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Nuclear Localization Mechanism of Hsp105β and its Possible Function in Mammalian Cells

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Hsp105α and Hsp105β are mammalian stress proteins of the Hsp105/110 family. We have shown that Hsp105β localizes to the nucleus, whereas Hsp105α localizes to the cytoplasm of mammalian cells. Hsp105α localizes in the cytoplasm, as the nuclear export signal (NES) activity rather than nuclear localization signal (NLS) activity dominates in Hsp105α, due to suppression of the NLS activity. In this study, we determined the mechanisms behind the nuclear localization of Hsp105β, and revealed that the NES was suppressed by the N-terminal (amino acids 3–10) or C-terminal (amino acids 699–756) region of Hsp105β, and the NLS activity rather than NES activity seemed to dominate in Hsp105β. Furthermore, as Hsp105β which localizes in the nucleus, functioned as an inducer of Hsp70 in mammalian cells, Hsp105 family proteins may play an important role in the protection of cells against deleterious stressor together with Hsp70.

Key words: Hsp105 Hsc70, Hsp70, nuclear export signal, nuclear localization signal.

Abbreviations: Hsp, heat shock protein; Hsc70, heat shock cognate 70; NLS, nuclear localization signal; NES, nuclear export signal; LMB, leptomycin B; CRM1, chromosome maintenance region 1; SO, spliced out.

Of the HSP105/110 family, Hsp105α is constitutively expressed and induced by various forms of stress, whereas Hsp105β an alternatively spliced form lacking 44 amino acids (the SO sequence) is specifically expressed during mild heat shock (*1–5*). These proteins exist as complexes associated with Hsp70 and Hsc70 (a constitutive form of Hsp70) in mammalian cells (*6*) and suppress the chaperone activity of Hsc70, suggesting that they act as a negative regulator of Hsp70 (*7–9*). Furthermore, Hsp105α and Hsp105β suppress the aggregation of heat-denatured proteins as does Hsp70 (*7*) and function as a substitute for Hsp70 family proteins to suppress the aggregation of denatured proteins in cells under severe stress such as ATP-depleted conditions (*10*).

The predicted secondary structure of Hsp105α and Hsp105β is composed of an amino-terminal ATP-binding domain, a β-sheet domain, a loop domain and a carboxyl-terminal α-helical domain, similar to HSP70 family proteins (*3*, *4*). HSP70 family proteins are expressed in the cytoplasm under normal conditions, and accumulate in the nucleus and nucleolus after heat shock (*11–14*). In contrast, Hsp105β localizes to the nucleus, whereas Hsp105α localizes to the cytoplasm of mammalian cells under normal and stressed conditions (*4*). Recently, we showed that the cytoplasmic localization of Hsp105α is dependent on the nuclear export signal (NES), and the SO sequence that Hsp105β lacks suppresses the activity of the nuclear localization signal (NLS) (*15*).

In this study, we revealed that the NES was suppressed by the N-terminal or C-terminal region of Hsp105β, resulting in the nuclear localization. Furthermore, we found that Hsp105β induced the expression of Hsp70 in mammalian cells.

MATERIALS AND METHODS

Plasmids—The mammalian expression plasmids for mouse Hsp105α (pcDNA105β), Hsp105β (pcDNA105β) and their mutants with substituted NLS (pcDNA105αmNLS and pcDNA105βmNLS, respectively) have been described previously (*15*, *16*). To construct expression plasmids for Myc-epitope/His-tagged Hsp105β and C-terminal deletion mutants of Hsp105β, polymerase chain reactions were performed with a template and specific 5′-end-phosphorylated primers (Table 1) and the PCR products were self-circularized. To construct expression plasmids for N-terminal deletion mutants of Hsp105β, PCR were performed with a template and specific primers (Table 1), and the products were cloned into the vector pcDNA3 (Invitrogen, Carlsbad, CA, USA). The plasmids for mutants with substituted NES of Hsp105β were made using a QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA) with a template and specific primers (Table 1). The plasmid pHBCAT containing the CAT reporter gene driven by a human *hsp70* promoter was kindly provided by Dr R.I. Morimoto (*17*). For construction of reporter plasmid containing *hsp70* promoter upstream of luciferase reporter gene (pGL70), a 2.6-kb fragment of the 5′-flanking region of the *hsp70* gene, derived from the plasmid pHBCAT was subcloned into *BamH*I site of the pGL2-control (Promega, Madison, WI, USA).

