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CHARACTERIZATION OF HIGH-MOLECULAR-MASS HEAT SHOCK PROTEINS AND 42°C-SPECIFIC HEAT SHOCK PROTEINS OF MURINE CELLS

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There are two isoforms of high-molecular-mass heat shock protein (HMM-HSP), hsp105A and hsp105B, in murine FM3A cells. To characterize the HMM-HSPs, we here purified hsp105A and hsp105B, as well as 42°C-specific HSPs that are specifically induced by continuous heating at 42°C, from the cytoplasmic extracts of the FM3A cells heat-shocked at 42°C for 8 h. Digestion of the hsp105A, hsp105B, and 42°C-specific HSPs with lysyl endopeptidase generated 17,000-Da polypeptide fragments in common, and the N-terminal amino acid sequences of the fragments revealed a homology with those of the adenosine binding domain of hsp70 family proteins and actin. Thus, the two isoforms of hsp105 and the 42°C-specific HSPs seemed to be very similar proteins having a ATP binding domain in common, and these HSPs may constitute a HMM-HSP family in murine cells.

Exposure to heat shock or other environmental stress induces a physiological reaction in cells called stress response [1-3]. This response is characterized by the induction of a set of proteins called heat shock proteins (HSPs). The HSPs of mammalian cells have been classified into several families according to their apparent molecular mass: high-molecular-mass HSP (HMM-HSP), hsp90, hsp70, hsp60, and low-molecular-mass HSP families [2, 3]. Most HSPs are expressed in considerable amounts in unstressed cells, and they are indispensable for normal cellular functions. HSPs are also involved in cell protection and the repair of cell damage caused by a variety of stress.

Mammalian major HSPs such as hsp70 and hsp90 have been studied extensively, whereas little is known about a mammalian HMM-HSP. Hsp110, separated by two-dimensional gel electrophoresis of heat-shocked Chinese hamster ovary cells, was used to prepare a rabbit antiserum [4]. The hsp110 is shown to be constitutively expressed at low levels and appears to increase with heat shock. By indirect immunofluorescence with the anti-hsp110 serum, the

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The abbreviations: HMM, high-molecular-mass; HSP, heat shock protein; hsp105, heat shock protein of 105,000-Da; 42°C-hsp, 42°C-specific heat shock protein: PAGE, polyacrylamide gel electrophoresis.

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protein is present essentially in nucleoli of non-stressed and heat-shocked murine cells [4, 5]. The hsp110 is released from the nucleoli by RNase treatment, suggesting that hsp110 participates in the ribosome assembly.

We have previously prepared a rabbit antiserum against hsp105 of murine FM3A cells, that was separated by two-dimensional gel electrophoresis [6]. The anti-hsp105 serum reacts with not only hsp105 but also with a specific HSP that is synthesized only when mammalian cells are heat-shocked continuously at 42°C (42°C-specific HSP, 42°C-hsp) [7-9]. By immunoprecipitation and immunoblot analysis using the anti-hsp105 serum, we revealed that there are two isoforms of hsp105 (hsp105A and hsp105B) in FM3A cells [7]. Furthermore, by indirect immuno-fluorescence analysis with the anti-hsp105 serum, the HMM-HSPs are localized in the cytoplasm and nuclei, and are never found in the nucleoli under both non-stressed and stressed conditions. Thus, hsp105s of FM3A cells and hsp110 of CHO cells seemed to be different proteins [7].

In this study, to characterize the HMM-HSPs, we purified these HSPs from the cytoplasmic extracts of the FM3A cells heat-shocked at 42°C, and revealed that hsp105A and hsp105B as well as 42°C-specific HSPs were similar proteins containing a particular amino acid sequence similar to an adenosine binding domain of hsp70 family proteins and actin.

EXPERIMENTAL PROCEDURES

Cell culture and radioisotope labeling---Murine FM3A cells were supplied from Japan Cancer Research Resource Bank, and were maintained in Eagle's minimal essential medium supplemented with 10% calf serum at 37°C in a CO_2 incubator (5% CO_2 in air).

