Ubiquitous and cell-specific members of the avian small heat shock protein family

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Abstract Small heat shock proteins (sHsps) have been suggested to act as molecular chaperones for many kinds of substrates and have protective roles in cells exposed to external stresses. Unlike other major Hsps such as Hsp70 and Hsp90, expression of many vertebrate sHsps is restricted to the muscle tissues and/or eye lens. Among the sHsps, the heat-inducible human Hsp27 (hHsp27) homologue is believed to be expressed ubiquitously in various cell types. Here, we distinguished the chicken homologue of hHsp27 (cHsp24) from the chicken major heat-inducible protein of molecular size 25 kDa (cHsp25). cHsp25 is not expressed in the absence of stress, but is highly expressed after hyperthermia in all tissues of developing embryos. In contrast, expression of cHsp24 is restricted to some specific tissues even in the presence of stress. Thus, cHsp25 is the first member of the sHsps in vertebrates the expression of which is ubiquitous in tissues exposed to external stresses similar to Hsp70.

Key words: Heat shock protein; Molecular chaperone; Stress response; Embryo

1. Introduction

Small heat shock proteins (sHsps) are characterized by the presence of a conserved sequence of about 100 residues called the α-crystallin domain or small heat shock protein domain [1]. Molecular sizes of sHsps range from 12 to 42 kDa, and characteristically they form large multimeric complexes in cells. They act as molecular chaperones for many kinds of substrates [2–5] by binding to denatured substrates and maintaining them in a folding-competent state [6,7]. In human, six different sHsps have been reported [8–15], all of which are highly expressed in muscle tissues such as the heart and soleus (Hsp27, HSPL27, p20, HSPB2/MKBP and αB-crystallin) and/or the eye lens (αA-crystallin and αB-crystallin), whereas most of these molecules show very low levels of expression in many other tissues [9,11,12,14,15]. Hsp27, which can be induced by heat shock, is believed to be ubiquitously expressed in various tissues in the absence of stress and has been speculated to act as a general molecular chaperone.

In chicken cells, a major heat-inducible protein of molecular size 25 kDa (cHsp25) has been analyzed extensively because this protein was shown to accumulate at tremendously high levels [17–19]. cHsp25 forms a perinuclear large aggregate under conditions of heat shock [18,19], suggesting that cHsp25 is a member of the small heat shock protein family. However, it has not been demonstrated whether cHsp25 is a chicken homologue of human Hsp27. Previously, we isolated a cDNA clone encoding a chicken homologue of human Hsp27 [20] and noticed a discrepancy between the protein level of cHsp25 and the mRNA level of a chicken homologue of human Hsp27. In this study, we distinguished cHsp25 from cHsp24, a chicken homologue of human Hsp27, using specific antisera. cHsp25 was not expressed in the absence of stress and was highly induced by heat shock in all of the tissues in the developing chicken embryo, whereas cHsp24 expression was restricted to some tissues.

2. Materials and methods

2.1. Generation of antisera

To generate antiserum against cHsp24, which is a chicken homologue of human Hsp27 [21], a full-length cHsp24 cDNA [20], which is identical to that encoding a 25-kDa inhibitor of actin polymerization (25-kDa IAP) [22], was modified using PCR mutagenesis to introduce *Eco*RI sites and then ligated into the pGEX-2T vector (Pharmacia) to create pGEX2T-cHsp24. After induction with 0.4 mM IPTG, GST-fused cHsp24 was gel purified and used to immunize rabbits as described previously [23]. To generate antiserum against cHsp25, spots of cHsp25 were excised from extracts of heat-shocked DT40 cells separated by two-dimensional gel electrophoresis. After electroelution, cHsp25 was used to immunize rabbits.

