博士（医学）論文
Doctor's Thesis

Studies on a Putative Mitochondrial Protein Import Factor Metaxin and a New Hsp70 Cochaperone dj4

（ミトコンドリア蛋白質輸送候補因子メタキシンと新規Hsp70コシャペロンdj4に関する研究）

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Studies on a Putative Mitochondrial Protein Import Factor Metaxin and a New Hsp70 Cochaperone dj4

Doctor's Thesis By

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For the Fulfillment of the Doctorate Degree in Medical Sciences

Under supervision of

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２００２年３月

CONTENTS

1. SUMMARY

Metaxin is an outer membrane protein of mammalian mitochondria which was found to be encoded between the glucocerebrosidase and thrombospondin 3 genes in the mouse, and was suggested to be involved in protein import into the organelle. Roles of metaxin in mitochondrial protein import were studied. RNA blot analysis showed that distribution of metaxin mRNA in human tissues differs from that of mRNA for the translocase component Tom20. Overexpression of metaxin resulted in impaired mitochondrial import of natural and chimeric preproteins and in their accumulation. Overexpression of the cytosolic domain of metaxin also caused inhibition of preprotein import, although less strongly than the full-length metaxin. In blue native PAGE, Tom40, Tom22 and a portion of Tom20 migrated as a complex of ~400 kDa. On the other hand, metaxin migrated at a position of ~50 kDa. These results confirm earlier *in vitro* results that metaxin participates in preprotein import into mammalian mitochondria, and indicate that it does not associate with the Tom complex.

DnaJ homologues are cochaperones of heat shock protein 70 (Hsp70) family. dj1 (Hsp40/hdj-1), dj2 (HSDJ/hdj-2) and dj3 (hdj-3/rdj-2) have been identified in mammalian cytosol and characterized. Here, I characterized newly found dj4 (DjA4) and compared with other chaperones. dj4 was expressed strongly in heart and testis, and moderately or weakly in other tissues in mice. In subcellular fractionation of mouse heart, dj4 was recovered mostly in the cytosol fraction. In immunocytochemical analysis of H9c2 heart muscle cells, dj4 and heat shock cognate 70 (Hsc70) colocalized in the cytoplasm under normal conditions, whereas they colocalized in the nucleus after heat shock. dj4 as well as Hsp70, dj1 and dj2 were induced in H9c2 cells by heat treatment, whereas Hsc70 and dj3 were not. Heat pretreatment promoted survival of cells after severe heat shock. H9c2 cells overexpressing Hsp70 were more resistant to severe heat shock, and a much better survival was obtained when dj4 or dj2 was co-overexpressed with Hsp70. Taking a high concentration of dj4 in heart into consideration, these results suggest that Hsc70/Hsp70-dj4 chaperone pair prevents heart muscle cells from various stresses.

2. LIST OF PUBLICATIONS

1. Khaleque Md. Abdul, Kazutoyo Terada, Masato Yano, Michael T. Ryan, Illo Streimann, Nicholas J. Hoogenraad, Masataka Mori. Functional analysis of human metaxin in mitochondrial protein import in cultured cells and its relationship with the Tom complex. Biochem. Biophys. Res. Commun. 276: 1028–1034 , 2000

2. Khaleque Md. Abdul, Kazutoyo Terada, Tomomi Gotoh, Rahman Md. Hafizur, Masataka Mori. Characterization and functional analysis of a heart-enriched DnaJ/Hsp40 homolog dj4/DjA4. Cell Stress and Chaperones. in press

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I would like to thank the Ministry of Education, Science, Sports and Culture of Japan and the authority of Kumamoto University School of Medicine, Kumamoto for giving me an opportunity to study in Japan.

Finally, I would like to dedicate this work to my family for their uncompromising love and support, and especially to my parents for their great wish and inspiration and to my elder brother who encouraged me and wished me an accomplishment on my scientific carrier.

