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Molecular and Functional Characterization of HSC54, a Novel Variant of Human Heat-Shock Cognate Protein 70

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ABSTRACT

A novel variant of human heat-shock cognate protein 70 (HSC70) transcript, named heat-shock cognate protein 54 (HSC54), was identified and characterized. The transcript encodes the protein lacking 153 amino acid residues of HSC70 in a part of the protein-binding and variable domains, resulting in a calculated molecular mass of 53.5 kDa. HSC54 mRNA was detected in all human cells and tissues examined. The protein was also detected in peripheral mononuclear cells and U937 human histiocytic lymphoma cells. Heat treatment of U937 cells up-regulated the expression of HSC54. The chaperoning activity of HSC54 was examined by luciferase renaturation assay. HSC70 recovered the luciferase activity in the presence of reticulocyte lysate as a source of cochaperones. However, HSC54 did not facilitate the recovery of denatured luciferase; besides, HSC54 significantly inhibited the HSC70-mediated chaperoning activity. In pull-down experiments, HSC54 interacted with cochaperones, p60, HSP40, and p48, as HSC70 did. The resonant mirror detection analysis showed that p60 binds to HSC54 with a higher association rate constant than HSC70 with a similar affinity constant. These results suggest that HSC54 is constitutively expressed and also inducible by stress and may function as an endogenous inhibitory regulator of HSC70 by competing the cochaperones.

The heat-shock cognate protein 70 (HSC70) is a member of the heat-shock protein 70 (HSP70) family (reviewed by Ellis and van der Vies, 1991; Gething and Sambrook, 1992; Hendrick and Hartl, 1993; Pratt and Toft, 1997; Smith et al., 1998; Li and Mivechi, 1999). The protein is constitutively expressed and functions as a molecular chaperone to mediate diverse important cellular processes, including protein folding, refolding, assembly, disassembly, and protein translocation across biological membranes. In physiological conditions, for example, HSC70 assists newly synthesized protein to fold correctly, serves the steroid-receptor complex assembly, and plays a role in the translocation of transcription factor nuclear factor κB to the nucleus (Fujihara and Nadler, 1999). Under several stress conditions, HSC70 is up-regulated to protect cells by preventing denaturation of proteins.

HSC70 consists of three domains: the N-terminal 44-kDa adenosine triphosphatase (ATPase) domain (residues 1–384), the middle 18-kDa protein-binding domain (residues 385–543), and the C-terminal 10-kDa variable domain (residues 544–646) (Wang et al., 1993; Tsai and Wang, 1994; Freeman et al., 1995; Hu and Wang, 1996). HSC70 binds to a substrate molecule via the protein-binding domain and undergoes a repeated cycle of binding and dissociation with substrates. The HSC70-substrate binding is controlled by a reaction cycle of ATP binding, hydrolysis, and ADP-ATP exchange at the ATPase domain (Palleros et al., 1994). The variable domain contributes to the stability of the HSC70-substrate complex (Tsai and Wang, 1994; Freeman et al., 1995; Hu and Wang, 1996). Several cochaperones, including p60, p48, and heat-shock protein 40 (HSP40) cooperate with human HSC70 in protein folding and assembly (reviewed by Frydman and Höhfeld, 1997).

The human HSC70 gene was previously isolated (Dworniczak and Mirault, 1987) and mapped to chromosome 11 (Tavaria et al., 1995). Although cDNA and amino acid sequences of HSC70 share some homology to other HSP70 family members, no variant of HSC70 has been described. In the present study, we identified a novel variant of the human HSC70, named heat-shock cognate protein 54 (HSC54). Sequencing analysis revealed that the transcript encodes the protein lacking 153 amino acid residues of HSC70 in a part of the protein binding and variable domains. To clarify the molecular and functional characterization of HSC54, we examined the expression of HSC54 in human cells and tissues. The chaperoning activity of HSC54 was assessed by the luciferase

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ABBREVIATIONS: HSC, heat-shock cognate protein; FCS, fetal calf serum; GST, glutathione *S*-transferase; HSP, heat-shock protein; HUVEC, human umbilical vein endothelial cells; RT, reverse transcription; PCR, polymerase chain reaction; nt, nucleotide(s); ECL, enhanced chemiluminescence.

renaturation assay. We further studied the interaction of HSC54 with cochaperones p60, HSP40, and p48 by a glutathione *S*-transferase (GST)-pull-down experiment, and also the dynamic interaction between HSC54/HSC70 and p60 was analyzed by the resonant mirror detection method.