Cells and Transfection—African monkey kidney COS-7 cells were maintained in Dulbecco's modified Eagle's

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Table 1. **Primers and template DNAs used for construction of expression plasmids**

Plasmid	Template DNA	Orientation	Sequence
pcDNA105β-myc/His	pcDNA105α-myc/His	Sense	5′-ATCCACATCCGAGCTCTCTGTTGGCC-3′′
		Antisense	5′-GCAAATGAAAAGAAAGTTGATCAG-3′
pcDNA105β(1–756)	pcDNA105β-myc/His	Sense	5′-GAACAAAAACTCATCTCAGAAGAG-3′
		Antisense	5′-AACAGGTTCACAAACATTGTTCAA-3′
pcDNA105β(1–698)	pcDNA105β-myc/His	Sense	5′-GAACAAAAACTCATCTCAGAAGAG-3′
		Antisense	5′-GTTGTATTTCTCATCCTTGCC-3′
pcDNA105β(1–641)	pcDNA105β-myc/His	Sense	5′-GAACAAAAACTCATCTCAGAAGAG-3′
		Antisense	5′-AGCCTGGTCCTCCCCTTCCT-3′
pcDNA105β(1–591)	pcDNA105β-myc/His	Sense	5′-GAACAAAAACTCATCTCAGAAGAG-3′
		Antisense	5′-GGCGTCGTTCGCTCCTTCT-3′
pcDNA105βΔα	pcDNA105β-myc/His	Sense	5′-GAACAAAAACTCATCTCAGAAGAG-3′
		Antisense	5′-GTCTCTCCCTAACTGCCATACC-3′
pcDNA105β(3–814)	pcDNA105β	Sense	5′-GGGGATCCACCATGGTGGTTGGGCTAGACGTAGGC-3′
		Antisense	5′-GCTCTAGACCTAGTCCAGGTAAAGGTCCATGTTGAC-3′
pcDNA105β(11–814)	pcDNA105β	Sense	5′-GGGGATCCACCATGCAGAGCTGCTACATTGCGGTG-3′
		Antisense	5′-GCTCTAGACCTAGTCCAGGTAAAGGTCCATGTTGAC-3′
pcDNA105β(81–814)	pcDNA105β	Sense	5′-GGGGATCCACCATGCAGAAGGAAAAGGAGAACCTG-3′
		Antisense	5′-GCTCTAGACCTAGTCCAGGTAAAGGTCCATGTTGAC-3′
pcDNA105βmNES	pcDNA105β	Sense	5′-GGGAGAGACGCTCTTAACGCGTATGCTGAGACAGAG-3′
		Antisense	5′-CTCTGTCTCAGCATACGCGTTAAGAGCGTCTCTCCC-3′
pcDNA105β(1–698)mNES	pcDNA105β(1-698)	Sense	5′-GGGAGAGACGCTCTTAACGCGTATGCTGAGACAGAG-3′
		Antisense	5′-CTCTGTCTCAGCATACGCGTTAAGAGCGTCTCTCCC-3′

medium supplemented with 10% fetal calf serum under a 5% CO_2 atmosphere at 37°C. Transfection was performed by lipofection using DMRIE-C reagent (Invitrogen) according to the manufacturer's instructions.