4. LIST OF ABBREVIATIONS

AII. arginase II

ADP. adenosine 5'- diphosphate

ATP. adenosine 5'-triphosphate

DIG, digoxigenin

DMEM. Dulbecco's modified Eagle's medium

$\Delta\Psi$. membrane potential

EDTA. ethylenediaminetetraacetic acid

ER. endoplasmic reticulum

FCS, fetal calf serum

GAPDH. glyceraldehyde 3-phosphate dehydrogenase

GFP, green fluorescent protein from jellyfish *Aequoria victoria*

dj1. dj2. dj3 and dj4. DnaJ homolog-1. 2. 3 and 4. respectively

H_6mdj4. hexahistidine-tagged mdj4

MTX. metaxin

Hsc70, heat shock cognate protein 70

HSF. heat shock factors

Hsp70. heat shock protein 70

IPTG. isopropyl-β-D-thiogalactopyranoside

ΔhMTX. human metaxin lacking the C-terminal hydrophobic region

mHsp70, mitochondrial Hsp70

MIP, mitochondrial intermediate peptidase

MPP, mitochondrial processing peptidase

OTC, ornithine transcarbamylase

pOTC, pre-ornithine transcarbamylase

pOTC-GFP, a fusion protein containing the presequence of human pOTC fused to GFP

PBS, phosphate buffered saline

PCR, polymerase chain reaction

PMSF, phenylmethylsulfonyl fluoride

SDS, sodium dodecylsulfate

SDS-PAGE, SDS-polyacrylamide gel electrophoresis

Tim, translocase(s) of the inner membrane of mitochondria

Tom, translocase(s) of the outer membrane of mitochondria

Tom20, Tom22, Tom40 and Tom70, translocases of the outer membrane of mitochondria of 20, 22, 40 and 70 kDa

Tom34, 34 kDa protein involved in mitochondrial protein import.

Tris, tris (hydroxymethyl)aminomethane

PART 1

Functional Analysis of a Mitochondrial Outer Membrane Protein Metaxin and its Relationship with the Tom Complex

5. INTRODUCTION

Molecular mechanism involved in protein import into mitochondria is well conserved from lower eukaryotes to higher animals, but some differences exist. In animals as well as in lower eukaryotes, most mitochondrial proteins are initially synthesized on free ribosomes as precursors, many with NH_2-terminal presequences which function as mitochondrial targeting and import signals, and released into the cytosolic pool. Newly synthesized preproteins associate with cytosolic chaperones which keep them loosely folded in the cytosol, bind to a mitochondrial surface receptor(s), and are then transported into or across the outer and inner membranes. Many presequences of transported preproteins are then proteolytically cleaved in the mitochondrial matrix, and the mature portions are folded into their native conformations. The whole process of synthesis of mitochondrial preproteins, their translocation, processing, folding and assembly involves many factors in the cytosol, mitochondrial membranes and matrix compartments (Neupert *et al*, 1997; Mori and Terada, 1998). An important step in this process is the interaction of the preproteins with the outer membrane of mitochondria. A number of proteins in the outer membrane that are responsible for recognizing and translocating preproteins into the organelle have been identified in yeast and *Neurospora* (Haucke and Schatz, 1997; Lill and Neupert, 1996; Ryan *et al*, 2000).

5-1. Cytosolic Factors

Mitochondrial precursor proteins cannot be imported into the mitochondria in a tightly

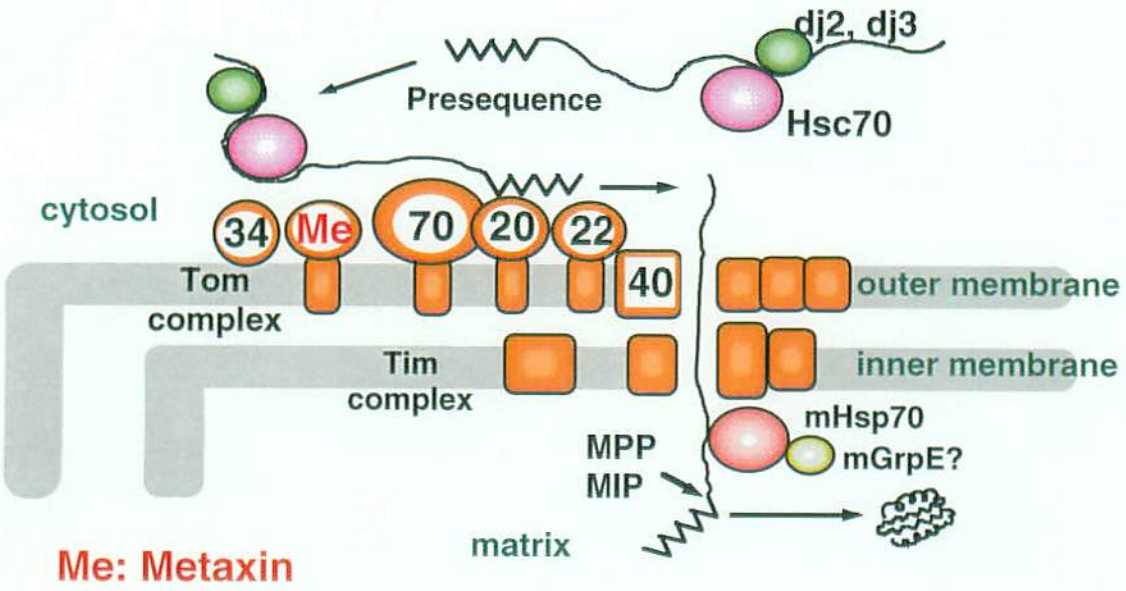


Fig. 1. **A model of protein import into the mitochondria in mammals.** Preproteins with an amino-terminal presequence are recognized by import receptors and translocated by the general import pore of the translocase of the outer membrane (Tom). The presequences are cleaved off by the mitochondrial processing peptidase (MPP) in the matrix, and also in some cases, by the mitochondrial intermediate peptidase (MIP).

state, rather they must be maintained in a loosely-folded, transport-competent state in the cytosol prior to import. Presequences might prevent or retard folding of the mature portion of mitochondrial precursor proteins. However, some precursor proteins was

isolated in an enzymatically-active form or could be folded into an enzymatically-active form. These results indicate that the presequence portion does not always interfere with much of the folding of the mature portion.

