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Heat Shock Protein 40/DjB1 Is Required for Thermotolerance in Early Phase

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DjB1 (Hsp40/DnajB1/Hdj1) is a member of the Hsp40/DnaJ family that functions as a co-chaperone of mammalian Hsp70s. DjB1 recognizes substrate proteins and facilitates the ATPase activity of Hsp70. We generated DjB1 deficient mice. The DjB1$^{-/-}$ mice were viable and fertile with no obvious abnormalities, thus indicating that DjB1 is dispensable for development and viability. No difference was found between the DjB1$^{-/-}$ and wild-type peritoneal macrophages regarding resistance against various types of apoptosis-inducing reagents. However, DjB1$^{-/-}$ cells showed decreased thermotolerance in the early phase after mild heat treatment, but not in the late phase. After the heat treatment, Hsp70 was induced similarly in wild-type and DjB1$^{-/-}$ cells. Immunofluorescence staining of wild-type cells revealed the accumulation of DjB1 and Hsc70 in the nucleus after heat treatment. DjB1 also accumulated in the centrosome. The accumulation of Hsc70 in the nucleus was also observed in DjB1$^{-/-}$ cells. These results suggest that the impaired thermotolerance of DjB1$^{-/-}$ cells is not due to a mislocation of the Hsp70 family.

Key words: DjB1 (Hsp40/DnajB1/Hdj1), Hsp70, knockout mice, molecular chaperone, thermotolerance.

Abbreviation: Hsp, heat shock protein.

The Hsp70 chaperone system is a highly conserved system that facilitates folding and assembly of newly synthesized polypeptides, translocation of proteins across organellar membranes, and degradation of denatured proteins (*1–4*). Hsp70 is associated with the target proteins that engage in various biological events such as endocytosis, exocytosis, apoptosis, and antigen presentation (*5–7*).

The folding activity is mediated through transient and reiterant interactions between Hsp70 and substrate proteins. Hsp40, a co-chaperone of Hsp70, recognizes unfolded proteins and presents them to the substrate-binding domain of Hsp70 (*8*, *9*). Hsp40 also enhances ATP hydrolysis at the nucleotide-binding domain of Hsp70, thereby inducing a conformational change in the substrate-binding domain of Hsp70 to substrate-bound form (*10*, *11*). To elucidate how the Hsp70 chaperone system plays multiple roles in various substrate proteins and their diverse folding states, we focused on members of Hsp40 homologues as a substrate determinant of Hsp70.

Members of the Hsp40 family are classified into three subtypes according to their domain structures (*12*, *13*). Type I (subfamily A) has four distinct domains, namely an N-terminal J domain, a glycine- and phenylalanine-rich (G/F-rich) domain, a zinc-finger domain, and a conserved C-terminal domain. The J domain includes a highly conserved His-Pro-Asp (HPD) motif, which accelerates the ATP-hydrolysis of Hsp70. Type II (subfamily B) contains J domain, G/F domain, a glycine- and methionine-rich (G/M rich) domain, and a C-terminal domain. Type III (subfamily C) has only the J domain as a common domain.

Up to now, the knockout mice of five Hsp40 homologues have been reported: DjA1 (DnaJA1/Hdj2), a type I Hsp40 in cytosol; DjA3 (DnaJA3/hTid1), a type I Hsp40 in cytosol and mitochondria; DjB6 (DnaJB6/Mrj), a type II Hsp40; DjC3 (DnaJC3/P58^{IPK}), a type III Hsp40; and DjC5 (DnaJC5/CSPα), a type III Hsp40 in synaptic vesicle. All these knockout mice show different phenotypes. The DjA1 knockout mice show defects in spermatogenesis with aberrant androgen receptor signaling (*14*). DjA3 deficiency results in embryonic lethality (*15*) and heart-specific DjA3 deficient mice show dilated cardiomyopathy (*16*). DjB6 deficient mice are embryonic lethal and show defects in placental development (*17*). DjC3 deficient mice develop diabetes (*18*). DjC5 deficient mice have progressive neurodegeneration (*19*). The difference in the knockout phenotypes of the Hsp40 homologues suggests that each member of the Hsp40 family recognizes a unique substrate protein and displays a specified *in vivo* function against the given substrate.

DjB1 (Hsp40/DnaJB1/Hdj1), a type II Hsp40 homologue, is a major Hsp40 member that regulates Hsp70 in mammalian cytosol, in addition to DjA1 and DjA2 (*20*, *21*). We have previously reported that both DjA1 and DjA2 enhance the protein folding and mitochondrial protein import *in vitro* in combination with Hsp70, whereas DjB1 does not (*22–24*).

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Both the Hsp70-DjA1 and Hsp70-DjB1 pairs prevent apoptosis by inhibiting the translocation of an apoptosis inducing factor Bax to the mitochondria (*25*, *26*). However, the details regarding the role sharing among these Hsp40 homologues and specific substrates of DjB1 *in vivo* remain to be elucidated. Based on many studies which have shown DjB1 to be required for efficient protein folding *in vitro* (*8*, *10*, *11*), we therefore speculated that DjB1 knockout mice may thus show severe defects.

To investigate the physiological functions of DjB1 *in vivo*, we generated DjB1 deficient mice. Unexpectedly, the *DjB1*$^{-/-}$ mice were viable and fertile, with no obvious abnormalities. At the cell level, *DjB1*$^{-/-}$ peritoneal macrophages showed normal responses to multiple apoptosis-inducing reagents. However, *DjB1*$^{-/-}$ peritoneal macrophages exhibited impaired thermotolerance in a proximal period after preconditioning. Wild-type and *DjB1*$^{-/-}$ cells exhibited similar distribution of Hsc70, which co-localized with DjB1 in the nucleus in wild-type cells after heat treatment. The defect of *DjB1*$^{-/-}$ cells in thermotolerance may not be due to a mislocalization of Hsc70.