Western Blotting—Cells were lysed with 0.1% SDS and boiled for 5 min. Aliquots (10 μg of protein) of cell extracts were subjected to 10% SDS–PAGE, then transferred onto nitrocellulose membranes, and incubated with rabbit anti-mouse Hsp105 antibody (*5*), anti-Hsp90 antibody (clone 3B6, Affinity Bioreagents, Golden, CO, USA), anti-Hsp70 (clone C92F3A-5, Stressgen, Victoria, BC, Canada), anti-Hsp60 antibody (clone LK-1, Medical & Biological Laboratories, Nagoya, Japan), anti-Hsp27 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and anti-α-tubulin antibody (clone DM1A, Sigma, St Louis, MO, USA), respectively. Then, membranes were further incubated with horseradish peroxidase-conjugated anti-mouse, rabbit or goat IgG antibody, and the antibody–antigen complexes were detected using Western Blotting Luminol Reagent (Santa Cruz Biotechnology).

Immunoprecipitation—Cells were washed with PBS, lysed in an extraction buffer (50 mM Tris–HCl, pH 8.0, 50 mM NaCl, 5 mM EDTA, 1% NP-40 and 2 mM PMSF). After centrifugation at 12,000*g* for 30 min at 4°C, the resulting supernatants (200 μg of protein) were incubated with anti-mouse Hsp105 antibody at 4°C for 1 h. After addition of Protein A sepharose, the mixtures were incubated at 4°C for 1 h, then the precipitates were subjected to SDS–PAGE, and Hsp105 and Hsc70 were detected by Western blotting using anti-myc (Invitrogen) and anti-Hsp70 antibody (clone BRM-22, Sigma), respectively.

Immunofluorescence Analysis—Cells were grown on cover glasses and fixed with cold methanol for 5 min. After treatment with 5% BSA in PBS at 37°C for 1 h, cells were incubated with rabbit anti-mouse Hsp105 antibody (1/50) (*5*) or anti-myc antibody (1/200, Invitrogen) for 1–2 h at 37°C. Then, cells were washed with PBS and incubated with fluorescein isothianate-conjugated anti-rabbit IgG (1/50, Vector, Burlingame, CA, USA) or rhodamine-conjugated anti-mouse IgG (1/50, Invitrogen) for 1–2 h at 37°C. After washing with PBS, cells were observed using a confocal laser-scanning microscope (LSM410, Zeiss, Jena, Germany).

Reporter Gene Assay—COS-7 cells were grown in 4-well plates and transfected with 0.5 μg of a reporter plasmid containing *hsp70* promoter upstream of luciferase reporter gene (pGL70) and 1.5 μg of pcDNA3, pcDNA105α, pcDNA105β, pcDNA105αmNLS or pcDNA105βmNLS using DMRIE-C reagent. After 48 h, cells were lysed in 20 μl of Cell Culture Lysis Reagent (Promega). After centrifugation at 20,000*g* for 5 min, aliquots (3 μl) of supernatants were mixed with 50 μl of Luciferase Assay Reagent (Promega), and the luciferase activity was measured in a luminometer (Mithras LB 940; Berthold Technologies Bad Wildbad, Germany).

RESULTS AND DISCUSSION

Nuclear Export of Hsp105β is Suppressed by N-terminal or C-terminal Region of Hsp105β—To understand the mechanism behind the distribution of Hsp105β in mammalian cells, we constructed a series of plasmids expressing C-terminal or N-terminal deletion mutants of mouse Hsp105β. These expression plasmids were transfected into monkey COS-7 cells, and the exogenously expressed mouse Hsp105β and its mutants were detected by indirect immunofluorescence using anti-myc epitope antibody or anti-mouse Hsp105 antibody which specifically reacts with mouse Hsp105 (*5*, *15*).