Materials and Methods

Identification and Isolation of HSC54 cDNA. HSC54 cDNA was identified by the RT-PCR method. Total RNA was isolated from U937 cells by using ISOGEN (Nippongene, Tokyo, Japan) and was reverse transcribed by the Superscript Preamplification System (Life Technologies, Rockville, MD) with oligo(dT) primers under the conditions recommended by the supplier. A primer set (F1: 5′-GCTTCCTTCGTTATTGGAGC-3′ sense and R: 5′-ACTTGGTTGGCTTAATCAACC-3′ antisense, corresponding to nt 43–62 and nt 2035–2015 of HSC70 cDNA, respectively) was designed to amplify the entire coding region of HSC70 cDNA (Dworniczak and Mirault, 1987) (Fig. 1B). Another primer flanking the deleted region in HSC54 cDNA (F2: 5′-TGGATGTCACTCCTCTTTCC-3′ sense, corresponding to nt 1264–1283 of HSC70 cDNA) was designed to confirm the existence of HSC54 mRNA. The PCR protocol was denaturation at 94°C for 5 min, followed by 30 cycles of amplification at 94°C for 1 min, 54°C for 1 min, and 72°C for 2 min, with a final elongation at 72°C for 10 min. The amplified products were analyzed by agarose gel electrophoreses and subcloned into T-vector (Novagen, Madison, WI) for sequencing. Both strands of isolated subclones were sequenced several times by the dideoxy chain terminator method (Sanger et al., 1977), using an SQ-5500 sequencer (Hitachi, Tokyo, Japan). The DNA sequence and deduced amino acid residues were analyzed with GENETYX-MAC (Software Development, Tokyo, Japan).

Construction of Plasmids. The entire coding regions of HSC70, HSC54, p60 (Honoré et al., 1992), HSP40 (Ohtsuka, 1993), and p48 (Prapapanich et al., 1996) were amplified by PCR using the sense/antisense primer set encoding initiation/stop codons. These primers also encoded the *Bam*HI site (for HSC70, HSC54, and HSP40) or *Eco*RI site (for p60 and p48) at the 5′-end. PCR was performed using isolated cDNA clones (for HSC70 and HSC54) or human cDNA (for p60, HSP40, and p48) as templates. The products were sequenced to ensure the absence of mutations and then were subcloned into pGEX-2T (Amersham Pharmacia Biotech, Buckinghamshire, England) at the *Bam*HI (for HSC70, HSC54, and HSP40) or the *Eco*RI (for p60 and p48) site. The resulting plasmids were used for expression of HSC70, HSC54, p60, HSP40, and p48 as GST fusion proteins in *Escherichia coli*. The plasmids pGEX-2T-HSC70/HSC54 were digested by *Bam*HI, and the resulting inserts were ligated into the *Bam*HI site of pFLAG-CMV-2 (Eastman Kodak, New Haven, CT), a C-terminal FLAG-fusion vector. The resulting plasmids were used for expression of HSC70 and HSC54 as FLAG fusion proteins in COS7 cells.

Cell Culture. U937 cells were grown in a stationary suspension culture in RPMI 1640 medium (Life Technologies) supplemented with 10% (v/v) fetal calf serum (FCS), 100 U/ml penicillin, and 100 μg/ml streptomycin. HepG2 cells were grown in Dulbecco's modified Eagle's medium (Life Technologies) supplemented with 10% (v/v) FCS, 4 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. The human umbilical vein endothelial cells (HUVEC) were grown in M199 medium (Life Technologies) supplemented with 10% (v/v) FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2.5 μg/ml fungizone. COS7 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) FCS, 4 mM glutamine, and 100 μg/ml kanamycin. The cells were incubated in a humidified 5% CO_2, 95% air atmosphere at 37°C .

RT-PCR Analysis for the Expressions of HSC54. To study the expression of HSC54 in human cells, total RNA was isolated from U937, HUVEC, and HepG2 cells and was reverse transcribed as described above. To study the expression of HSC54 in human tissues, total RNAs from human brain, kidney, liver, lung, and trachea (Clontech, Palo Alto, CA) were reverse transcribed. The effect of heat shock on the expression of HSC70 and 54 was examined in U937 cells. For heat treatments, U937 cells (1×10^6/ml) were seeded in a 6-cm culture dish and placed in a CO_2 incubator at 43°C for 30 min. After an exposure to heat, the cells were allowed to recover in the CO_2 incubator at 37°C for 1 to 6 h. Then, total RNA was isolated and was reverse transcribed. RT-PCR was performed with F1/R or F2/R primer sets (Fig. 1B). Amplification of the cDNA with the primers for human β-actin [βF: 5′-CCCAGATCATGTTTGAGACC-3′ sense (nt 400–419) and βR: 5′-TAGCTCTTCTCCAGGGAGGA-3′ antisense (nt 760–741)] (Ponte et al., 1984) served as a control for the quantity of cDNAs. The PCR protocol was denaturation at 94°C for 5 min, followed by 30 cycles (for study of the expression of HSC70 and 54 in human cells and tissues), 22 cycles (for study of the effect of heat shock on the expression of HSC70 and 54), or 25 cycles (for β-actin) of amplification at 94°C for 1 min, 54°C for 1 min, and 72°C for 2 min, with a final elongation at 72°C for 10 min. The PCR products were electrophoresed in a 0.7 or 2% agarose gel and stained with ethidium bromide. The digital images of stained gels were obtained by the Fluor-S MultiImager (Bio-Rad, Hercules, CA), and the density of bands was analyzed by a Macintosh computer with image-analyzing software (Molecular Analyst, Bio-Rad).