For labeling of cells, cells were incubated in a methionine-deficient medium supplemented with 10% calf serum and 20 μCi/ml [^{35}S]protein labeling mix (a 77:18% mixture of [^{35}S]methionine:[^{35}S]cysteine, 1,000 Ci/mmol; NEN). After labeling, the cell proteins were recovered by solubilization with 0.1% SDS.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE)---The protein samples were electrophoresed on SDS-7.5% or 15% polyacrylamide slab gels [10]. The gels were stained with Coomassie brilliant blue R-250, and destained. Some gels were stained with silver using silver-staining kit (Daiichi Kagaku). The molecular masses of the proteins were calibrated with low molecular mass markers (Pharmacia Fine Chemicals).

Two-dimensional gel electrophoresis---Two-dimensional gel electrophoresis was performed as described by O'Farrell [11]. An isoelectrofocusing gel containing 1.6% ampholytes (pH 5-7)/0.4% ampholytes (pH 3.5-10) was used for the first dimension, and a SDS-7.5% polyacrylamide gel served for the second dimension. The gels containing labeled proteins were processed for fluorography with Amplify (Amersham), then dried, and fluorographed at -80°C.

Purification of HMM-HSPs---FM3A cells (2 x 10^6 cells/ml, 12 ml/ø 10 cm dish) were heated in a water bath set at 42°C for 6-8 h, and then harvested and washed twice with phosphate-buffered saline. The heat-shocked FM3A cells (approximately 10 g wet weight/ 500 dishes) were homogenized in 20 ml of buffer A containing 20 mM Tris-HCl (pH 7.5), 0.1 mM dithiothreitol, 0.1 mM ethylenediaminetetraacetic acid and 1 mM phenylmethylsulfonyl fluoride. The homogenates were centrifuged for 10 min at 12,000 xg at 4°C, and the supernatant was further centrifuged for 1 h at 105,000 xg at 4°C. The second supernatant was applied to a DEAE-Sepharose CL-6B column (ø 1.5 x 30 cm) equilibrated with buffer A at 4°C. After washing the column with the buffer, the adsorbed proteins were eluted with a linear gradient of 20-500 mM sodium chloride in the buffer (total 200 ml) at a flow rate of 48 ml/h, and 5 ml each fraction was collected. Aliquots (100 μl) of the eluted fractions were freeze-dried, and separated by SDS-7.5% PAGE. Hsp105s were detected by immunoblotting using anti-hsp105 serum, as described previously [7].

Fractions (approximately 30 ml) from the DEAE-Sepharose CL-6B column containing hsp105 proteins were pooled and dialyzed against 3 l of buffer B containing 20 mM potassium phosphate (pH 7.5), 0.1 mM ethylenediaminetetraacetic acid and 0.1 mM dithiothreitol overnight, and applied to a hydroxylapatite column (ø 1.0 x 10 cm) equilibrated with buffer B at 4°C. After

washing the column with the buffer, the adsorbed proteins were eluted with a linear gradient of 20-300 mM phosphate in the buffer (total 100 ml) at a flow rate of 10 ml/h, and 2 ml each fraction was collected. Aliquots (100 μl) of the eluted fractions were precipitated with 10% trichloroacetic acid, and were separated by SDS-7.5% PAGE. The hsp105s were detected by immunoblotting using anti-hsp105 serum.

Fractions from the hydroxylapatite column containing hsp105 proteins were pooled. This pooled fraction (12 ml) was concentrated to 50 μl using Centricon-30 (Amicon), and to this fraction 4 ml of SDS-sample buffer was added and heated at 100°C for 2 min. The sample (500 μl/a 2mm thick gel) was then separated by SDS-7.5% PAGE. After staining with 0.1 % Coomassie brilliant blue, 10% methanol and 0.5% acetic acid for 2 h, the gels were destained with 10% methanol for 2 h. The protein bands of hsp105A, hsp105B, 42°C-hspA, and 42°C-hspB were cut out, and the proteins were electro-eluted from the stained bands in a 2-fold diluted SDS running buffer using Centriluter (Amicon) at 100 V for 12 h. The eluted proteins were concentrated using Centricon-30. Protein concentration of the purified proteins was estimated by the comparison of density of the silver-stained bands with those of bovine serum albumin.