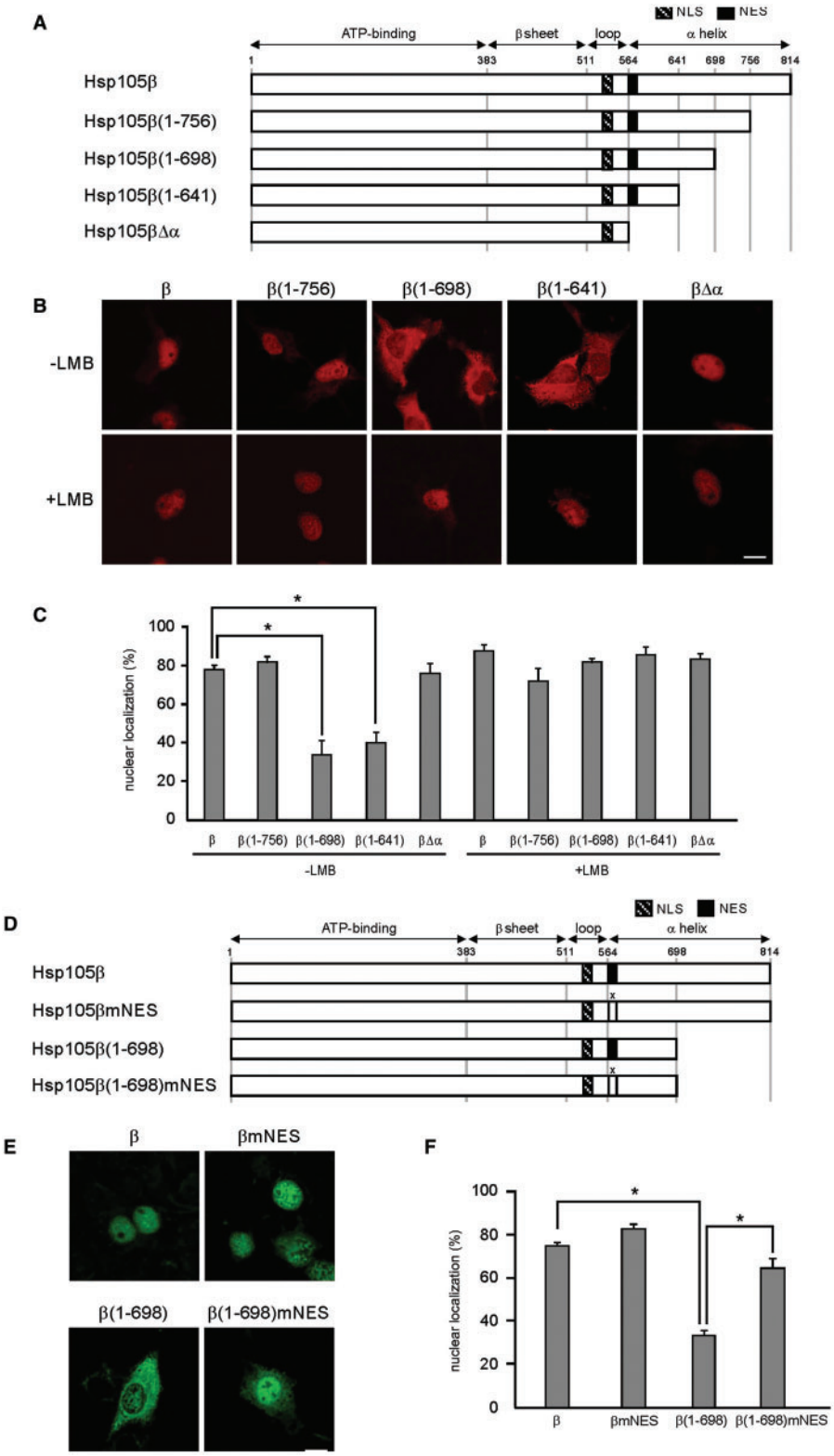

Fig. 1. **C-terminal (amino acids 699–756) region of Hsp105β is required for nuclear localization of Hsp105β.** (A and D) Schematic representation of the structure of Hsp105β and its mutants. (B and E) COS-7 cells were transfected with the expression plasmid for Hsp105β or its mutants, and incubated for 48 h. Then, cells were incubated with or without LMB (10 ng/ml) for 24 h. The intracellular distribution of myc-tagged Hsp105β and C-terminal deletion mutants (B, *red*) or Hsp105β and Hsp105β(1–698) without or with mutated NES (E, *green*) was determined by indirect immunofluorescence microscopy using anti-myc or anti-Hsp105 antibody, respectively. Scale bars, 20 μm. (C and F) Proportions of cells in which most Hsp105β or its mutants were found in the nucleus were obtained from at least 100 cells. Each value represents the mean ± SE for three independent experiments. Statistical significance was determined by unpaired Student's *t*-test ($^*P < 0.005$).