2.2. Cell culture and two-dimensional gel electrophoresis

DT40 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10^{-5} M β-mercaptoethanol, 10% fetal bovine serum and 1% chicken serum. Chicken embryo fibroblasts were maintained in DMEM containing 5% fetal bovine serum, 5% tryptose phosphate broth and 2% chicken serum. Cells were incubated at 37°C with 5% CO_2. To prepare cell extracts, cells were washed with phosphate-buffered saline (PBS) and suspended in 200 μl of lysis buffer (1% Nonidet P-40, 0.15 M NaCl, 50 mM Tris-HCl, pH 8.0, and 5 mM EDTA). After being kept on ice for 20 min, samples were centrifuged at 14 000 rpm for 15 min. The supernatant was removed and kept at −20°C until use after addition of an equal volume of glycerol. Non-equilibrium pH gradient gel electrophoresis (NEPHGE) was performed in the first dimension using pH 3.5–10 ampholine [24] and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed in the second dimension. Gels were stained with a silver staining kit (Wako, Osaka). To determine the p*I* gradient of the non-equilibrium pH gradient gel, the gel was immediately cut into pieces (0.5 cm each), soaked in distilled water for 30 min and pH was determined using a pH meter.

2.3. Isolation of tissue extracts from developing chick embryos

Fertilized chicken eggs were incubated in a humidified incubator at 38°C and rotated four times a day. To examine the effects of whole-embryo hyperthermia, eggs were placed in plastic bags and submerged in a water bath at 45°C. Tissues were dissected after heat treatment, washed in ice-cold PBS, and then suspended in 10 volumes of Laemmli's SDS-sample buffer, followed by sonication. To isolate red blood cells, the main vessels were cut and blood fluid was collected.

2.4. Western blotting

Proteins were separated by SDS-PAGE and electrophoretically

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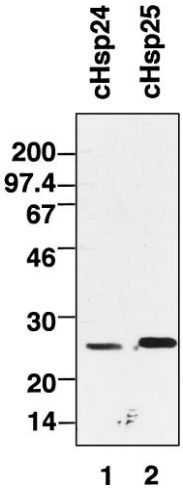

Fig. 1. Generation of antisera specific for the chicken homologue of human Hsp27 (cHsp24) and chicken Hsp25 (cHsp25). CEFs were heat shocked at 45°C for 1 h and then allowed to recover for 37°C for 2 h. Cell extract was prepared, aliquots of protein (30 μg) were subjected to 12% SDS-PAGE and transferred onto a nitrocellulose membrane. A piece of the membrane was blotted using anti-cHsp24 (lane 1) or anti-cHsp25 (lane 2) as the primary antibody and peroxidase-conjugated goat anti-rabbit IgG as the second antibody. Signals were detected using the ECL system (Amersham). Molecular weight standards were as indicated: 200 kDa, myosin; 97.4 kDa, phosphorylase b; 69 kDa, bovine serum albumin; 46 kDa, ovalbumin; 30 kDa, carbonic anhydrase; 20 kDa, trypsin inhibitor; 14 kDa, lysozyme.
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transferred onto nitrocellulose membranes in a buffer containing 5 mM sodium tetraborate. The filters were blocked with 5% dry milk in PBS for 1 h at room temperature and incubated with a 1:1000 dilution of rabbit antiserum against cHsp70 (Kawazoe et al., submitted) in PBS with 2% dry milk. After washing with PBS, the filters were incubated with a 1:1000 dilution of horseradish peroxidase-conjugated goat anti-rabbit IgG (Cappel) for 30 min at room temperature, and then signals were detected using the ECL system (Amersham). After estimating the levels of cHsp70s, aliquots containing the same amounts of cHsp70s were subjected to SDS-PAGE, followed by Western blotting using antiserum for cHsp24 or cHsp25 as described above.