Peptide mapping---For peptide mapping with lysyl endopeptidase (Wako chemicals), 10 μg of the electro-eluted protein was digested with 0.1 μg of the enzyme in a digestion buffer (100 μl) containing 100 mM Tris-HCl (pH 9.0) and 0.1 % SDS at 37°C for 3 h.

Determination of amino acid sequences---The lysyl endopeptidase-digests were freeze-dried, and dissolved in 20 μl of a two-fold concentrated SDS-sample buffer. After prerunning with SDS-running buffer containing 0.1 mM thioglycolate, the sample (10 μl/lane) was separated by SDS-15% PAGE using a mini-gel (1 mm thick). After electrophoresis, the gel was shaked in solution C (25 mM Tris, 40 mM 2-aminocapronic acid, 20% methanol, and 0.02% SDS) for 15 min. The gel was placed on a sheet of PVDF membrane (Bio-Rad, 0.2mm pore), under which 2 layers of blotting paper containing solution A (0.3 M Tris, 0.02% SDS, and 20% methanol) and 2 layers of blotting paper containing solution B (25 mM Tris, 0.02% SDS, and 20% methanol) were layered successively on the lower electrode, and then assembled into a blotting apparatus for electro-elution at 1 mA/cm^2 for 3 h. After blotting, the membrane was rinsed with water, washed with 25 mM sodium chloride and 10 mM sodium borate (pH 8.0) for 5 min, stained with 0.1% Coomassie brilliant blue R in 50% methanol, destained in 50% methanol, and dried. Each polypeptide on the membrane was then subjected to Edman degradation for the determination of N-terminal amino acid sequence using an Applied Biosystem 477A/120A protein sequencer system.

RESULTS

HMM-HSPs of murine cells---We have shown that by immunoprecipitation and immunoblot analysis using the anti-hsp105 serum there are two isoforms of HMM-HSP having a molecular mass of 105,000 Da (hsp105A and hsp105B) in FM3A cells [7]. Figure 1 shows the two-dimensional gel electrophoretic analysis of the HMM-HSPs of FM3A cells. By metabolic labeling, two proteins (d-1 and d-2) of molecular mass of 105,000-Da that increased a little by heat-shock at either 42 or 45°C were observed (Fig. 1A-C). By Coomassie brilliant blue-staining, two stained spots corresponding to the two labeled proteins (d-1 and d-2) were observed (Fig. 1D-F). In non-stressed cells, a considerable amount of d-1 protein was found, whereas d-2 protein was present in a small amount. By heat shock, d-1 protein increased a little, whereas d-2 protein increased considerably. Thus, the two proteins, d-1 and d-2, seemed to correspond to hsp105A and hsp105B, respectively [7]. In additions, two protein spots (e-1 and e-2) in the 90,000-Da molecular mass regions were specifically synthesized by heating at 42°C but not at 45°C, as has been reported previously [8, 9] (Fig. 1B). The 42°C-specific HSPs accumulated corresponding to their synthesis during the continuous heat shock at 42°C (Fig. 1E). The e-1 and e-2 proteins also corresponded to 42°C-hspA and 42°C-hspB, respectively.

Purification of hsp105s and 42°C-hsps---To characterize these HMM-HSPs, we purified them from FM3A cells heat-shocked at 42°C for 6-8 h, as described in Experimental Procedures. The