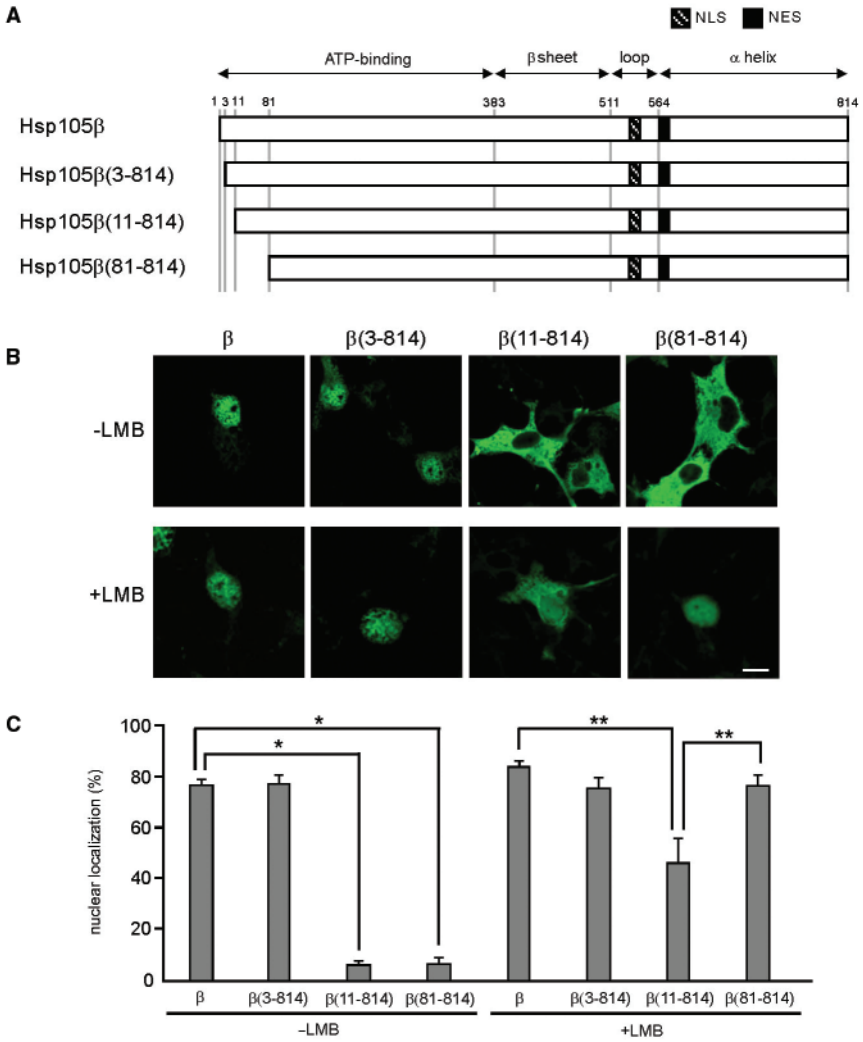

Fig. 2. **N-terminal (amino acids 3–10) region of Hsp105β is required for nuclear localization of Hsp105β.** (A) Schematic representation of the structure of Hsp105β and its mutants (B) The intracellular distribution of Hsp105β or these mutants was determined as described in Fig. 1 using anti-Hsp105 antibody. Scale bar, 20 μm. (C) Proportions of cells in which most Hsp105β or its mutants were found in the nucleus were obtained from at least 100 cells. Each value represents the mean ± SE for three independent experiments. Statistical significance was determined by unpaired Student's *t*-test ($^{*}P < 0.005$, $^{**}P < 0.05$).