3. Results

We generated antiserum specific for the chicken homologue of human Hsp27 (hHsp27). This antiserum recognized a single band of 24 kDa in extracts of chicken embryo fibroblasts (CEFs) (Fig. 1, lane 1). We designated this protein chicken Hsp24 (cHsp24). To identify bands of cHsp24 on two-dimensional gels, we performed two-dimensional gel electrophoresis consisting of NEPHGE in the first dimension and 10% SDS-

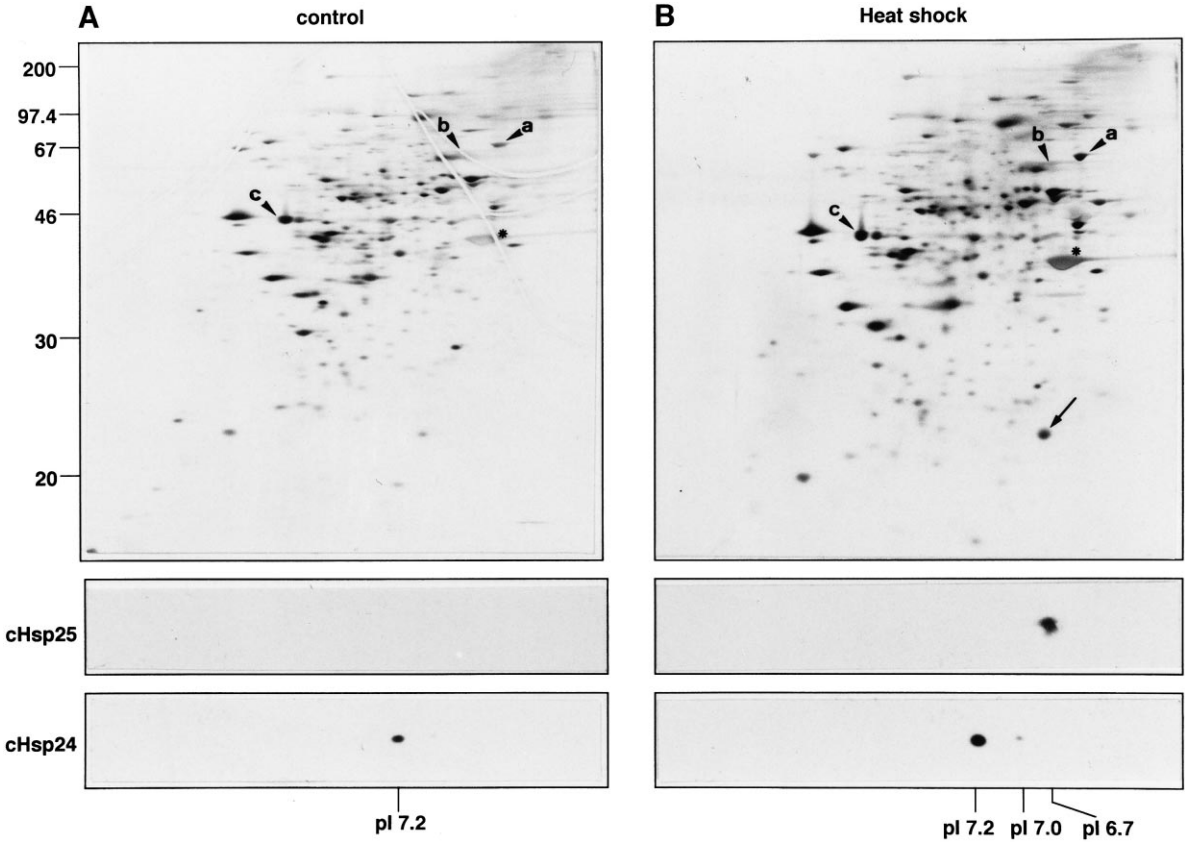

Fig. 2. Identification of cHsp24 and cHsp25 on two-dimensional gel. Cell lysates were prepared from control (A) and heat-shocked (45°C for 1 h and recovered for 37°C for 24 h) (B) CEFs and subjected to two-dimensional gel analysis consisting of NEPHGE and 12% SDS-PAGE, followed by silver staining (upper panels) and Western blotting using anti-cHsp25 (middle panels) and anti-cHsp24 (lower panels). The arrow on the upper panel in B indicates cHsp25. Letters a–c indicate heat shock proteins: a, Hsp90; b, Hsp70; c, Hsp47. Asterisks indicate actin. The p*I* of cHsp25 was 6.7. cHsp24 was detected as two spots with different p*I*s of 7.2 and 7.0. Estimated p*I*s of actin Hsp90 were 6.1 and 6.2, respectively.