MATERIALS AND METHODS

Generation of DjB1 Deficient Mice—To construct the DjB1 targeting vector, the mouse DNA fragments containing the target region were cleaved from a BAC clone (Invitrogen, Tokyo, Japan, clone ID: 212H13), and were subcloned into a pcDNAII plasmid (Invitrogen). This BAC clone is derived from RPCI-23 genomic library of C57BL/6J mouse. The 1.3 kb neomycin resistance gene (*Neo*) was inserted into a *Sac*II site of exon 1. The homologous region for the 5′-arm was 4.4 kb (*Xba*I/*Sac*II) and the 3′-arm was 4.8 kb (*Sac*II/*Sph*I). The 10.5 kb construct (5′-arm/*Neo*/3′-arm) for targeting was ligated into a pMCDT-A plasmid (*27*) to generate the targeting vector. The vector was linearized by *Not*I digestion at the 3′-end of the diphtheria toxin A (*DT-A*) cassette, and then was electroporated into TT2 embryonic stem (ES) cells (*28*). G418-resistant clones were screened for homologous recombination by PCR using a forward primer in the *Neo* gene (5′-ATCGCCTTCTTGACGAGTTCTTCTG-3′) and a reverse primer in the 3′-side of non-homologous region (5′-TTCAAACCTGGAAGCCAAAGTTACA-3′). The selected clones were confirmed by genomic Southern blot analyses after *Bst*XI and *Bam*HI–*Eco*RV digestion. A 1059 bp-fragment of the *DjB1* gene that was proximal to the 3′-end of the homologous region of the targeting vector and a 614 bp-fragment of the *Neo* gene were used as probes for Southern blot analyses. Seven independent ES cell clones were injected into ICR eight-cell stage embryos to generate chimeric mice. Male chimeras were bred to C57BL/6J female mice to achieve germline transmission of the targeted allele. Mice homozygous for the DjB1-targeted allele were obtained by intercrossing F_1 heterozygotes. Genotyping of the offspring was performed by two sets of PCRs with a genomic DNA sample obtained from tail tissue specimens. Two forward primers were set in the 5′-untranslated region (5′-GGGCGGGGTGCCTATTTTTATCC-3′) and in the *Neo* gene (5′-ATCGCCTTCTTGACGAGTTCTTCTG-3′), respectively. A reverse primer in the intron 1 (5′-CCCTGGGTGAGCGAACACTGAGC-3′) was used for both sets of PCRs. The mice were housed in an environmentally controlled room at the Center for Animal Resources and Development (CARD) in Kumamoto University according to the guidelines of Kumamoto University for animal and recombinant DNA experiments.

Northern Blot Analysis—Total RNA was isolated from testis using an RNeasy Mini Kit (Qiagen, Tokyo, Japan). The RNAs (2 μg each) were electrophoresed in 1% agarose-formaldehyde gels and blotted onto nylon membranes. Digoxigenin-labeled antisense RNA probes were synthesized from mouse DjA1, DjA2, DjA4, and DjB1 cDNAs under the control of the T7 or Sp6 promoter using a DIG RNA labeling kit (Roche diagnostics, Tokyo, Japan).

Western Blot Analysis—The cell or testis tissue specimens were homogenized in RIPA buffer (150 mM NaCl, 50 mM Tris-HCl, pH 7.4, 0.1% SDS, 1% sodium deoxycholate) supplemented with complete mini (Roche diagnostics) protease inhibitor mix on ice. The lysates were mixed with 2× SDS sample buffer (50 mM Tris-HCl, pH 6.8, 4% SDS, 10% glycerol, 100 mM DTT, 0.05% bromophenol blue) and processed for SDS-PAGE. A Western blot analysis was performed using rabbit antisera against DjB1 (*22*), and DjA4 (*29*), mouse monoclonal antibodies against Hsp70 (SPA-810, Stressgen Bioreagents, Victoria, Canada), Hsp90 (SC-13119, Santa Cruz, CA, USA), and DjA1 (MS-225P, Labvision Corp., Fremont, CA, USA), rat monoclonal antibody against Hsc70 (*22*), and rabbit polyclonal antibodies against BiP (SPA-826, Stressgen Bioreagents), DjA2 (*23*), and Hsp25 (SPA-801, Stressgen Bioreagents).

Preparation of Peritoneal Macrophages—*DjB1*$^{+/+}$ and *DjB1*$^{-/-}$ mice were injected intraperitoneally with 4 ml of 4% thioglycollate solution. Three days after the injection, peritoneal macrophages were aseptically harvested and cultured for three days in RPMI 1640 medium with 10% fetal bovine serum.

Heat Treatment and Detection of Apoptosis—For heat treatment, the medium was changed to RPMI 1640 with 10% fetal bovine serum and 20 mM HEPES-NaOH, pH 7.3. Culturing plates were tightly sealed and immersed in a water bath for the indicated periods, and then were returned to CO_2 incubator at 37°C. After the heat shock at 45°C for 15 min, the cells were maintained at 37°C for 24 h, and then were processed for apoptosis detection. To detect a disruption of the mitochondrial membrane potential, the cells were stained with a membrane potential–dependent dye DePsipher (Trevigen Inc., Gaithersburg, MD, USA). To assess the morphological changes in the nucleus, the cells were stained with 8 μg/ml of Hoechst 33258 dye for 15 min.