Western Blot Analysis. U937 cells and peripheral mononuclear cells, isolated from human blood by the standard Ficoll gradient method, were rinsed with PBS, lysed with sample buffer [50 mM Tris-HCl (pH 6.8), 10% glycerol, 2% SDS, 6% β-mercaptoethanol, and 0.1% bromphenol blue], and boiled for 10 min. After centrifugation (16,000*g*, 30 min), supernatants (30 μg protein/lane) were electrophoresed in 10% acrylamide gels and transferred electrophoretically onto Hybond-ECL (Amersham Pharmacia Biotech). An anti-human HSC70 antibody (1B5; Stressgen, Victoria, BC, Canada) was hybridized and detected with the ECL kit (Amersham Pharmacia Biotech) by the protocol provided by the manufacturer. The Fluor-S MultiImager (Bio-Rad, Hercules, CA) was used for analyses.

Preparation of Recombinant Proteins. HSC70, HSC54, p60, HSP40, and p48 proteins were produced as GST fusion proteins in *E. coli*. *E. coli* (BL21) was transformed with the plasmids pGEX-2T-HSC70/HSC54/p60/HSP40/p48, and the GST-fusion proteins were induced by isopropyl-β-D-(−)-thiogalactopyranoside. The fusion proteins were purified by glutathione-Sepharose 4B beads (Amersham Pharmacia Biotech), under the conditions recommended by the supplier. The purified GST fusion proteins were used for the GST-pull-down assay.

For the luciferase renaturation assay and the binding assay by resonance mirror detection, the purified GST fusion proteins were cleaved with thrombin, and the proteins were further purified using glutathione-Sepharose 4B. To verify the purity, the purified proteins were electrophoresed in 10% SDS-acrylamide gel and stained with Coomassie Brilliant Blue R-250 (data not shown).

Luciferase Renaturation Assay. The chaperoning activity of HSC70 and HSC54 were determined by the refolding of thermally denatured firefly luciferase as previously described (Minami et al., 1996) with several modifications. Briefly, firefly luciferase (10 ng/ml) (Sigma, St. Louis, MO) in 25 mM Tricin-HCl, pH 7.75, 8 mM $MgSO_4$, 0.1 mM EDTA, 10 mg/ml BSA, 10% glycerol, and 1% Triton X-100 was denatured by an incubation at 40°C for 5 min. Recombinant HSC70 and HSC54 (100 μg/ml) were incubated in 10 mM Tris-HCl, pH 7.5, 3 mM $MgCl_2$, 100 mM KCl, 2 mM dithiothreitol, 5% rabbit reticulocyte lysate (Promega, Madison, WI), 2 mM ATP, and an ATP-regenerating system [10 mM phosphocreatine (Sigma) and 3.5 U of creatine kinase, type I rabbit muscle (Sigma)] for 1 h at 4°C. After incubation, denatured luciferase (1 ng/ml) was added and allowed to renature at 30°C for 60 min. Luciferase activity present in aliquots was determined by a luciferase kit (Promega) using a Bio-Orbit luminomater (Bio-Orbit, Turku, Finland). The percentage activities were calculated against the activity of nondenatured luciferase.

GST-Pull-Down Assay. COS7 cells were transfected with the plasmids pFLAG-CMV-2-HSC70/HSC54 using Superfect Transfection Reagent (Qiagen, Hilden, Germany) under the conditions recommended by the supplier. After 48 h, the cells were lysed in a lysis buffer (20 mM HEPES, pH 7.5, 150 mM NaCl, 1 mM EDTA, 10 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride and 1% Triton X-100). The lysates were incubated with 5 μg of p60-GST, HSP40-GST, or p48-GST fusion protein prebound to 20 μl of glutathione-Sepharose 4B beads for 60 min at 4°C in a binding buffer (50 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 100 mM NaCl, 10% glycerol, 0.5 mg/ml BSA, and 5 mM β-mercaptoethanol). Nonspecific binding was determined by GST protein prebound to beads. Then, the beads were