When the cellular distribution of C-terminal deletion mutants of Hsp105β was examined, Hsp105β(1–756) lacking 58 C-terminal amino acids was found in the nucleus, whereas Hsp105β(1–698) and Hsp105β(1–641) lacking more than 118 amino acids at the C-terminus were detected in both the cytoplasm and nucleus of cells (Fig. 1A). Hsp105βΔα which lacked NES was found in the nucleus. When cells were treated with leptomycin B (LMB), a specific inhibitor of NES- and CRM-1-dependent nuclear export, Hsp105β(1–698) and Hsp105β(1–641) were localized in the nucleus. Furthermore, Hsp105β (1–698)mNES which was mutated at the NES sequence was mainly localized in the nucleus, as Hsp105β and Hsp105βmNES (Fig. 1B). Thus, the C-terminal region between amino acids 699 and 756 of Hsp105β seemed to suppress the NES-dependent nuclear export of Hsp105β.

When the cellular distribution of N-terminal deletion mutants of Hsp105β was examined, Hsp105β(3–814) lacking the two N-terminal amino acids was accumulated in the nucleus similar to Hsp105β (Fig. 2). However, Hsp105β(11–814) and Hsp105β(81–814) lacking the 10 and 80 amino acids, respectively, were found in the cytoplasm of cells. When the cells were treated with LMB, Hsp105β(11–814) was partially accumulated in the nucleus and Hsp105β(81–814) was completely accumulated in the nucleus. Thus, the N-terminal region between amino acids 3 and 10 of Hsp105β seems to be essential for the nuclear localization of Hsp105β. As the deletion of N-terminal 10 amino acids almost abolished the nuclear localization of Hsp105β, the N-terminal region of Hsp105β seemed to be dominant for suppression of the NES activity of Hsp105β than the C-terminal region. Furthermore, as Hsp105β(11–814) was located in not only the nucleus but also the cytoplasm of cells in the presence of LMB, the region between amino acids 11 and 80 may partially suppress the NLS activity of Hsp105β.

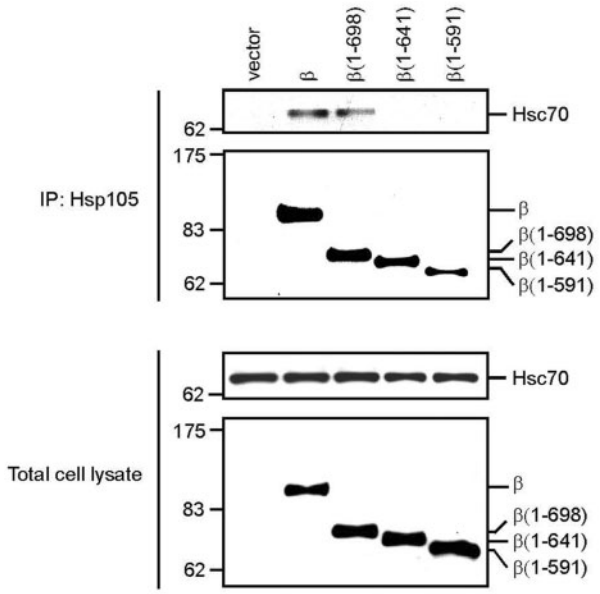

Fig. 3. **The region (amino acids 642–698) of Hsp105β is necessary for the association of Hsp105β with Hsc70 *in vivo*.** COS-7 cells were transfected with control vector or expression plasmid for myc-tagged Hsp105β or C-terminal deletion mutants. At 48 h after transfection, lysates from these cells were immunoprecipitated with anti-Hsp105 antibody. Aliquots of the precipitates and total cell lysates were analysed by Western blotting using anti-myc antibody and anti-Hsp70 antibody.

The N-terminal (amino acids 3–10) or C-terminal (amino acids 699–756) region seemed to suppress the NES-dependent nuclear export of Hsp105β. However, as the cytoplasmic localization of these mutants was suppressed by LMB treatment or by mutation of the NES, the NLS activity rather than NES activity seems to dominate in Hsp105β. On the other hand, Hsp105α localizes in the cytoplasm, as the NES activity rather than NLS activity dominates in Hsp105α, due to suppression of the NLS activity by the SO sequence (*15*).