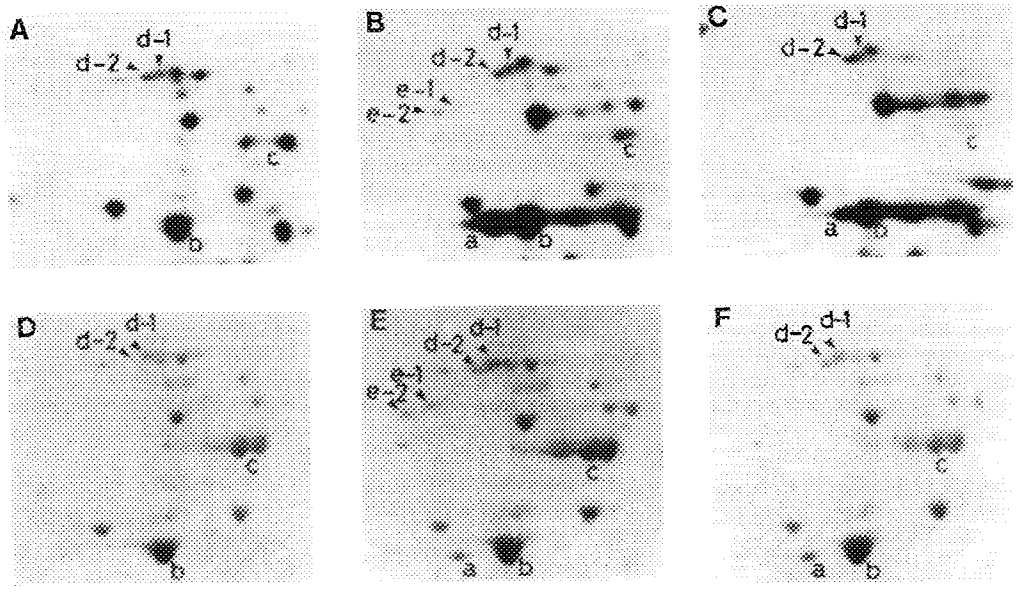

Fig. 1. Two-dimensional gel electrophoretic analysis of HMM-HSPs of murine FM3A cells. Upper panels: FM3A cells were incubated at 37°C (A) or 42°C (B) for 3 h, or at 37°C for 3 h after heat shock at 45°C for 10 min (C) in the presence of [^{35}S]protein labeling mix. The labeled proteins (1 x 10^6 cpm) were separated by two-dimensional gel electrophoresis and detected by fluorography. Lower panels: FM3A cells were incubated at 37°C (D) or 42°C (E) for 6 h, or at 37°C for 6 h after heat shock at 45°C for 10 min (F). The proteins (400 μg) were separated by two-dimensional gel electrophoresis and detected by Coomassie brilliant blue-staining. Isoelectric focusing was performed for the first dimension (horizontal axis) and SDS-7.5% PAGE for the second dimension (vertical axis). Fluorographs and Coomassie brilliant blue-stained gels are shown with the acidic end to the right. a, hsp70; b, hsp73; c, hsp90; d-1, hsp105A; d-2, hsp105B; e-1, 42°C-hspA; e-2, 42°C-hspB.

cytoplasmic extracts from the FM3A cells were separated by DEAE-Sepharose CL-6B column chromatography (Fig. 2A). Hsp105A and hsp105B were simultaneously eluted at fractions 43-46 (between 150 and 180 mM NaCl). These fractions were collected, dialyzed, and further separated by hydroxylapatite column chromatography (Fig. 2B). The hsp105A and hsp105B were also simultaneously eluted at fractions 43-48 (between 125 and 150 mM phosphate).

Fig. 3A shows the protein patterns, separated by SDS-PAGE, of fractions at each purification step. Since 42°C-specific HSPs were coeluted with hsp105s through the two column chromatographies, the hydroxylapatite fraction contained not only hsp105A and hsp105B, but also 42°C-hspA and 42°C-hspB, with some other proteins. Figs. 3D and E show the two-dimensional gel patterns of the cytoplasmic extracts and the hydroxylapatite fraction, respectively. Since there seemed to be no contaminating proteins having similar apparent molecular masses to these HSPs in the hydroxylapatite fraction, we further purified hsp105A, hsp105B, 42°C-hspA, and 42°C-hspB by electro-elution from the Coomassie brilliant blue-stained bands in gels separated by SDS-PAGE shown in Fig. 3B. Each electro-eluted protein preparation contained a single protein (Fig. 3C). These protein bands were surely reacted with anti-hsp105 serum (not shown).