Immunofluorescence Staining—Peritoneal macrophages were cultured on coverslips and fixed with methanol as previously described (*23*). Cytoskeletal proteins were detected by mouse monoclonal antibodies against α-tubulin (T5168, Sigma-Aldrich, Inc., Saint Luis, USA) and γ-tubulin (T6557, Sigma-Aldrich, Inc.). Secondary antibodies used were Cy3-labeled goat anti–rabbit IgG (Jackson ImmunoReserch Lb. Inc., West Grove, PA, USA), Cy2-labeled goat anti–mouse IgG (Amersham Pharmacia Biotech, Buckinghamshire, UK), and Alexa Fluor 488–labeled goat anti–rat IgG (Invitrogen). Images were captured by an Olympus BX50 fluorescence microscope equipped with Penguin 600 CL camera system (Pixera, Yorkville, IL, USA). Cy3-fluorescent images were

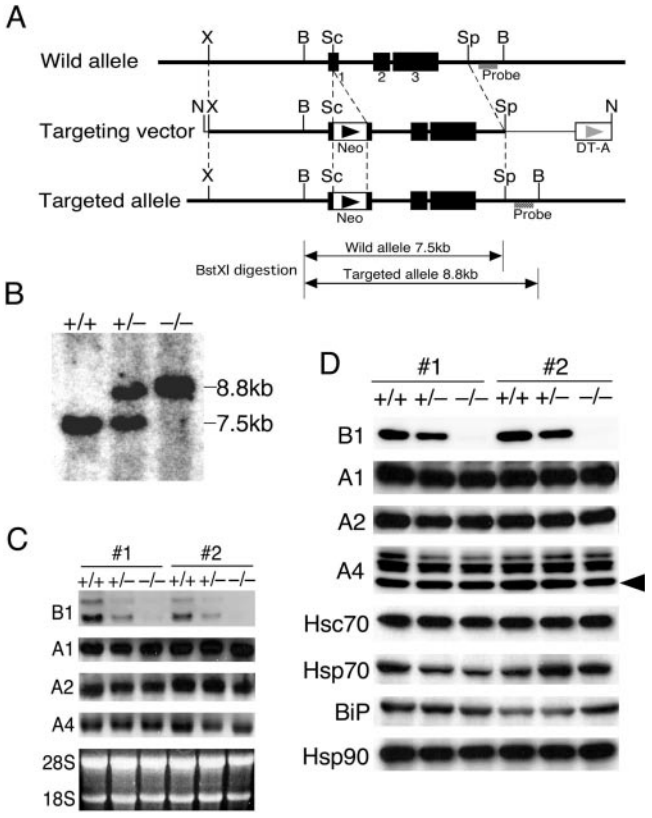

Fig. 1. **Generation of DjB1 deficient mice.** (A) Gene targeting strategies and a restriction map of the DjB1 gene. Exons 1–3 are indicated by filled rectangles. The targeting vector contains a neomycin resistance gene (*Neo*) for positive selection and a diphtheria toxin A gene (*DT-A*) for negative selection. The predicted sizes of wild and targeted alleles and the location of the probe used in a Southern blot analysis (B) are shown. The restriction enzymes were designated as follows: B, *Bst*XI; N, *Not*I; Sc, *Sac*II; Sp, *Sph*I; X, *Xba*I. (B) Southern blot analysis of *Bst*XI-digested genomic DNAs (10 μg each). (C) A Northern blot analysis of DnaJ chaperones from testes. The mouse lines #1 and #2 were derived from two independent ES cell clones. Total RNA samples were analyzed using digoxigenin-labeled probes. (D) A Western blot analysis of Hsps from the testes (2 μg of protein for Hsc70; 5 μg of protein for DjB1, DjA1, DjA2, DjA4 and Hsp90; 10 μg of protein for Hsp70 and BiP). An arrowhead indicates position of DjA4 protein of 46 kDa species. Additional larger species of DjA4 protein were also detected in the testis (*14*, *24*, *29*).

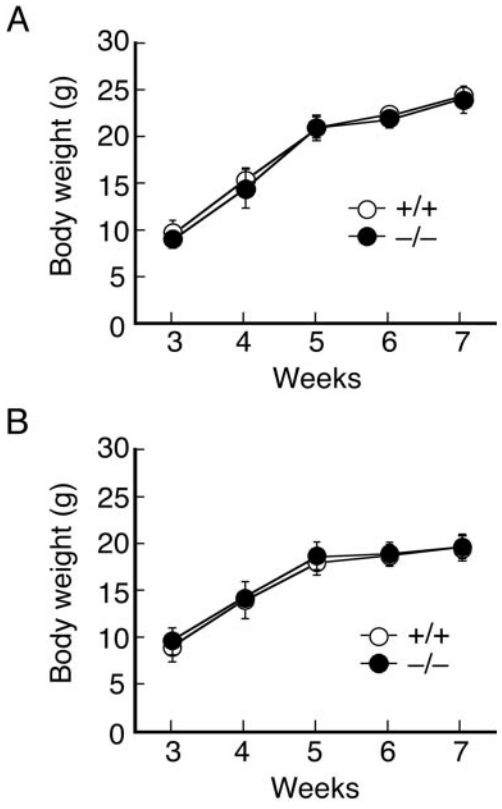

Fig. 2. **Growth curves of wild-type (open circle) and DjB1$^{-/-}$ (closed circle) mice.** Body weights of male (A) and female (B) mice were measured every week. Data are shown as the mean ± SD (n = 9). The mice were derived from three independent ES cell clones.

