Interaction between hsp70 and hsp40, eukaryotic homologues of DnaK and DnaJ, in human cells expressing mutant-type p53

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Abstract We have recently identified a novel 40-kDa heat-shock protein hsp40 as a mammalian homologue of bacterial DnaJ protein. Here we demonstrate the physical interaction between hsp70 (DnaK homologue) and hsp40 in human cells as determined by immunoprecipitation methods. Co-immunoprecipitation of hsp70 with hsp40 was dependent on the presence of ATP or unfolded protein (reduced carboxymethylated α-lactalbumin). A mutant type of tumor suppressor gene product, mtp53, was co-immunoprecipitated not only with hsp70 but also with hsp40. These results suggest the existence of a hsp70(DnaK)/hsp40(DnaJ) chaperone system in mammalian cells.

Key words: hsp70 (DnaK); hsp40 (DnaJ); Molecular chaperone; p53;Immunoprecipitation

1. Introduction

A set of proteins induced by elevated temperature or other environmental stresses are called heat-shock (stress) proteins (hsps) [1,2]. It has become clear in the last several years that significant amounts of these hsps are expressed in cells even in the absence of stress. These hsps have recently been shown to have basic and indispensable cellular functions as molecular chaperones at normal growth temperature in addition to protecting cells from stress-related deleterious effects. An hsp70 (70-kDa hsp) is known to have an affinity for the hydrophobic region of stress-induced partially unfolded proteins and that of nascent polypeptide being synthesized on the ribosomes, and assists in their correct folding [3–8]. Also, genetic and biochemical studies have suggested that hsp70 maintains mitochondria- or ER-targeted proteins in a transport-competent (partially unfolded) state, so as to facilitate their entry into each organelle [9,10]. A bacterial heat-shock protein, DnaK, a homologue of eukaryotic hsp70, has similar molecular chaperone activity. However, DnaK protein usually acts together with other hsps DnaJ and GrpE as the chaperone system (or chaperone machine) in these cellular processes [11].

Ohtsuka et al. have previously found a novel 40-kDa heat-shock protein hsp40 in mammalian and avian cells [12]. Amino acid sequencing of N-terminal and cDNA cloning of human hsp40 have revealed that it is a mammalian homologue of bacterial DnaJ heat-shock protein [13,14]. We also showed that both hsp70 and hsp40 translocate and accumulate in the nuclei and nucleoli upon heat shock and they colocalize in the nucleoli of heat-shocked mammalian cells [13,15]. Thus, we suggested that there is an hsp70 (DnaK)/hsp40 (DnaJ) chaperone system involved in repairing (refolding) stress-damaged proteins in mammalian cells.

In this report, we investigated the physical interaction between hsp70 and hsp40 and also examined whether hsp40 could interact with hsp70/mutant p53 complex in human cells by immunoprecipitation methods.

2. Materials and methods

2.1. Cells, cell culture and heating

A human oral squamous cell carcinoma cell line, HOC815 [16], was used in this study. Cells were grown in Dulbecco's modified Eagle's minimal essential medium (Nissui, Tokyo) supplemented with 10% fetal bovine serum (JRH Biosciences, Lenexa, KS) in a CO_2-incubator at 37°C. Since the amounts of hsps, especially hsp40, were very low in non-heat-shocked control cells, it was difficult to detect coimmunoprecipitation between hsp70 and hsp40 (see Fig. 1). Therefore, cells were heated at 45°C for 15 min, then recovered at 37°C for 16 h, to increase the cellular amount of hsps. In most of the present studies, we used the hsp-enriched cells. We have already reported that a majority (80–90%) of both hsp70 and hsp40 are localized in the cytoplasm in the hsp-enriched cells as determined by cell fractionation methods [17].