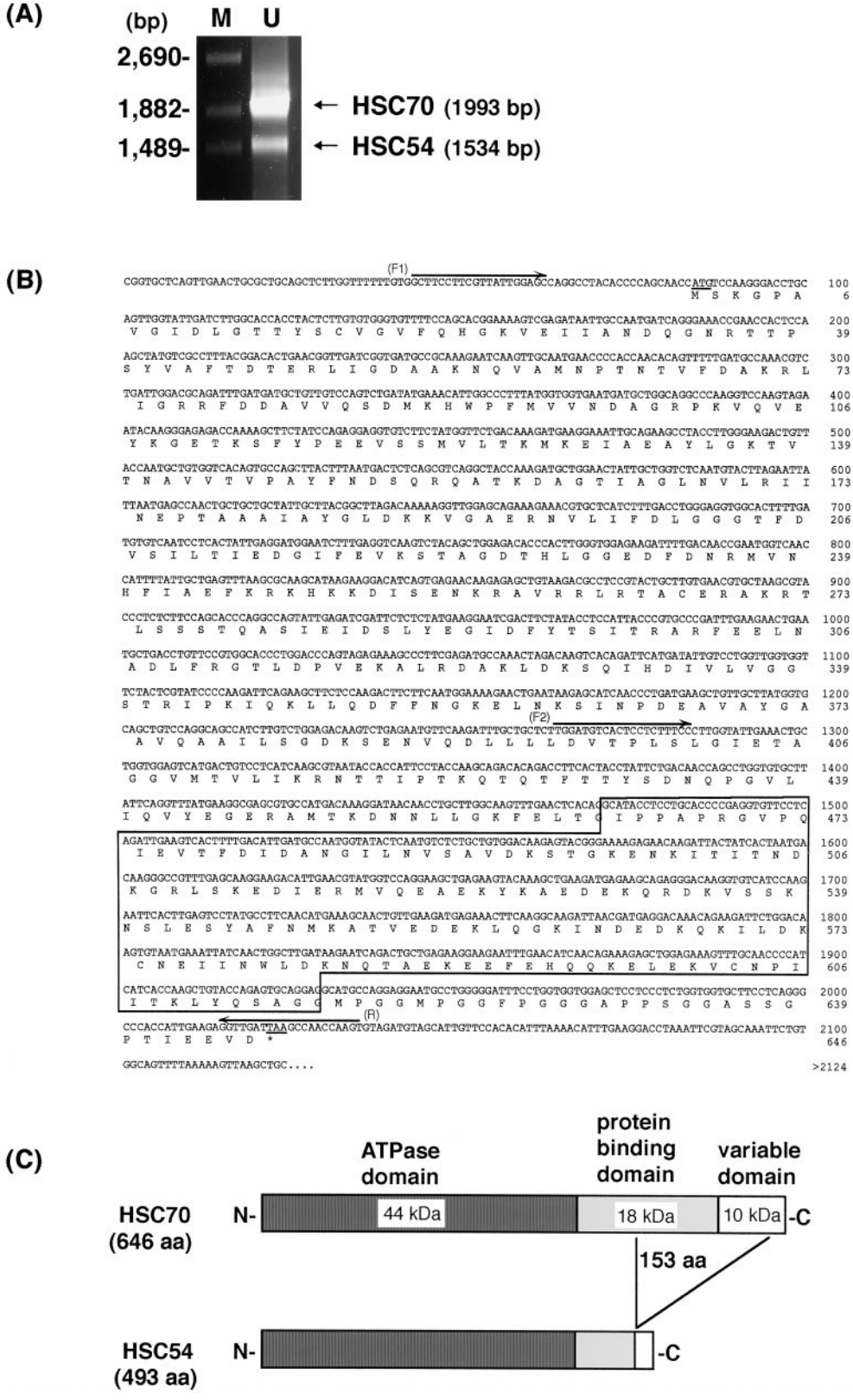

Fig. 1. A, RT-PCR of total RNA from U937 cells using a primer pair (F1/R) to amplify an entire coding region of HSC70. An expected band of 1993 bp and an unexpected band of 1534 bp were amplified. The sizes of molecular markers (M) are given on the left. U, U937 cells. B, nucleotide and amino acid sequences of HSC70 and HSC54. The ATG initiation (nt 84–86) and the TAA stop codons (nt 2022–2024) of HSC70 cDNA are underlined. Deleted sequences in HSC54 cDNA (nt 1471–1929) and protein (464–616 amino acids) are boxed. F1 and R are primers used in the PCR, and F2 is another primer used in the study to confirm HSC54 (shown in Fig. 2). Nucleotide and amino acid residues are numbered on the right, and one-letter amino acid designation is used. C, schematic structure of HSC54 protein. HSC70 consists of three domains, including an NH_2-terminal 44-kDa ATPase domain (residues 1–384), a middle 18-kDa protein binding domain (residues 385–543), and a COOH-terminal 10-kDa variable domain (residues 544–646). HSC54 lacked a portion of HSC70 at protein binding and variable domains (residues 464–616). aa, amino acids.

precipitated washed five times with a binding buffer, and proteins bound to beads were eluted with an elution buffer (50 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 100 mM NaCl, 10% glycerol, 5 mM β-mercaptoethanol, and 10 mM reduced glutathione). The proteins were separated by 10% SDS-polyacrylamide gel electrophoresis, electrotransferred onto a Hybond-ECL membrane, and immunochemically detected by an anti-FLAG antibody (M2 monoclonal antibody, Sigma) and ECL kit.