Table 1. **Offspring of DjB1 deficient mice.**

Mated genotype[a]	Litter size (offspring/litter)
Female (*DjB1*$^{+/+}$) × Male (*DjB1*$^{+/+}$)	7.2 ± 1.9
Female (*DjB1*$^{-/-}$) × Male (*DjB1*$^{-/-}$)	7.6 ± 2.1

[a]Appropriate genotypes of adult mice (3 months old) were mated. Data are shown as the mean ± SD (n = 5 litters).

pseudocolored in magenta and merged images were generated by DP Manager software (Olympus, Tokyo, Japan).

RESULTS

Generation of DjB1 Deficient Mice—To investigate the physiological functions of DjB1 *in vivo*, we generated DjB1 deficient mice by gene targeting strategy. A targeting vector was constructed by inserting the neomycin phosphotransferase gene (*Neo*) into 40 bp downstream of the initial ATG. The diphtheria toxin A fragment gene (*DT-A*) was also included in the targeting vector for negative selection of homologous recombinants (Fig. 1A). The vector was introduced into the TT2 ES cells by electroporation, and 12 homologous recombinants were isolated from 258 G418-resistant clones. Their genotype was confirmed by a Southern blot analysis. Seven independent clones were injected into ICR embryos to obtain chimeric male mice. The chimeric mice were backcrossed to C57BL/6J female mice to generate heterozygous (*DjB1*$^{+/-}$) mice. The *DjB1*$^{+/-}$ mice displayed no obvious defect and were interbred to generate *DjB1*$^{-/-}$ mice. Generation of *DjB1*$^{-/-}$ mice was confirmed by a genomic Southern blot analysis, a Northern blot analysis, and a Western blot analysis (Fig. 1, B–D).

Phenotype of DjB1 Deficient Mice—Interbreeding of the *DjB1*$^{+/-}$ mice produced litters with predicted Mendelian frequencies. Both male and female *DjB1*$^{-/-}$ mice developed without growth retardation (Fig. 2) and showed no gross abnormality under normal conditions within the 18-month observation period. The clinical chemistry parameters were normal (data not shown). *DjB1*$^{-/-}$ mice were fertile and produced normal litter sizes (Table 1). The lack of any noticeable alterations in *DjB1*$^{-/-}$ mice suggests no essential physiological role of DjB1. However, the role of DjB1 *in vivo*

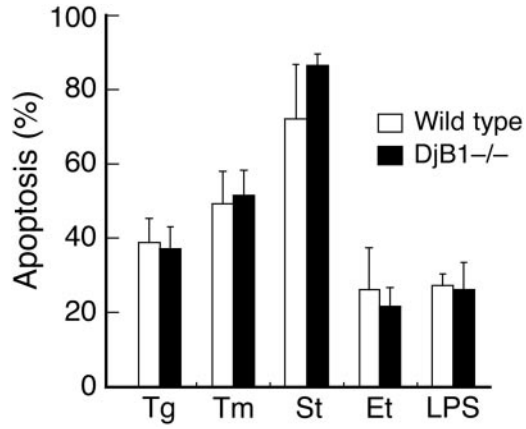

Fig. 3. **Normal responses of DjB1$^{-/-}$ peritoneal macrophages to multiple apoptotic stimuli.** The cells were exposed to apoptosis-inducing reagents for 24 h. Apoptosis was assessed by Hoechst 33258 staining. Tg, thapsigargin (2 μM); Tm, tunicamycin (2 μg/ml); St, staurosporine (1 μM); Et, etoposide (5 μM); LPS, lipopolysaccharide (500 μg/ml). Apoptotic cell death was <3% without inducing reagent. Data are shown as the mean ± SD (n = 3).

may be concealed by a compensatory function of other members of Hsp40 homologues. To elucidate the effect of DjB1 deficiency on the expression of other Hsps in cytosol, we checked the expression of DjA1, DjA2, and DjA4 in the testis by Northern blot and Western blot analyses (Fig. 1, C and D). In addition, the expressions of Hsc70, Hsp70, Hsp90, and BiP were investigated by a Western blot analysis (Fig. 1D). No significant difference was observed between the wild-type and DjB1 deficient littermates.

Responses of DjB1$^{-/-}$ Peritoneal Macrophages to Multiple Apoptotic Stimuli—Although DjB1 deficient mice showed no obvious abnormality under normal conditions, DjB1 may play a role under protein-damaging conditions. To clarify this issue, we examined the effect of DjB1 deficiency on cell viability after exposure to various proteotoxic stress conditions at the cellular level.

Previous studies showed that the Hsp70-DjB1 pair protects cells from various apoptotic stimuli (*30*, *31*). We also showed that the expression of either Hsp70-DjA1 or Hsp70-DjB1 pair prevents apoptosis in mouse RAW 264.7 macrophage cell line (*25*, *26*). Therefore, the effect of DjB1 deficiency on the apoptosis in macrophages was of great interest to us. To evaluate the role of DjB1 on the anti-apoptotic effects, we tested the resistance of peritoneal macrophages to multiple apoptosis-inducing reagents, including ER stress inducers thapsigargin and tunicamycin, a protein kinase inhibitor staurosporine, a topoisomerase inhibitor etoposide (VP-16), and an immunostimulant lipopolysaccharide. After exposure to these reagents, apoptotic cells were detected by Hoechst 33258 staining. Contrary to our expectation, wild-type and *DjB1$^{-/-}$* cells underwent comparable levels of apoptosis (Fig. 3). These results indicate that a DjB1 deficiency can thus effectively protect cells from apoptosis under these conditions.