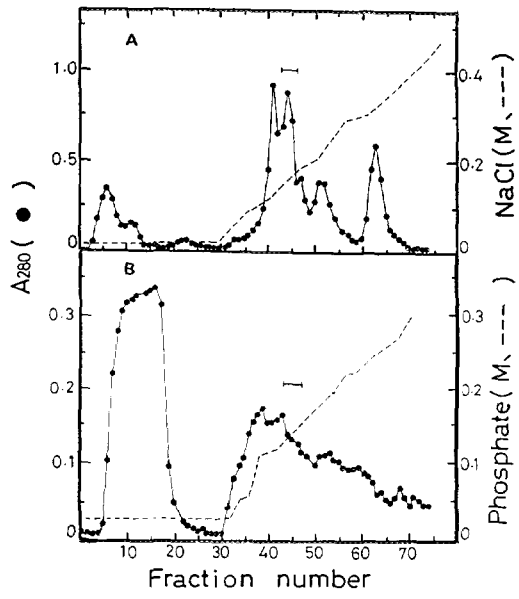

Fig. 2. Purification of HMM-HSPs by DEAE-Sepharose and hydroxylapatite column chromatographies. HMM-HSPs were partially purified from the cell extracts of approximately 10 g of heat-shocked FM3A cells by sequential column chromatographies of DEAE-Sepharose CL-6B (A) and hydroxylapatite (B), as described in Experimental Procedures. The filled circle and the broken line indicate an absorbance at 280 nm and salt or phosphate concentration determined by conductivity, respectively.

Peptide-mapping analysis of HMM-HSPs---When the purified HSPs were digested with lysyl endopeptidase, there was one major peptide fragment of 17,000-Da common to the four HSPs (Fig. 4). There were no apparent differences between the peptide maps of 42°C-hspA and 42°C-hspB, and the peptide maps were also very similar to that of hsp105B. When hsp73 and bovine serum albumin were electro-eluted in a similar manner from SDS-polyacrylamide gels and digested with lysyl endopeptidase, the peptide maps were completely different from those obtained with the HMM-HSPs.

Amino acid sequence analysis of HMM-HSPs---When N-terminal amino acid sequences of hsp105A, hsp105B, 42°C-hspA and 42°C-hspB were analyzed by gas-phase protein sequencer, all of the four HSPs seemed to be blocked at N-terminal amino acid. Hence, we next analyzed the 17,000-Da peptide fragments of these HMM-HSPs digested with lysyl endopeptidase. As shown in Fig. 5, the N-terminal sequences determined were the same among hsp105A, hsp105B, 42°C-hspA and 42°C-hspB. When the sequence of hsp105A was subjected to the homology search using the data bases of NBRF-PDB and SWISS-PROT, the sequence showed a strong homology to dnaK of *B. subtilis*, Chinese hamster BIP, SSA1 of *S. cerevisiae*, human hsc70, murine hsp70 and actin of *S. cerevisiae*. The homology of the sequences was the adenosine binding motif of ATPase domain of hsp70 family proteins and actin [12, 13]. When the other peptide fragments, 13,000-Da fragments of hsp105A and hsp105B digested with lysyl endopeptidase, were