PAGE in the second dimension. CEFs maintained at 37°C were incubated at 45°C for 1 h and then allowed to recover at 37°C for 3 h. Cell extracts were subjected to two-dimensional gel electrophoresis. Proteins were silver-stained or transferred onto nitrocellulose membranes followed by Western blotting using antiserum for cHsp24 (Fig. 2, lowest panels). Unexpectedly, a major heat shock protein of 25 kDa (Fig. 2B, arrow in upper panel), which we called cHsp25, was not recognized by this antiserum. Instead, cHsp24 was identified as two spots with estimated isoelectric points (p*I*s) of 7.2 and 7.0 (Fig. 2A,B, lowest panels), whereas p*I* of cHsp25 was 6.7 (Fig. 2B, middle panel). Although Western blot analysis showed that the expression of cHsp24 was induced severalfold by heat shock, the cHsp24 level was too low to be detected by silver staining even after heat shock in CEFs (Fig. 2A,B). To distinguish cHsp25 from cHsp24 more clearly, we generated antiserum specific for cHsp25. The bands of cHsp25 in two-dimensional gels were excised and the electro-eluted protein was used to immunize a rabbit. This antiserum detected a single band of 25 kDa in extracts of heat-shocked CEFs (Fig. 1, lane 2). cHsp25 was specifically recognized by this antiserum on two-dimensional gel electrophoresis, whereas cHsp24 did not cross-react (Fig. 2B). cHsp25 was not expressed in cells under normal growth conditions (Fig. 2A).