An Essential Role of DjB1 in Early Phase of Thermotolerance—We next examined thermotolerance of peritoneal macrophages. As shown in Fig. 4A, wild-type macrophages express DjB1 under normal conditions. The expression level increased immediately after preconditioning at 42°C for 15 min (2.1 ± 0.5 fold), and then decreased to 1.4 ± 0.1 fold after 3 h of recovery. Similarly, the expression levels of DjA1 and DjA2 increased approximately 1.5- and 2.0-fold, respectively. After 3 h of recovery, the expression levels of these two type I Hsp40 members decreased to 1.3- and 1.1-fold, respectively. The other type I Hsp40 member, DjA4, was not detected in the peritoneal macrophages. Hsc70, a constitutive member of Hsp70, was expressed under normal conditions and it did not change significantly after the preconditioning. Hsp70, an inducible member of Hsp70 family, was not detected under normal conditions, but it was strongly induced by the preconditioning. Hsp90 was expressed under normal conditions, and its level in the wild type macrophages decreased to 0.3-fold immediately after the preconditioning. However, that of the DjB1$^{-/-}$ macrophages remained essentially unchanged (0.9-fold). The expression levels of Hsp90 during the recovery periods increased to 5.7- and 2.5-fold for the wild type and the DjB1$^{-/-}$ macrophages, respectively. The representative member of small heat shock proteins in rodent is Hsp25, while that in primate is Hsp27 (*32*). We thus investigated the expression level of Hsp25. However, Hsp25 was not detected in the peritoneal macrophages. In addition, certain murine samples have been reported to not express Hsp25 under certain conditions (*33*).

After the preconditioning and following recovery period at 37°C for 3 h or 6 h, wild-type and the *DjB1$^{-/-}$* cells were heat shocked at 45°C for 15 min. Cell death was assayed with a mitochondrial membrane potential-dependent dye. When wild-type cells were heat shocked without preconditioning, then cell death was 70%. In *DjB1$^{-/-}$* cells, cell death was similar to that in wild-type cells. After the preconditioning and a 3-h recovery period, the cell death decreased to 45% in wild-type cells. However, cell death remained 70% in *DjB1$^{-/-}$* cells. After a 6 h-recovery period, however, cell death was less than 30% in wild-type and the *DjB1$^{-/-}$* cells. Similar results were obtained with Hoechst 33258 staining (Fig. 4, B and C). These results indicate that DjB1 plays a role in thermotolerance in the early phase.

Intracellular Localization of DjB1 and Hsp70—The impaired thermotolerance of *DjB1$^{-/-}$* cells after 3 h-recovery period may be due to a dysfunction of Hsp70 and Hsc70 by the absence of partner DjB1. Since previous studies have demonstrated that DjB1 and Hsp70 accumulate in the nucleoli after heat shock stimuli (*34*, *35*), we hypothesized that the DjB1 deficiency could thus possibly affect the localization of Hsp70 and Hsc70. To confirm the intracellular distribution of Hsp70 and Hsc70, each protein was detected by immunofluorecence staining (Fig. 5). In wild-type cells, DjB1 localized mainly in the cytoplasm under normal conditions, whereas it accumulated in the nucleus and a specific region of the cytoplasm after heat treatment. Hsp70 was hardly detected under normal conditions, but it was strongly induced during 3 h-recovery period. Hsp70 localized in both the cytoplasm and nucleus after heat-treatment. The level of Hsp70 induction varied from cell to cell. Approximately 53% of the total cells strongly expressed Hsp70 in wild-type macrophages and 56% in *DjB1$^{-/-}$* macrophages (Fig. 5A). This may represent different populations in the peritoneal macrophages, but the degree of Hsp70 expression in each cell does not explain