Interaction of Hsp105β with Hsc70 is Not Necessary for Nuclear Localization of Hsp105β—Many proteins shuttle between the cytoplasm and nucleus of cells. Some transcription factors containing both an NLS and an NES localize in the nucleus of cells by the inhibition of NES activity (*18*, *19*). NES activity is suppressed due to the complex formation of proteins itself or with other proteins, or the intra-molecular interaction of proteins (*18*, *19*). As Hsp105β exists as high molecular complexes associated with Hsc70 in mammalian cells (*6*), we next examined whether the association of Hsp105β with Hsc70 is necessary for the nuclear localization of Hsp105β (Fig. 3). When the association of Hsp105β or its mutants with Hsc70 in COS-7 cells was analysed by immunoprecipitation experiments, Hsc70 was found to associate with Hsp105β and Hsp105β(1–698), whereas no association of Hsc70 with Hsp105β(1–641) or Hsp105β(1–591) was observed. Thus, as the complex formation of Hsp105β with Hsc70 was not necessary for the nuclear localization of Hsp105β, the intramolecular interactions in Hsp105β may suppress the NES activity, although it may be possible that proteins other than Hsc70 may interact and suppress the NES activity

Hsp105β but Not Hsp105α Induces the Expression of Hsp70—As Hsp105β is localized in the nucleus of cells in contrast to Hsp105α that localizes in the cytoplasm of cells, Hsp105β may have different function from that of Hsp105α (*15*). During these experiments, we noticed that Hsp105β enhanced the expression of Hsp70 in mammalian cells. As shown in Fig. 4A, when Hsp105α or Hsp105β was over-expressed in COS-7 cells, the expression of Hsp70 was increased in cells over-expressed Hsp105β but not Hsp105α. However, Hsp105β did not enhance the expression of other heat shock proteins such as Hsp90, Hsp60 or Hsp27. The similar results were obtained using stably transformed HeLa-tet cell lines that express Hsp105α or Hsp105β upon the removal of doxycycline (data not shown).

Furthermore, the effect of Hsp105α and Hsp105β on *hsp70* promoter activity was analysed using cells containing a construct of the human *hsp70* promoter fused to a luciferase reporter gene (Fig. 4B). Over-expression of Hsp105β but not Hsp105α markedly enhanced the *hsp70* promoter activity. However, the enhancement was abolished when NLS of Hsp105β was mutated. Thus, Hsp105β seemed to induce specifically the transcription of *hsp70* gene, and its nuclear localization seemed to be necessary for the induction of Hsp70 in mammalian cells.

Hsp70 family proteins are well-characterized of stress proteins that play important role in protection of cells against various stresses. As Hsp105β induced specifically the transcription of *hsp70* gene, Hsp105 family proteins may play an important role in the protection of cells against deleterious stressor together with Hsp70. Although further study is needed to elucidate the precise mechanism by which Hsp105β induces the expression of Hsp70 in cells, the present findings may provide clues as to the physiological functions of HSP105 family proteins in mammalian cells.