2.2. Immunoprecipitation, gel electrophoresis and immunoblotting

Cells at subconfluence in 100 mm plastic dishes were washed with cold phosphate buffered saline (PBS) and harvested by treatments with 0.05% trypsin and 0.02% EDTA. Cells were washed with PBS and pelleted by centrifugation. The same number of cells were used in each experiment. Cells were then lysed with 500 μl of lysis buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% Triton X-100, 1% Na-deoxycholate) containing 1 μg/ml leupeptin, 1 μg/ml pepstatin and 1 mM phenylmethylsulfonyl fluoride (PMSF). Also, 5 units of apyrase (Sigma, St. Louis, MO) was added to the lysate to deplete endogenous ATP where indicated. The cell lysates were sonicated and clarified by centrifugation. The supernatants were preabsorbed with 50 μl of insoluble protein A (20% v/v of Zysorbin, non-viable *Staphylococcus aureus*; Zymed, San Francisco, CA), which was previously boiled for 15 min in SDS-sample buffer (62.5 mM Tris-HCl, pH 6.8, 2.3% SDS, 5% β-mercaptoethanol, 10% glycerol) [18] and then washed with the lysis buffer. After 10 min incubation, the supernatants were clarified. Preabsorption was repeated once again. The supernatants were then incubated with 30 μl of anti-hsp70 antibody (KO4, No. 4 rabbit serum described in [13,19]), 30 μl of anti-hsp40 antibody [13,15] or 30 μl of anti-p53 monoclonal antibody (PAb1801; Oncogene Sci. Inc., Cambridge, MA) for 60 min at 4°C. Reduced carboxymethylated α-lactalbumin (RCMLA, Sigma) which is known to be permanently unfolded protein, was added to the supernatant before the addition of the antibody where indicated. Zysorbin (100 μl) was then added and the incubation continued for 30 min. The immunoprecipitates were collected by centrifugation and washed five times with the lysis buffer. The precipitated proteins were released from

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the Zysorbin by the addition of SDS-sample buffer followed by boiling for 5 min. The immunoprecipitates were then analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) [18] followed by immunoblotting with anti-hsp70 antibody (KO4), a mixture of anti-hsp70 antibody (KO4) and anti-hsc70 (p73) monoclonal antibody (1B5; see [15]), anti-hsp40 antibody or anti-p53 antibody (PAb1801). Peroxidase activity was detected using 4-chloro-1-naphthol as a substrate or enhanced chemiluminescence method (ECL, Amersham, UK).

3. Results and discussion

It is known in human cells that both hsc70 (p73, constitutive form) and hsp70 (p72, inducible form) are expressed at normal growth temperature, and hsp70 (p72) is induced remarkably upon heat shock [3,20] (see lanes 1 and 3, upper panel of Fig. 1). In non-heat-shocked control cells, it was difficult to detect hsp40 precipitated with anti-hsp70 antibody (lane 1 in Fig. 1). The hsp70 (p73/p72) precipitated with anti-hsp40 antibody was faintly detected (lanes 2 and 2′ in Fig. 1). In contrast, co-immunoprecipitation between hsp70 (p73/p72) and hsp40 was observed more evidently in hsp-enriched cells (lanes 3, 4 and 4′ in Fig. 1). Both hsc70 (p73) and hsp70 (p72) were precipitated with anti-hsp40 antibody (lanes 2′ and 4′ in Fig. 1), indicating that hsp40 has an affinity for both p73 and p72 in human cells. Under our experimental conditions, approximately 10% of total hsp70 (p73/p72) and hsp40 was precipitated with anti-hsp70 antibody and anti-hsp40 antibody, respectively. In Fig. 1, the density of hsp70 (p73/p72) in lane 4 was approximately 10% of that in lane 3, and the density of hsp40 in lane 3 was approximately 5% of that in lane 4 as determined by densitometry. Therefore, 5–10% of total hsp70 (p73/p72) and hsp40 was associated with each other in hsp-enriched cells. In the following experiments, we used hsp-enriched cells to detect the interaction between hsp70 (p73/p72) and hsp40.

It has been shown that eukaryotic hsp70 (DnaK in prokaryote) has an ATPase activity and that interaction between hsp70 and target proteins is disrupted through conformational change of hsp70 by the addition of ATP [3,6,21]. We therefore examined the effect of ATP on the interaction of hsp70 with

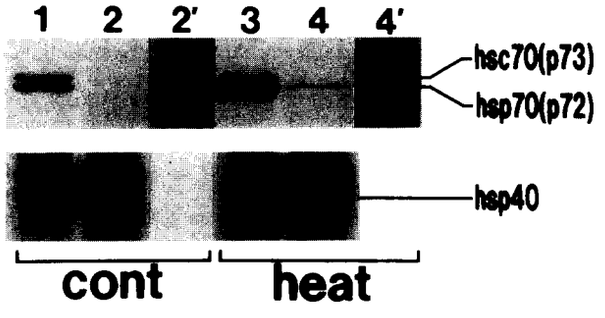

Fig. 1. Co-immunoprecipitation between hsp70 and hsp40 in hsp-enriched HOC815 cells. Non-heat-shocked control cells (lanes 1, 2 and 2′) and hsp-enriched cells (lanes 3, 4 and 4′) were lysed in the presence of apyrase and subjected to immunoprecipitation as described in section 2. Lanes 1 and 3, precipitated with anti-hsp70 antibody (KO4); lanes 2, 2′, 4 and 4′, precipitated with anti-hsp40 antibody. Immunoprecipitates were analyzed by SDS-PAGE followed by immunoblotting probed with anti-hsp70 antibody (KO4) (lanes 1, 2, 3 and 4 in upper panel) or anti-hsp40 antibody (lower panel). Lanes 2′ and 4′ indicate the immunoblot probed with a mixture of anti-hsp70 antibody (KO4) and anti-hsc70 (p73) monoclonal antibody (1B5) of the same sample of lanes 2 and 4, respectively, in order to detect hsp70 (p73/p72) more clearly.