Binding Assay by Resonance Mirror Detection. The binding characteristics of HSC70 and HSC54 with p60 were studied using the resonant mirror detection method of the IAsys system (Affinity Sensors, Cambridge, UK) as described previously (Nunomura et al., 1997). Recombinant p60 (200 μg/ml in PBS; 10 mM Na_2HPO_4/NaH_2PO_4, pH 7.4, containing 0.15 M NaCl) was covalently immobilized on an aminosilane matrix of the cuvette surface with bis(sulfosuccinimidyl suberate) (Pierce, Rockford, IL), and the unoccupied sites on the aminosilane matrix were blocked with 1 M ethanolamine. The running buffer used in this experiment was 20 mM HEPES buffer, pH 7.4, containing 150 mM NaCl, 5 mM EDTA, and 0.05% Tween 20. This buffer was also used for dilution of the samples. Various concentrations (10–1000 nM) of recombinant HSC70 and HSC54 were added to the cuvette, and time-dependent changes in arc seconds were monitored to detect the protein-protein interactions at 37°C. The resulting binding curve (arc seconds versus time) was analyzed using the software package FASTfit (Affinity sensors) to calculate k_{ass}, k_{diss}, and K_D. The association rate constant, k_{ass} (the gradient) and the dissociation rate constant, k_{diss} (the extrapolated intercept on the y-axis), were obtained from a plot of k_{on} (the on-rate constant for the particular concentration of HSC70 and HSC54) against HSC70 and HSC54 concentrations. The affinity constant, K_D, is equivalent to k_{diss}/k_{ass}. Results are expressed as the means ± S.E., and statistical significance of differences was evaluated by Student's t test.

Results

Identification and Sequence Analysis of HSC54. RT-PCR of total RNA from U937 cells using the F1/R primer set, which amplifies the entire coding region of HSC70 cDNA, amplified two distinct bands (Fig. 1A). Sequence analysis revealed that the larger product of 1993 bp corresponds to HSC70, and the smaller product of 1534 bp is a novel variant that has a deletion from nt 1471 to nt 1929 in HSC70 cDNA (Fig. 1B). The variant encodes a predicted 493 amino acid residues with a calculated molecular mass of 53.5 kDa; therefore, it was designated HSC54. The putative protein lacks 153 amino acid residues (464–616) of HSC70 in the protein binding and the variable domains partly (Fig. 1C).

Expression of HSC54. The expression of HSC54 was studied in human cells and tissues by RT-PCR. Using F1/R primer set, two bands corresponding to HSC70 and HSC54, respectively, were detected in all human tissues and cells, including brain, heart, kidney, liver, lung, trachea, U937, HepG2, and HUVEC (Fig. 2A). The existence of this variant was further confirmed by RT-PCR using another primer set, F2/R, which produced two bands of 772 and 313 bp, corresponding to HSC70 and the variant, respectively (Fig. 1B and Fig. 2B). The ratio of the amount of PCR products for HSC70 and HSC54 was variable in each cell and tissue. The intensities of the bands for HSC70 and 54 were almost the same in brain, kidney, trachea, U937, HepG2, and HUVEC. The band of HSC54 was faint in heart, liver, and lung. By Western blot analysis, two bands migrating at 73 and 54 kDa, corresponding to HSC70 and HSC54, respectively, were detected in peripheral mononuclear and U937 cells using anti-HSC70 antibody (1B5) (Fig. 2D). The intensities of the bands for HSC54 were uniformly less than those for HSC70.

Heat-Shock Response of HSC70 and HSC54 Expression. The effect of heat shock on the expression of HSC70 and HSC54 was studied in U937 cells by RT-PCR. Heat treatment (43°C for 30 min) of U937 cells up-regulated HSC70 mRNA levels, which was consistent with the previous report (Marunouchi and Hosoya, 1993). The increase in HSC54 mRNA level was also demonstrated after the heat shock (Fig. 3). An increase in mRNAs of both HSC70 and HSC54 was observed 1 h after heat shock and increased further at 6 h.

Measurement of Chaperoning Activity of HSC54. The chaperoning activities of HSC70 and HSC54 were compared by the luciferase renaturation assay. Heat treatment of firefly luciferase (40°C for 5 min) reduced its activity to about 14% of the initial value. Consistent with previous reports (Schumacher et al., 1994; Minami et al., 1996), the luciferase activity was not recovered by incubation with HSC70 in the absence of reticulocyte lysate as a source of cochaperones. In the presence of reticulocyte lysate, addition of HSC70 clearly enhanced recovery of the enzyme activity (Fig. 4). In contrast, addition of HSC54 did not enhance the recovery. Besides, HSC54 significantly inhibited HSC70-mediated refolding of luciferase when HSC70 and HSC54 were simultaneously given in the complete reaction system.