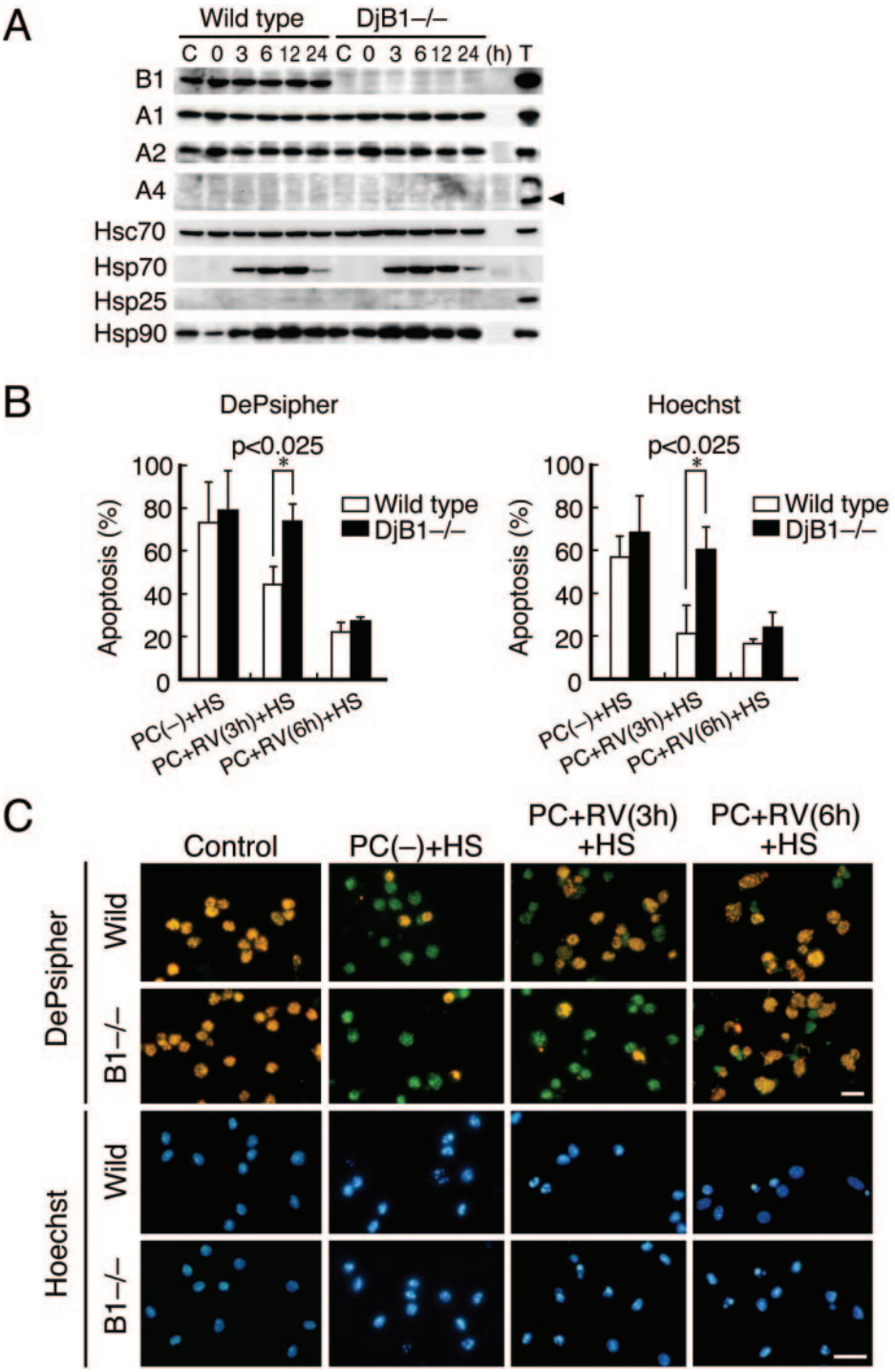

Fig. 4. **Apoptotic cell death of DjB1$^{-/-}$ peritoneal macrophages after heat shock.** (A) Induction of Hsps after heat treatment. Cells were heat shocked at 42°C for 15 min, followed by recovery at 37°C for the indicated times. Total cell lysate was subjected to a Western blot analysis. An arrowhead indicates position of DjA4 protein of 46 kDa species. C, control sample without heat shock treatment; T, sample lysate from mouse testis. (B and C) Thermotolerance of wild-type and *DjB1*$^{-/-}$ peritoneal macrophages. The cells were preconditioned at 42°C for 15 min (PC), followed by recovery at 37°C for 3 h or 6 h (RV), and then heat shocked at 45°C for 15 min (HS). Apoptotic cells were detected with a mitochondrial membrane potential–dependent dye DePsipher (Trevigen). Cells shown in green are apoptotic cells. Apoptotic cell death was also confirmed by chromatin condensation and nuclear fragmentation with Hoechst 33258 staining. In each experiment, 70 to 300 cells were counted. Data are shown as the mean ± SD (n = 3 or 4). Bar = 20 μm.

the difference in apoptotic cell death between the wild-type and *DjB1*$^{-/-}$ macrophages. On the other hand, Hsc70 was readily detected under normal conditions, and localized in the cytoplasm. Hsc70 co-localized with DjB1 in the nucleus after heat treatment (Fig. 5B). However, the localization patterns of Hsp70 and Hsc70 in *DjB1*$^{-/-}$ cells were comparable to those of wild-type cells (Fig. 5, A and B).

In the heat-shocked wild-type macrophages, DjB1 noticeably accumulated not only in the nucleus but also in the cytoplasm (Fig. 5, A and B). To clarify the localization of the cytoplasmic DjB1, we co-stained cytoskeletal proteins and other intracellular organelles. Interestingly, DjB1 strongly co-stained with α-tubulin at centrosome (Fig. 6A). γ-Tubulin was readily detected at centrosomes under normal conditions (Fig. 6B). However, it was not detected in the heat-shocked cells (Fig. 6C). The loss of the γ-tubulin staining after heat treatment is consistent with previous observations (*36*, *37*). DjB1 did not significantly co-localize with other cytoskeletal proteins and organellar markers (data not shown).