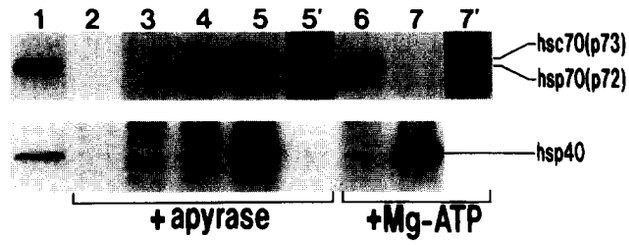

Fig. 2. Effect of ATP on the interaction between hsp70 and hsp40. After cell lysis of hsp-enriched cells, apyrase (5 units) (lanes 2, 3, 4, 5 and 5′) or Mg-ATP (2.5 mM) (lanes 6, 7 and 7′) was added to the lysate and processed for immunoprecipitation. Lane 1, whole cell lysate; lanes 2, precipitated with preimmune serum of anti-hsp70 antibody; lane 3, with preimmune serum of anti-hsp40 antibody; lanes 4 and 6, with anti-hsp70 antibody (KO4); lanes 5, 5′, 7 and 7′, with anti-hsp40 antibody. Immunoprecipitates were analyzed as described in Fig. 1.

hsp40. As shown in Fig. 2, the amount of hsp70 (p73/p72) precipitated with anti-hsp40 antibody was decreased four- to five-fold by the addition of ATP as compared to the addition of apyrase to deplete endogenous ATP (lanes 5 and 7 in Fig. 2). Also, the addition of ATP decreased the amount of hsp40 precipitated with anti-hsp70 antibody to almost background level (similar level of the result of preimmune serum) (lanes 4 and 6 in Fig. 2). However, the amount of hsp70 (p73/p72) precipitated with anti-hsp70 antibody also decreased approximately two-fold by ATP addition (compare between lanes 4 and 6 in Fig. 2). This seems to be due to reduced accessibility of anti-hsp70 antibody to hsp70 which conformation was altered by ATP. In contrast, the amount of hsp40 precipitated with anti-hsp40 antibody were almost the same in the presence and absence of ATP (compare between lanes 5 and 7 in Fig. 2). These results suggest that interaction between hsp70 and hsp40 is ATP-dependent.

Since hsp70 is known to have an affinity for the hydrophobic region of unfolded protein [3,6], we next tested whether addition of permanently unfolded protein RCMLA to the cell lysate would enhance the interaction between hsp70 and hsp40. As shown in Fig. 3, the amount of hsp70 (p73/p72) precipitated with anti-hsp40 antibody was increased three- to four-fold by the addition of RCMLA (lanes 4 and 6 in Fig. 3). The amount of hsp40 precipitated with anti-hsp70 antibody, however, increased only slightly (1.1-fold) by RCMLA addition (lanes 3 and 5 in Fig. 3). These results might be interpreted as follows. Some portion of both hsp70 and hsp40 indeed bound to RCMLA and ternary complexes were formed. Thus, total amount of hsp70/hsp40 complex increased by the addition of RCMLA. However, while the anti-hsp70 antibody might have reduced affinity for hsp70 in the ternary complex, anti-hsp40 antibody could recognize hsp40 irrespective of its complex formation.

It has been reported that HOC815 cells used in this study have a mutant-type p53 tumor suppressor gene product ($Tyr^{205} \rightarrow Cys$) [22]. The mutant-type p53 (mtp53) is known to interact with hsp70 [23–25]. We here demonstrated that the mtp53 interacted not only with hsp70 (p73/p72) but also with hsp40 (Fig. 4). Thus, anti-hsp70 antibody could precipitate both hsp40 and mtp53 (lane 3 in Fig. 4), and anti-p53 antibody precipitated both hsp70 (p73/p72) and hsp40 (lane 5 in Fig. 4). However, anti-hsp40 antibody immunoprecipitated only hsp70 (p73/p72), not p53 (lane 4 in Fig. 4). This might be

due to the antibody's interference with the interaction of hsp40 with hsp70/mtp53 complex, or the antibody's inability of recognition of hsp70/mtp53/hsp40 ternary complex. Even when cells were treated with cross linker DSP (3,3′-dithiobis-[succinimidyl propionate]) before cell lysis, anti-hsp40 antibody could recognize only hsp70 (p73/p72) but not mtp53 (data not shown). Addition of ATP disrupted the interaction of these proteins (data not shown). To our knowledge, this is the first report on the interaction of hsp40 (DnaJ homologue) with hsp70/mtp53 complex. Interaction between hsp70 and hsp40 in cells which express wild-type p53 was less than that in cells with mutant-type p53; and, of course, wild-type p53 did not interact with hsp70 nor hsp40 (data not shown). Since it has been suggested that hsp70 plays a role in regulation of conformational change of the temperature-sensitive mutant of p53 in an ATP-dependent manner [26], the present results suggest that hsp40 together with hsp70 is involved in this process.

