* **62**, 939-944.

Towbin, H., Staehelin, T. and Gordon, J. (1979), Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedures and some applications. *Proc. Nat. Acad. Sci. USA* **76**, 4350-4354.

Welch, W. J. (1990). The mammalian stress response: Cell physiology and biochemistry of stress proteins. In *Stress Proteins in Biology and Medicine* (ed. R.I. Morimoto, A. Tissières and C. Georgopoulos), pp. 223-278. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Welch, W. J. and Feramisco, J. R. (1984). Nuclear and nucleolar localization of 72,000-dalton heat shock protein in heat-shocked mammalian cells. *J. Biol. Chem.* **259**, 4501-4513.

Welch, W. J. and Feramisco, J. R. (1985). Rapid purification of mammalian 70,000-dalton stress proteins: Affinity of the proteins for nucleotides. *Mol. Cell. Biol.* **5**, 1229-1237.

Welch, W. J. and Mizzen, L. A. (1988). Characterization of the thermotolerant cells. II. Effects on the intracellular distribution of heat-shock protein 70, intermediate filaments and small nuclear ribonucleoprotein complexes. *J. Cell Biol.* **106**, 1117-1130.

Wickner, S., Hoskins, J. and McKenney, K. (1991). Monomerization of RepA dimers by heat shock proteins activates binding to DNA replication origin. *Proc. Nat. Acad. Sci. USA* **88**, 7903-7907.

Zylicz, M., Ang, D., Liberek, K. and Georgopoulos, C. (1989). Initiation of DNA replication with purified host- and bacteriophage-encoded proteins: the role of the dnaK, dnaJ and grpE heat shock proteins. *EMBO J.* **8**, 1601-1608.

(Received 21 October 1992 - Accepted 2 December 1992)