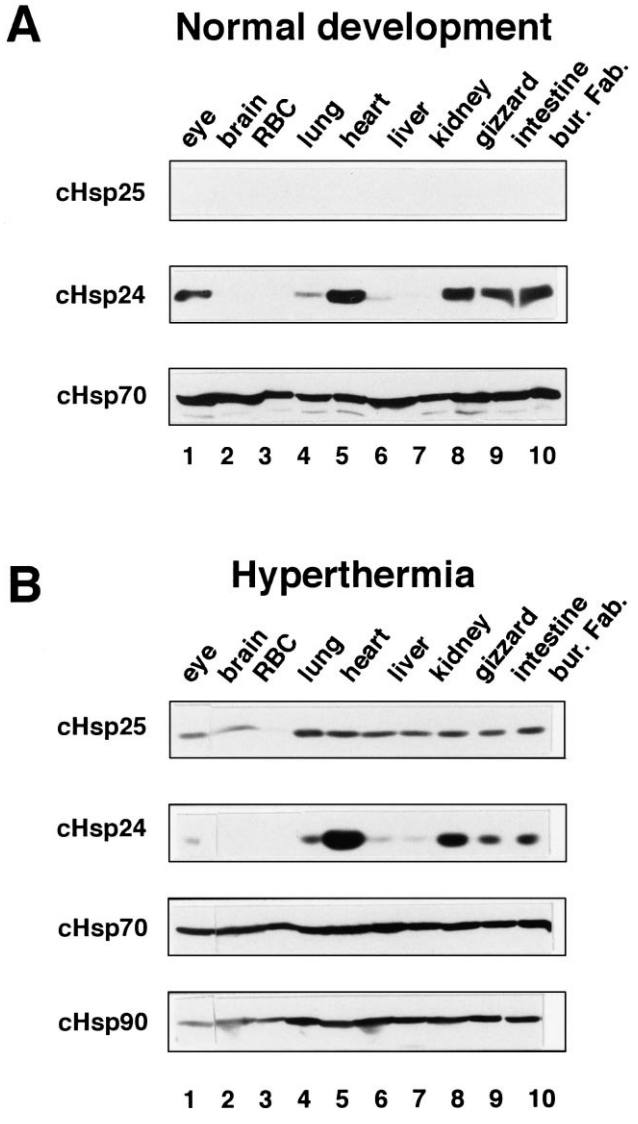

Fig. 3. Tissue distributions of cHsp25 and cHsp24. A: Tissues were dissected from chicken embryos on day 12. Samples were suspended directly in Laemmli's SDS-sample buffer. Aliquots containing the same amounts of Hsp70s were subjected to SDS-PAGE, followed by Western blotting with specific antisera for cHsp25, cHsp24, cHsp70 and cHsp90. The eye tissue contained the sensory layer of the retina and pigmental epithelium, but not the lens. The extract of red blood cells (RBC) contained mostly red blood cells with small amounts of other blood cells. B: Eggs maintained at 38°C were incubated at 45°C for 1 h and allowed to recover at 38°C for 3 h. Tissues were dissected immediately and expressions of Hsps were analyzed by Western blotting as in A.

We next examined the tissue distributions of cHsp24 and cHsp25. Tissues from embryos on day 12 were dissected, immediately suspended in Laemmli's SDS-sample buffer and homogenized by sonication. Aliquots containing the same amount of Hsp70s were subjected to SDS-PAGE followed by Western blotting using antiserum for cHsp24 or cHsp25. cHsp24 was found to be expressed at high levels in the heart, gizzard, intestine, bursa Fabricius and eye tissues, but at very low levels in the lung, liver, kidney and brain (Fig. 3A, middle panel). We detected no cHsp24 in red blood cells (lane 3). In marked contrast, cHsp25 was not detected in any of the tissues of normally developing embryos (Fig. 3A, upper panel). To examine the levels of cHsp24 and cHsp25 after whole-body hyperthermia, day 12 embryos were incubated at 45°C for 1 h and then allowed to recover at 38°C for 3 h. cHsp25 was expressed at substantial levels in all of the tissues similarly to cHsp70s and cHsp90 (Fig. 3B, third panel). In contrast, the pattern of expression of cHsp24 after hyperthermia was similar to that in normally developing embryos. Remarkably, little expression was observed in the brain and red blood cells (Fig. 3B, second panel, lanes 2 and 3). To examine the heat shock induction of cHsp24 in hematopoietic cells, we compared the induction in CEFs with that in the B lymphoblast cell line DT40. cHsp24 was expressed in CEFs under normal conditions and the expression was induced by about threefold by heat shock (Fig. 4B, lanes 1–4). In contrast, in DT40 cells cHsp24 was not expressed under either normal or heat shock conditions (Fig. 4B, lanes 5–8). This suggested that cHsp24 is cell-specific, whereas cHsp25 is ubiquitously expressed only after heat shock.

4. Discussion

In chicken cells, many laboratories have characterized the major heat-inducible protein cHsp25 as a small heat shock protein. However, there are two marked differences between chicken Hsp25 and the well-characterized human Hsp27. First, the level of cHsp25 after heat shock is almost as high as that of the major heat shock protein Hsp70 [16], whereas that of hHsp27 is much lower in fibroblasts [8]. In human fibroblast cells there is no heat-inducible protein of small molecular weight that is easily detected by Coomassie staining. Second, cHsp25 is detected as a single band on two-dimensional gel electrophoresis (Fig. 2), whereas hHsp27 is detected as four bands with different p*I*s [8]. The apparent p*I* of cHsp25 is lower than that of hHsp27. Nevertheless, cHsp25 has not been distinguished from the chicken homologue of hHsp27 (cHsp24 in this study). Edington and Hightower examined the induction of transcripts of the chicken homologue of hHsp27 (cHsp24) using hHsp27 cDNA [25]. Miron et al.