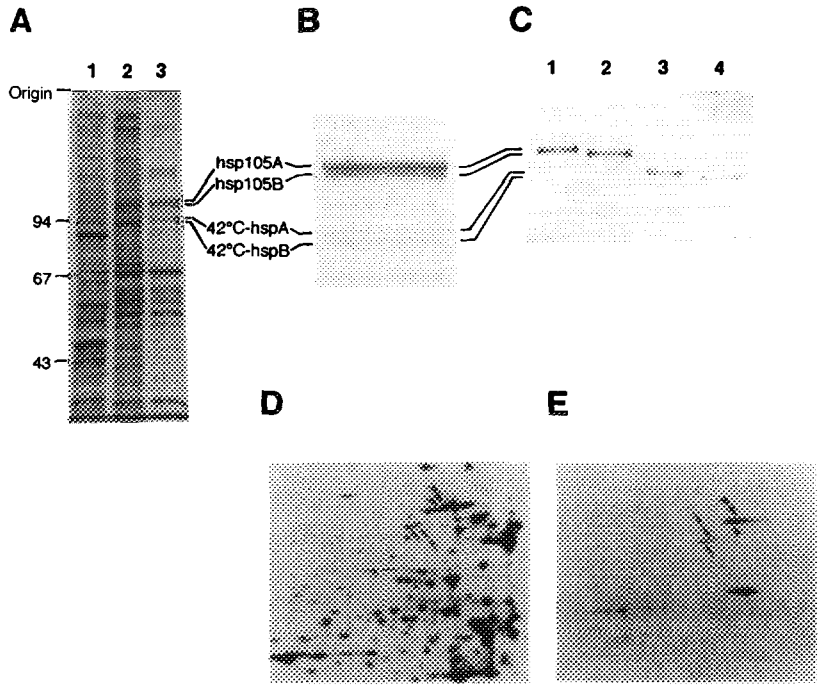

Fig. 3. Purification of HMM-HSPs from murine FM3A cells. (A) Proteins at each purification step were analyzed by SDS-7.5% PAGE and detected by Coomassie brilliant blue-staining: lane 1, cytoplasmic extracts (40 μg); lane 2, DEAE-Sepharose fraction (20 μg); lane 3, hydroxylapatite fraction (10 μg). (B) The hydroxylapatite fraction was separated by SDS-7.5% PAGE and stained with Coomassie brilliant blue. The protein bands of hsp105A, hsp105B, 42°C-hspA, and 42°C-hspB were cut out, and the proteins were electro-eluted from the gel bands, as described in Experimental Procedures. (C) The electro-eluted proteins (0.2 μg each)were separated by SDS-7.5% PAGE and stained with silver; lane 1, hsp105A; lane 2, hsp105B; lane 3, 42°C-hspA; lane 4, 42°C-hspB. (D, E) The cytoplasmic extracts (100 μg, D) and the hydroxylapatite fraction (10 μg, E) were separated by two-dimensional gel electrophoresis and detected by silver-staining. The double arrowhead, single arrowhead, double arrow, and single arrow indicate hsp105A, hsp105B, 42°C-hspA, and 42°C-hspB, respectively.

sequenced, both sequences were similar but showed no homology to hsp70 family proteins and actins, indicating that these HMM-HSPs were different from hsp70 family proteins and actins.

DISCUSSION

We have shown that there are two isoforms of HMM-HSP, hsp105A and hsp105B, in murine FM3A cells [7]. To characterize the HMM-HSPs, we purified hsp105A and hsp105B, as well as 42°C-hspA and 42°C-hspB, from the cytoplasmic extracts of the heat-shocked FM3A cells. Digestion of hsp105A, hsp105B, and 42°C-specific HSPs with lysyl endopeptidase showed very similar peptide maps among the four HSPs. Furthermore, N-terminal amino acid sequences of the 17,000-Da polypeptide fragments of the four HSPs digested with lysyl endopeptidase revealed a homology with those of the adenosine binding domain of hsp70 family proteins and actin. However, since the lysyl endopeptidase-digestion of hsp73, a constitutive hsp70, did not

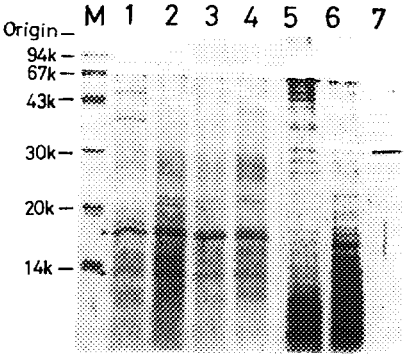

Fig. 4. Peptide mapping of HMM-HSPs. Ten μg each of electro-eluted hsp105A (lane 1), hsp105B (lane 2), 42°C-hspA (lane 3), or 42°C-hspB (lane 4) was digested with 0.1 μg of lysyl endopeptidase at 37°C for 3 h. The digests (1 μg) were separated by SDS-15% PAGE and detected by silver-staining. Hsp73 (lane 5) and bovine serum albumin (lane 6) were similarly electro-eluted from SDS-polyacrylamide gels and digested with lysyl endopeptidase. Lanes 7 and M represent lysyl endopeptidase (0.2 μg) and marker proteins, respectively. The numbers on the side of the gel represent the molecular masses of the marker proteins. The arrowhead and arrow indicate 17,000 and 13,000-Da peptide fragments, respectively.

generate the 17,000-Da fragment, these HSPs seemed to be different from hsp70 family proteins. The other 13,000-Da peptide fragments of hsp105A and hsp105B also did not show any homology to the hsp70 family proteins and actins. Thus, the two isoforms of hsp105, as well as