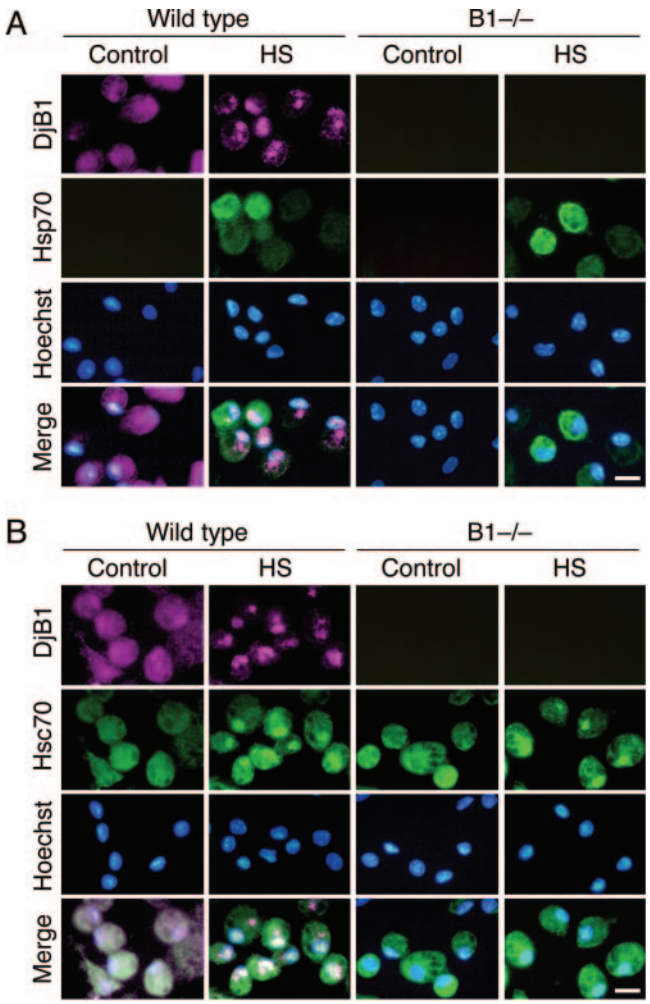

Fig. 5. **Intracellular localization of DjB1, Hsp70, and Hsc70 after heat shock.** Wild-type and *DjB1*$^{-/-}$ peritoneal macrophages were preconditioned at 42°C for 15 min, followed by recovery at 37°C for 3 h, and then heat shocked at 45°C for 15 min (HS). Control cells were maintained at 37°C. The cells were stained with rabbit anti-DjB1 antiserum, mouse monoclonal antibody against Hsp70, and Hoechst 33258 (A), and DjB1 antiserum, rat monoclonal antibody against Hsc70, and Hoechst 33258 (B). Appropriate fluorophore-labeled secondary antibodies were used for detection. Cy3-signals were pseudocolored in magenta to improve the visualization of the merged images. Bar = 10 μm.

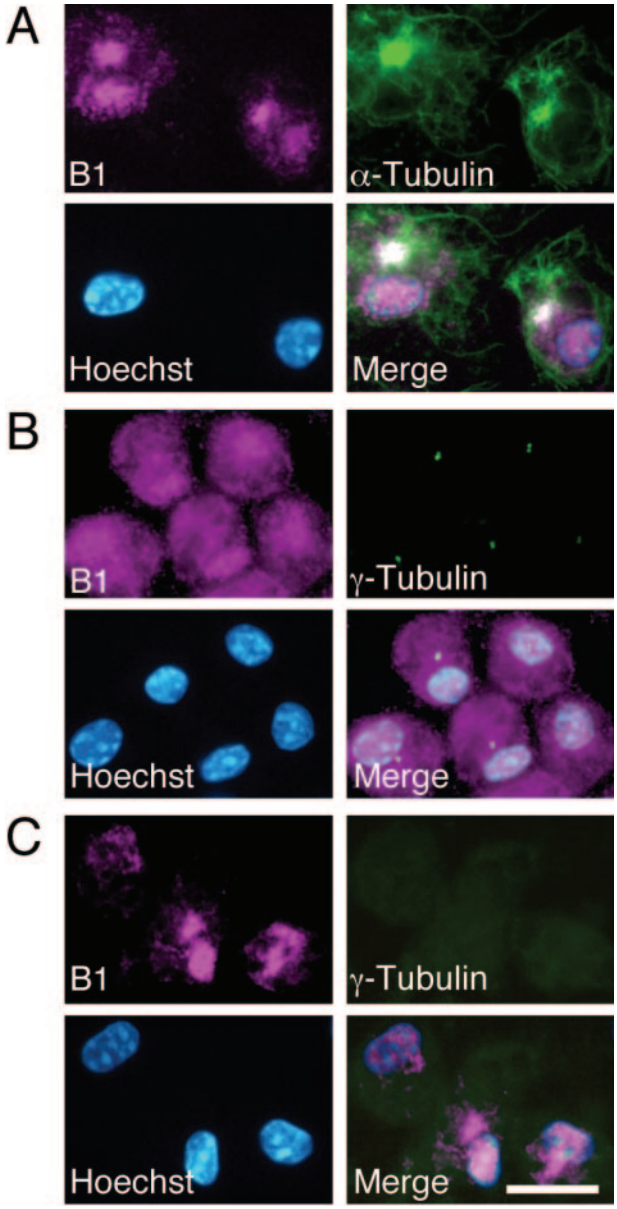

Fig. 6. **Cytoplasmic localization of DjB1 after heat shock.** Wild-type peritoneal macrophages were preconditioned at 42°C for 15 min, followed by recovery at 37°C for 3 h, and then heat shocked at 45°C for 15 min (A and C). Control cells (B) were maintained at 37°C. The cells were stained with rabbit anti-DjB1 antiserum and mouse monoclonal antibody against α-tubulin (A), and rabbit anti-DjB1 antiserum and γ-tubulin (B and C). Appropriate fluorophore-labeled secondary antibodies were used for detection. The nuclei were visualized by Hoechst 33258 staining. Cy3-signals were pseudocolored in magenta. Bar = 10 μm.