Binding of HSC54 to Cochaperones p60, HSP40, and p48. The interaction of HSC54 to cochaperones p60, HSP40, and p48 was studied by GST-pull-down assay. The FLAG fusion protein of HSC54 or HSC70 expressed in COS7 cells was incubated with GST fusion proteins of p60, HSP40, or p48, in vitro. The proteins bound to GST fusion proteins were analyzed by Western blot with FLAG-specific antibody. As

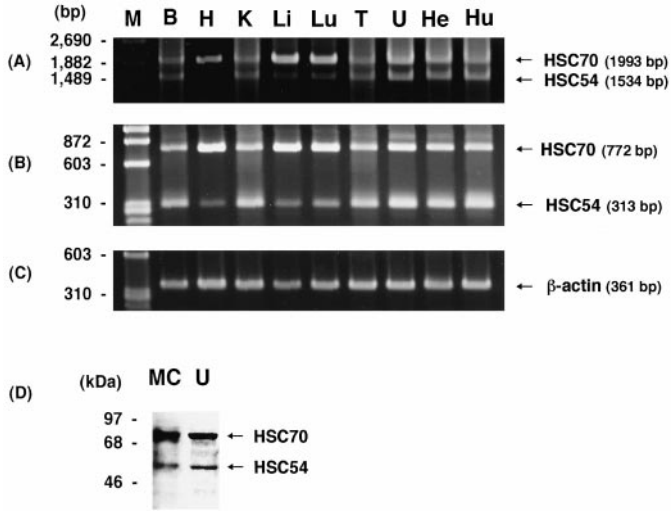

Fig. 2. Expressions of HSC70 and 54 in human tissues and cells. A to C, RT-PCR analysis. RT-PCR was performed with F1/R (A) and F2/R (B) primer sets, which amplify HSC70 and HSC54 (1993 and 1534 bp for F1/R and 772 and 313 bp for F2/R), respectively, and a βF/βR (C) primer set, which amplifies β-actin (361 bp). PCR products were electrophoresed in a 0.7% (A) or 2% (B and C) agarose gel and stained with ethidium bromide. The sizes of molecular markers (M) are given on the left. B, brain; H, heart; K, kidney; Li, liver; Lu, lung; T, trachea; U, U937 cells; He, HepG2 cells; Hu, HUVEC. D, Western blot analysis. Western blot was performed with HSC70-specific antibody (1B5). The sizes of molecular markers are given on the left (kDa). MC, peripheral mononuclear cells; U, U937 cells.

shown in Fig. 5, HSC70-bound p60, HSP40, and p48 were detected in the reaction containing GST-HSC70 fusion protein, as previously reported (Frydman and Höhfeld, 1997). The bindings of HSC54 and these three cochaperones were also demonstrated. GST controls (devoid of fusion proteins) showed no interactions with these proteins.

Kinetic Analysis of HSC70/54-p60 Interactions. Binding characteristics of HSC70/HSC54 to p60 were further studied by the resonant mirror detection method. Consistent with the results obtained by the pull-down assay, HSC70 or HSC54 showed a binding response (arc seconds) to p60 in a concentration-dependent manner. The binding data were then analyzed by FASTfit program using a single exponential function. As shown in Table 1, HSC54 bound p60 with almost the same affinity (K_D = 1.33 ± 0.45 μM) as HSC70 (K_D = 2.58 ± 1.23 μM). k_{ass} of HSC54 (1.78 ± 0.14 × 10^4 M^{-1} s^{-1}) was significantly higher than that of HSC70 (8.55 ± 2.93 × 10^3 M^{-1} s^{-1}), whereas k_{diss} was not different between HSC70 and 54.

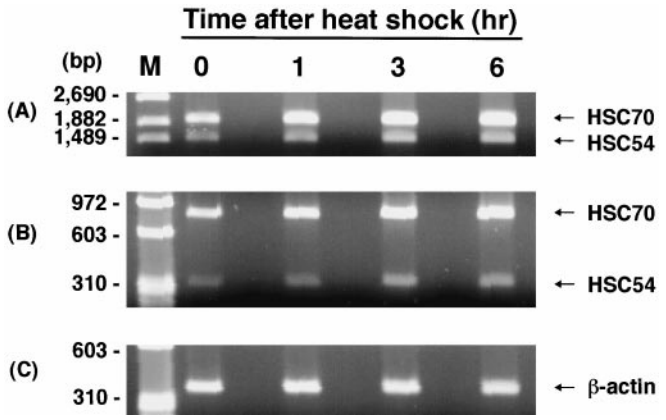

Fig. 3. Effect of heat shock on the expression of HSC54 in U937 cells. U937 cells were subjected to a heat shock for 30 min at 43°C and incubated for 1, 3, and 6 h at 37°C. The length of time (h) after the heat shock is given on each lane. RT-PCR was performed with F1/R (A) and F2/R (B) primer sets, which amplify HSC70 and HSC54, and βF/βR (C) primer set, which amplifies β-actin (same as Fig. 2). PCR products were electrophoresed in a 0.7% (A) or 2% (B and C) agarose gel and stained with ethidium bromide. The sizes of markers (M) are given on the left.