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(A)
hsp105A          1  AXLXS XIEIV GGATR IPAVK ERI  23
                    * * *  **** ***** ***
hsp105B            AXLVS AIEIV GGATR IPA
                    * *** ***** *** * **
42°C-hspA          AXLVS AIEIV GGAXR IP
                    * *** *****   *
42°C-hspB          AXLVS AIEIV XXA

(B)
hsp105A            1  AXLXSXIEIVGGATRIPAVKERI  23
                         . .***.******.* *
dnaK  (B.sub.)    301  ASEIDKVILVGGSTRIPAVQEAIKKETGKE  330
BIP   (hamster)   353  KSDIDEIVLVGGSTRIPKIQQLVKEFFNGK  382
SSA1  (S.cerev.)  325  KSQVDEIVLVGGSTRIPKVQKLVTDYFNGK  354
hsc70 (human)     328  KSQIHDIVLVGGSTRIPKIQKLLQDFFNGK  357
hsp70 (murine)    328  KAQIHDLVLVGGSTRIPKVQKLLQDFFNGR  357

hsp105A            1  AXLXSXIEIVGGATRIPAVKERI  23
                        * . * . **.*..*.. **.
Actin (S.cerev.)  291  KELYGNIVMSGGTTMFPGIAERMQKEITAL  320

(C)
hsp105A            1  VMYFA XXXMV  10
                      *****    **
hsp105B               VMYFA XTDMV
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Fig. 5. Partial amino acid sequences of murine HMM-HSPs. (A) The N-terminal sequences of the 17,000 Da fragments of hsp105A, hsp105B, 42°C-hspA and 42°C-hspB digested with lysyl endopeptidase were determined by gas-phase protein sequencer, as described in Experimental Procedures. X indicates unidentified amino acid. Star indicates the same amino acid between the HMM-HSPs. (B) Alignment of amino acid sequence of hsp105A with those of adenosine binding domains of DnaK of *B. subtilis*, hamster BIP, SSA1 of *S. cerevisiae*, human hsc70, murine hsp70, and actin of *S. cerevisiae*. Star and point indicate the same and similar amino acids, respectively, between hsp105A and the other proteins. (C) The N-terminal sequences of the 13,000 Da fragments of hsp105A and hsp105B digested with lysyl endopeptidase were also shown.

the 42°C-specific HSPs, seemed to be very similar proteins having a ATP binding domain in common. We are inclined to think that these HSPs may constitute a HMM-HSP family in mammalian cells.

In yeast cells, a HMM-HSP hsp104 is induced by a shift to a high temperature, by the transition to stationary growth phase, or by early sporulation [14, 15]. The hsp104 has two putative nucleotide-binding sites, and mutagenesis of these binding sites causes the loss of thermotolerance [14, 16]. Hsp104 exhibits homology to bacterial ClpA/ClpB proteins [16]. Although the hsp105s of murine FM3A cells seemed to have the adenosine binding motif of the ATPase domain of hsp70 family proteins and actin, the sequences did not show a homology to that of yeast hsp104.

We have previously shown that HeLa and FM3A cells synthesize a specific HSP that is synthesized only when these cells are heat-shocked continuously at 42°C [8, 9]. The 42°C-specific HSP consists of at least two polypeptides having a molecular mass of approximately 90,000, a basic and an acidic one. The basic one (42°C-hspB) is synthesized by mRNA transcription, and transition of basic to acidic one (42°C-hspA) seems to occur by post-transcriptional modification [9]. Hsp105s are induced by heat shock either at 42°C or 45°C, whereas 42°C-specific HSPs are synthesized only when heated continuously at 42°C, at which temperature splicing of RNA may be critically affected [17, 18]. It is interesting to determine whether the hsp105A, hsp105B and 42°C-specific HSPs originate from different genes, or hsp105s and 42°C-specific HSPs may originate from the primary transcript of a hsp105 gene by alternative splicing. To further clarify these points and to determine the structure and function of the HMM-HSP family proteins of mammalian cells, it is essential that the HMM-HSP genes be isolated and analyzed.

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