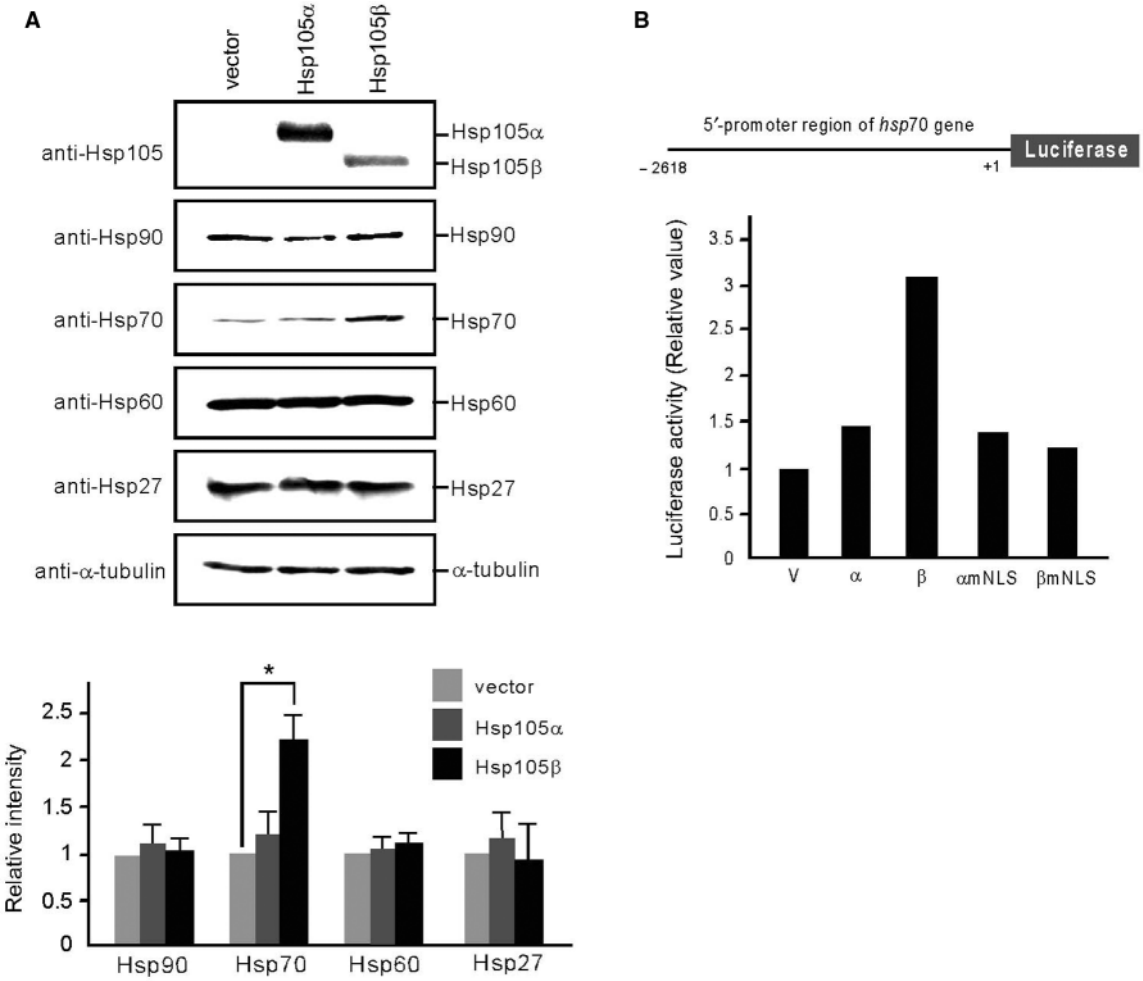

Fig. 4. **Hsp105β but not Hsp105α induces the expression of Hsp70.** (A) COS-7 cells were transfected with control vector or expression plasmid for Hsp105α or Hsp105β, then incubated for 48 h. Proteins (10 μg) from these cells were analysed by Western blotting using the antibodies indicated. The intensity of the bands was quantified by densitometry, and normalized to α-tubulin. Relative intensity of each band is expressed as a ratio to that of control cells. Each value represents the mean ± SE for three independent experiments. Statistical significance was determined by unpaired Student's *t*-test (*$P < 0.05$). (B) COS-7 cells were transfected with the plasmid containing 5′-promoter region of *hsp70* gene connected with luciferase and control vector (V) or expression plasmid for Hsp105α (α), Hsp105β (β) or these NLS mutants. At 48 h after transfection, cells were lysed and luciferase activity was assayed. Values are shown as ratios to that of cells transfected with control vector, and are the mean of two independent experiments.

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CONFLICT OF INTEREST

None declared.

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