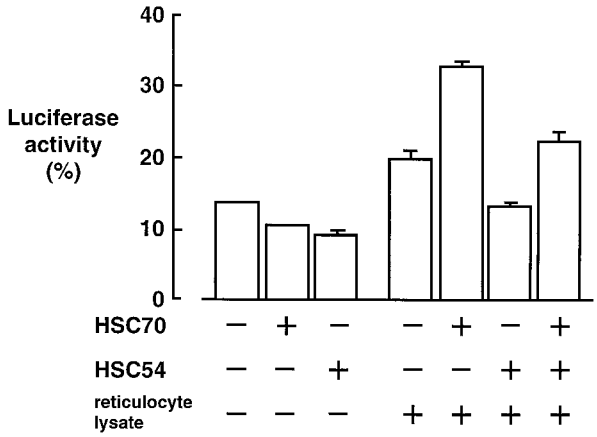

Fig. 4. Effect of HSC54 on HSC70-mediated luciferase refolding. Recombinant HSC70 and HSC54 (100 μg/ml) in 10 mM Tris-HCl, pH 7.5, 3 mM $MgCl_2$, 100 mM KCl, 2 mM dithiothreitol, 2 mM ATP, and an ATP-regenerating system were prepared for renaturation assay. Samples supplemented with (+) or without (−) 5% reticulocyte lysate were incubated at 4°C for 1 h before an addition of denatured luciferase. Samples were then incubated at 30°C for 1 h to allow renaturation. Columns and bars represent the means ± S.E. for the result performed in triplicate.

Discussion

During an effort to obtain cDNA of human HSC70, RT-PCR of total RNA from human-derived cells using the primer set F1/R, which amplifies the entire coding region of the cDNA, demonstrated two distinct unexpected PCR products. The sequence analysis of these PCR products confirmed that the smaller product has complete homology to the larger expected product (i.e., HSC70) except that there is a 459-nucleotide deletion. The second set of PCR primers (F2/R) further confirmed the presence of the variant of HSC70 mRNA, named HSC54. Although cDNA and amino acid sequences of human HSC70 share some homology to other family members of HSP70, no variant of HSC70 has been described. Therefore, HSC54 is a novel variant of human HSC70 transcript.

Analysis of nucleotide sequence revealed that HSC54 mRNA encodes the putative protein lacking 153 amino acid residues (464–616) in the protein binding and variable domains of HSC70. The calculated molecular mass of HSC54 is 53.5 kDa. The Western blot showed that there is a protein of ~54 kDa that is immunologically reactive to HSC70 antibody in peripheral mononuclear and U937 cells, indicating that HSC54 mRNA is actually translated.

The lack of amino acid residues in HSC54 predicts different physiological functions between HSC70 and 54. We further analyzed the chaperoning activity of HSC70 and 54 using thermally denatured luciferase as a model protein (Schumacher et al., 1994; Minami et al., 1996). In the presence of reticulocyte lysate as a source of cochaperones, HSC70, but not HSC54, promoted the recovery of luciferase activity. On the contrary, HSC54 rather attenuated the chaperoning activity of HSC70. These results suggest that the deleted region in HSC54 is critical for chaperoning activity of

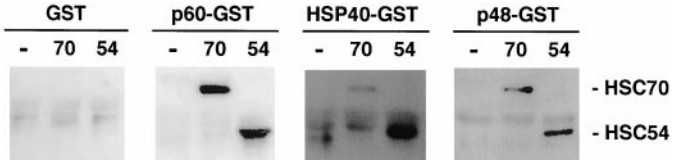

Fig. 5. Binding of HSC70/HSC54 to cochaperones p60, HSP40, and p48. COS7 cells were transfected with the plasmids pFLAG-CMV-2-HSC70/HSC54. Forty-eight hours after transfection, cell lysates were incubated with GST, GST-p60, GST-HSP40, or GST-p48. Proteins bound to GST fusion proteins were eluted with glutathione and were analyzed by anti-FLAG immunoblotting.