In this report, we showed the co-immunoprecipitation of hsp70 (p73/p72) with hsp40 in human cells, suggesting the existence of an hsp70 (DnaK)/hsp40 (DnaJ) chaperone system in mammalian cells. We also observed the ternary complex of hsp70/mtp53/hsp40 in cells which express mutant-type p53. It remains unclear whether hsp70 could interact, either directly or indirectly, through a target protein. In a bacterial system, since the affinity of DnaK for DnaJ is very weak, it is difficult to detect any direct interaction of these proteins. Under some experimental conditions, however, DnaK/DnaJ complex would be observed in the in vitro system using purified proteins [27,28]. Recently, Frydman et al. have shown that hsc70 (p73) in rabbit reticulocyte lysate is immunoprecipitated with anti-hsp40 antibody in the presence of translating ribosomes [29]. In the light of these results, the data in the present study suggest that at least some portion of these two hsps interacts directly and this interaction is enhanced in the presence of target proteins such as unfolded proteins and mutant-type p53. We are now in the process of investigating direct interaction between hsp70 and hsp40 using purified proteins.

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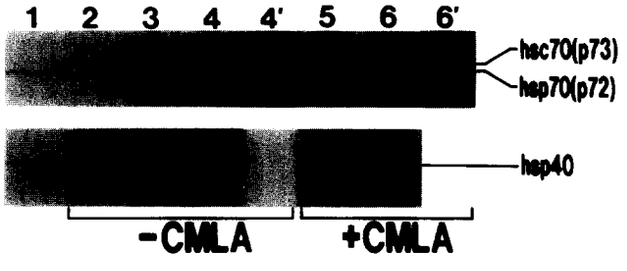

Fig. 3. Effect of the addition of permanently unfolded protein RCMLA on the interaction between hsp70 and hsp40. Apyrase was added to all samples. After preabsorption step, the cleared lysate was incubated in the absence (lane 2, 3, 4 and 4′) or in the presence (lanes 5, 6 and 6′) of RCMLA (final 10 μM) for 30 min, then subjected to immunoprecipitation. Lane 1, whole cell lysate; lane 2, precipitated with preimmune serum of anti-hsp40 antibody; lanes 3 and 5, with anti-hsp70 antibody (KO4); lanes 4, 4′, 6 and 6′, with anti-hsp40 antibody. Immunoprecipitates were analyzed as described in Fig. 1.

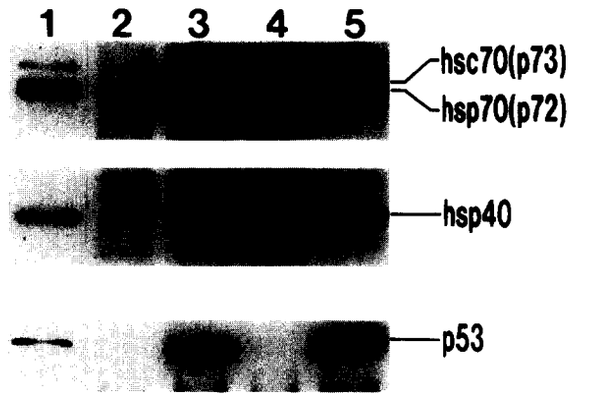

Fig. 4. Complex formation of hsp70, mutant-type p53 and hsp40 in hsp-enriched cells. Cells were washed with PBS, then lysed. Apyrase was added to all samples. Immunoprecipitation was performed as described in section 2. Lane 1, whole cell lysate; lane 2, precipitated with preimmune serum of anti-hsp40 antibody; lane 3, with anti-hsp70 antibody (KO4); lane 4, with anti-hsp40 antibody; lanes 5, with anti-p53 monoclonal antibody (PAb1801). Immunoprecipitates were analyzed by SDS-PAGE followed by immunoblotting probed with a mixture of anti-hsp70 antibody (KO4) and anti-hsc70 (p73) monoclonal antibody (1B5) (upper panel), anti-hsp40 antibody (middle panel) or with anti-p53 antibody (lower panel). p53 was detected with ECL system.

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