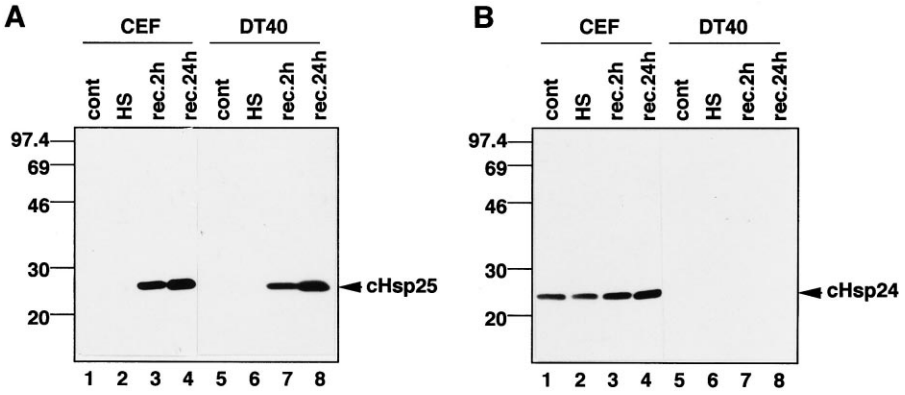

Fig. 4. Expression of cHsp25 and cHsp24 in CEFs and DT40 cells. CEFs and chicken B lymphoblast DT40 cells maintained at 37°C (lanes 1 and 5) were heat-shocked at 45°C for 1 h (lanes 2 and 6), and then allowed to recover for 37°C for 2 h (lanes 3 and 7) and 24 h (lanes 4 and 8). Cell extracts were prepared and levels of cHsp25 (A) and cHsp24 (B) proteins were determined by Western blotting using specific antisera. DT40 cells lacked expression of cHsp24 in the absence or presence of heat stress.

succeeded in molecular cloning of a chicken homologue of hHsp27 (cHsp24) as an inhibitor of actin polymerization [22]. However, both studies failed to compare the transcription/translation product from the cHsp24 cDNA with cHsp25. Thus, this is the first study to identify cHsp25 as a small heat shock protein distinct from the chicken homologue of human Hsp27 (cHsp24).

cHsp24 expression in the absence of stress is observed at very high levels in some tissues such as the heart and digestive organs (Fig. 3A; for mRNA level see [22]) similarly to its mouse homologue [15]. Even after hyperthermia, little expression of cHsp24 was observed in the brain or red blood cells (Fig. 3B). Furthermore, cHsp24 was not induced at all in some cells such as DT40 cells (Fig. 4) and embryonic red blood cells (data not shown) in which cHsp24 is not expressed under normal growth conditions (Fig. 4). In marked contrast, cHsp25 was induced by heat shock at high levels in all cells examined although cHsp25 expression was not observed at any of the cell types examined under normal growth conditions (Fig. 4, data not shown) or in tissues of unstressed developing embryos (Fig. 3A). These observations suggested that cHsp25 plays general roles in protein folding/assembly under stress conditions, whereas cHsp24 is involved in more specific cellular processes such as actin polymerization [22,26]. In agreement with our observations showing the tissue-specific expression of the chicken homologue of cHsp24, Lee et al. reported a lack of expression of hHsp27 in human L929 fibroblasts even after heat shock [27]. Murashov et al. examined expression of the mouse homologue of hHsp27 in the mouse brain and showed a restricted pattern of expression in the absence or presence of thermal stress [28]. Expression of the mouse homologue of hHsp27 is specifically restricted to the motoneurons of the facial, trigeminal, ambiguus and hypoglossal nuclei, whereas it was not observed in the cortex or hippocampus even after hyperthermia [28]. Taken together, these observations indicate that the hHsp27 homologue is not an ubiquitous molecular chaperone and is not necessary for normal cell growth. cHsp25 may play major roles specifically in the protection of cells from external stresses in general.

Molecular cloning of vertebrate cDNAs encoding at least six members of the small heat shock protein family has been reported. These include Hsp27, p20 [10], HSPL27 [13,14], MKBP/HSPB2 [11,12], αA-crystallin and αB-crystallin, which form highly ordered heterooligomers under normal growth conditions [29,30]. Among these, Hsp27 and αB-crystallin are induced when cells are incubated at high temperature. Other members are not induced by heat shock, and their expression is highly restricted to the muscle tissues and/or the eye lens. Chicken Hsp25 is quite unique among vertebrate sHsps in terms of its pattern of expression because cHsp25 is not expressed in any tissues under normal growth conditions and burst accumulation of cHsp25 was observed after heat shock in all of the cell types and tissues examined.

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