TABLE 1

Determination of k_{ass}, k_{diss}, and K_D values of HSC70 and HSC54 for p60

The binding response of HSC70 and HSC54 to p60 immobilized on the sensor surface was studied using the resonant mirror detection method of the IAsys system. From the binding curves, k_{ass}, k_{diss}, and K_D values were determined using the FASTfit software package as described under *Materials and Methods*. Results are the means ± S.E. of five independent experiment.

	k_{ass}	k_{diss}	K_D
	M^{-1} s^{-1}	s^{-1}	M
HSC70	8.55 ± 2.93 × 10^3	1.55 ± 0.47 × 10^{-2}	2.58 ± 1.23 × 10^{-6}
HSC54	1.78 ± 0.14 × 10^{4a}	2.20 ± 0.75 × 10^{-2}	1.33 ± 0.45 × 10^{-6}

[a] P <.05 versus HSC70.

HSC70. Furthermore, HSC54 acts as an inhibitory regulator for the chaperoning activity of HSC70.

HSC70 requires cochaperones, such as p60, HSP40, and p48, for efficient protein folding (Höhfeld et al., 1995; Freeman and Morimoto, 1996; Gross and Hessefort, 1996; Frydman and Höhfeld, 1997). We examined whether HSC54, like HSC70, interacts with these cochaperones. The pull-down experiments using GST-fusion proteins clearly showed that HSC54 as well as HSC70 bound p60, HSP40, and p48. The ATPase domain in the N terminus of HSC70 interacts with p60 and p48 (Höhfeld et al., 1995; Gross and Hessefort, 1996), and the EEVD motif in the C terminus of HSC70 interacts with HSP40 (Freeman et al., 1995). Both the ATPase domain and the EEVD motif are preserved in HSC54. Although ATPase activity of the HSC54 has not been tested in this study, it is reasonable to observe the binding of HSC54 to these cochaperones. The resonant mirror detection method characterized that HSC54 has affinity to p60 similar to HSC70. In protein folding, p60 catalyzes the dissociation of HSC70-bound ADP in exchange for ATP and promotes the recycling of HSC70 (Gross and Hessefort, 1996). HSP40 stimulates the ATPase activity of HSC70 to generate the ADP-bound form, which has a high affinity for substrate binding (Freeman and Morimoto, 1996; Minami et al., 1996). p48 prevents the dissociation of ADP from HSC70 to stabilize the HSC70-substrate complex (Höhfeld et al., 1995). Taken together, HSC54 seems to compete with HSC70 for these cochaperones and to prevent association of HSC70 with cochaperones, leading to inhibition of HSC70-mediated refolding activity.

The human HSC70 gene is composed of nine exons (Dworniczak and Mirault, 1987) and is mapped to chromosome 11 (Tavaria et al., 1995). The gene has a heat-shock response element (Marunouchi and Hosoya, 1993); several stresses, including heat shock and hypoxia, activate heat-shock factor by phosphorylation, and the expression of HSC70 is up-regulated. The existence of HSC54 mRNA was confirmed in all human cells and tissues examined by RT-PCR using two sets of primers. Heat treatment of U937 cells up-regulated the expression of HSC54. These results indicate that HSC54 is constitutively expressed and also inducible by stress, like HSC70. The deleted region of HSC54 was different from that of a splice variant of rat HSC70, HSC49, which was recently identified in the rat brain (Yamada et al., 1999). Although an exact mechanism of the generation of HSC54 remains to be clarified, HSC54 mRNA appears to be produced by alternative splicing from the HSC70 gene. In such a case, the 5′- and 3′-splice junctions of HSC54 pre-mRNA are located in exons 7 and 9 of the human HSC70 gene, respectively. Interestingly, the intron begins with GC and ends with AG, which does not conform to the mammalian consensus (GT-AG rule). There appear to be specific regulation factors for the turnover rates of HSC70 and 54 mRNAs in each human cell and tissue, because the relative abundance of HSC54 mRNA compared with HSC70 mRNA was variable in each cell and tissue. However, differential regulation of HSC70 and 54 protein expression was not observed in the current experimental settings (data not shown).

HSC70 plays an essential role in the maintenance of physiological cell function and has cytoprotective roles against cell death triggered by heat (Manzerra et al., 1997; Mosser et al., 1997), metabolic stress (Williams et al., 1993), hypoxia (Mestril et al., 1994), cytokines (Jäättelä et al., 1992; Kim et al., 1997; Yokoo and Kitamura, 1997), nitric oxide (Bellmann et al., 1996; Hirvonen et al., 1996), and apoptosis-inducing agents (Samali and Cotter, 1996). Our results show that an excessive HSC54 apparently inhibited the HSC70-mediated chaperoning process. The properties of HSC54 will serve as a useful tool not only for molecular understanding of HSC70-mediated chaperoning activity but also for development of molecules to pharmacologically modify the chaperoning activity. The protein levels of HSC54 are relatively low in physiological conditions; however, if it is up-regulated in several stress conditions, HSC54 may have a pathophysiological role via inhibition of HSC70-mediated chaperoning activity. Further characterization of HSC54 in pathophysiological conditions may clarify the exact role of this variant.

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