DISCUSSION

In the present study, we generated DjB1 deficient mice to clarify the physiological functions of DjB1 *in vivo*. Unexpectedly, the *DjB1*$^{-/-}$ mice were found to be viable and fertile, and had no obvious alteration under normal conditions. Considering that *DjA1*$^{-/-}$ mice (*14*) and *DjA2*$^{-/-}$ mice (Terada, K., unpublished observations) exhibit abnormal phenotypes, the lack of any gross abnormality in *DjB1*$^{-/-}$ mice was therefore striking. In yeast, a DjB1 homologue Sis1p is essential (*38*), whereas a type I homologue Ydj1p is not essential under normal conditions (*39*). We previously demonstrated that either DjA1 or DjA2, but not DjB1, is sufficient for *in vitro* protein folding and for mitochondrial protein import (*22–24*). Therefore, the existence of distinct phenotypes of DjA1 and DjA2 knockout mice were unexpected. In mammals, there are more than 30,000 proteins and some of these proteins may be strictly-dependent substrates for the Hsp70 chaperone system. Knockout mice of the respective member of Hsp40 proteins open a way to explore the proteins dependent on each Hsp40 protein in a physiologically reliable context. Our present results indicate that DjB1 is dispensable for mouse development and normal physiology, but it is required for a limited period of a proteotoxic and heat-stressed conditions. After this limited

period, other members of Hsps may thus compensate for the DjB1 deficiency.

Previous studies showed that an overexpression of Hsp70-DjB1 protects the cells against several apoptotic stimuli (*25*, *30*, *31*). Hsp70 is an anti-apoptotic protein and several mechanisms have been suggested to explain its anti-apoptotic function, such as the regulation of the JNK kinase pathway, the inhibition of caspase activation through the assembly of apoptosome, and the suppression of apoptosis-inducing factor (AIF) release from mitochondria (*6*). In the present study, DjB1 deficiency did not affect the resistance to multiple apoptosis-inducing reagents in peritoneal macrophages and embryonic fibroblasts (data not shown). DjB1 is thus considered to be dispensable for these anti-apoptotic effects.

The DjB1 gene as well as the Hsp70 gene has typical heat shock elements (*40*). Heat shock mediates the phosphorylation and trimerization of heat shock factor 1, and the activated heat shock factor 1 binds to the heat shock elements to induce the transcription of the Hsp genes (*41*). DjB1 and Hsp70 are simultaneously induced in most cultured cells after heat shock (*34*). According to our data, mouse peritoneal macrophages and embryonic fibroblasts (data not shown) expressed DjB1 under normal conditions. After mild heat treatment, the expression level of DjB1 slightly increased while Hsp70 was highly induced. In *DjB1*$^{-/-}$ cells, the expression of Hsp70 was slightly higher than that in wild-type cells. This may be the result of feedback regulation to compensate for the defective function of Hsp70 by the loss of DjB1 (*41*, *42*).

We herein showed that DjB1 is required for thermotolerance at an early phase. Previous studies have shown that DjB1 accumulates in the nucleoli of heat-stressed cells (*23*, *34*), and we also confirmed the colocalization of DjB1 and Hsc70 in the nucleus of wild-type macrophages. Kodiha *et al*. (*43*) reported inhibition of Hsc70 export from the nucleus to the cytoplasm in heat-stressed cells, and identified association of Hsc70 to several nuclear and nucleolar proteins. In this process, Hsc70 initially accumulates in the nucleus, then transiently relocates to nucleoli, and finally diffuses back to the cytoplasm during recovery period. In the present study, the accumulation of Hsc70 in the nucleoli was not clearly detected, probably because cells were observed immediately after the heat stress. Since DjB1 and Hsc70 accumulate in the nucleus after the heat shock, we hypothesized that DjB1 deficiency would affect the retention of Hsp70 and Hsc70 to the nucleus. However, the intracellular localization of Hsp70 and Hsc70 did not differ between wild-type and *DjB1*$^{-/-}$ cells.

DjB1 accumulated also in the centrosome after heat-shock. Earlier studies reported that heat shock causes functional and structural abnormalities of centrosomes, and that Hsp70 can protect the heat-stressed centrosomes (*36*, *37*, *44*). It is therefore possible that DjB1 may play a role in the protection of centrosomes. However, the staining patterns of the microtubules did not differ significantly between wild-type and *DjB1*$^{-/-}$ cells (data not shown).

DjB1 was found to be dispensable for the late phase of thermotolerance. In the late phase, other members of Hsp40s and other Hsps may compensate for DjB1. In addition to Hsp70 as mentioned above, small heat shock protein Hsp25 is a potent chaperone that is induced by heat shock to prevent protein aggregation (*45*). However, peritoneal macrophages did not express Hsp25 under our conditions. On the other hand, Hsp90 was induced after the heat treatment, and the induction was more rapid and stronger in *DjB1*$^{-/-}$ cells than in wild-type cells. In cooperation with Hsp70, Hsp90 mediates the folding and degradation of signal transduction proteins (*46*, *47*). The functions of DjB1, which were prominent at an early phase, may therefore be concealed by these Hsps at a late phase.

Up to now, DjB1 is the only Hsp40 homologue that is known to accumulate in the nucleoli after heat shock. Two ubiquitous type I Hsp40, DjA1 and DjA2 do not accumulate in the nucleoli after heat shock (*23*). This indicates that DjB1 thus plays an exclusive role in the nucleoli under heat-stressed conditions. Since the nucleolus has a variety of functions, including the assembly of ribosomal subunits and the processing of pre-rRNAs (*48*), the Hsc70-DjB1 pair may protect these nucleolar functions by preventing the aggregation of proteins, such as ribosomes and small nucleolar RNA protein, under heat-stressed conditions. Further studies to clarify the DjB1-specific substrates and the role of DjB1 in the nucleoli are presently underway.

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