It was thus assumed that there is a component(s) in the cytosol. the function of which is to maintain newly-synthesized precursor proteins in a conformation enabling transport across mitochondrial membranes. We found that a protein component(s) in the rabbit reticulocyte lysate markedly stimulate the uptake of *in vitro*-synthesized pOTC by isolated mitochondria (Miura *et al.* 1983). Recently, several cytosolic factors that facilitate mitochondrial protein import have been found in mammals as well as in lower eukaryotes, including molecular chaperones and presequence-specific factors (Ryan *et al*, 1997).

The roles of 70-kDa cytosolic heat shock protein (Hsp70) in intracellular protein traffic were first studied using *Saccharomyces cerevisiae* Hsp70 (Craig *et al.* 1993. Rassow *et al.* 1997). In mammals, two Hsp70 family members. Hsp70 and Hsc70, are present and their amino acid sequence are about 87% identical. Sheffield *et al* (1990) studied effects of reticulocyte Hsp70 (presumably Hsc70) on folding, aggregation and import competence of a recombinant hybrid precursor protein. They showed that Hsp70 prevented aggregation of the hybrid precursor, in an ATP-dependent manner. and retarded the apparent rate and extent of its folding. Terada *et al* (1995) have shown that Hsc70 is required during pOTC synthesis and not during import into mitochondria.

5-2. The Tom Complex

Mitochondrial precursor proteins, recognized by a specific import receptor on the mitochondrial surface, are imported through an import channel in the outer membrane. In yeast, the receptor and channel are composed of several non-identical subunits referred as Tom (translocase of the outer membrane of mitochondria) subunits (Lithgow *et al*, 1995; Lill and Neupert, 1996; Schatz *et al*, 1996; Haucke and Schatz, 1997) (Fig. 1). The receptor consists of Tom70 (Hines *et al*, 1990, Sollner *et al*, 1990), Tom37 (Dekker *et al*, 1998), Tom22 (Kiebler *et al*, 1993, Lithgow *et al*, 1994) and Tom20 (Ramage *et al*, 1993, Moczko *et al*, 1994) and can be divided into the Tom70- Tom37 and Tom22- Tom20 subcomplexes. The Tom70-Tom37 subcomplex recognizes only limited numbers of precursor proteins, while the Tom22-Tom20 subcomplex directly interact with majority of precursor proteins. The Tom22-Tom20 subcomplex is involved in import of the precursor proteins that initially interact with the Tom70-Tom37 subcomplex (Neupert *et al*, 1997, Lill and Neupert, 1996). The receptor bound precursors are then delivered to the protein channel in the outer membrane. The channel is composed of at least four hydrophobic proteins, Tom40, Tom7, Tom6 and Tom5. Tom40 appears to be a core subunit and small Tom proteins seem to regulate conformation of the Tom channel (Alconada *et al*, 1995, Honlinger *et al*, 1996, Dietmeier *et al*, 1997). In blue native PAGE, the yeast Tom complex migrates as a ~400 kDa complex containing Tom40, Tom22 and small Toms and a ~120 kDa complex containing Tom70 (Dekker *et al*, 1998). Tom20 migrates in a band of 40 to 100 kDa and is partly associated with the ~400 kDa complex. Tom37 has not been found in

association with the Tom complex.

In mammals, several counterparts have been identified and their roles have been studied: Tom20 (Hanson *et al*, 1996, Iwahashi *et al*, 1997, Schleiff and Turnbull, 1998, Yano *et al*, 1998), Tom22 (Yano *et al*, 2000, Saeki *et al*, 2000), and Tom40 (Suzuki *et al*, 2000) among the nine Tom subunits have been identified. In addition, Tom34 and metaxin have been identified as unique components of the mammalian mitochondrial import system. cDNA for human homolog of yeast and *Neurospora* Tom20 was isolated (Seki *et al*, 1995, Goping *et al*, 1995, Hanson *et al*, 1996). Human Tom20 has an overall sequence identity of 50% with *S. cerevisiae* Tom20 and 61% with *N. crassa* Tom20. This protein was shown to be the human counterpart of Tom20, because it can assemble with the yeast receptor complex (Seki *et al*, 1995), and can complement the respiratory defect of *tom20*-deficient yeast cells (Goping *et al*, 1995). Furthermore, *in vitro* import of precursor proteins into isolated mitochondria was inhibited by the soluble domain of human Tom20 (Hanson *et al*, 1996) and by antibody of human Tom20 (Goping *et al*, 1995, Hanson *et al*, 1996).

Metaxin, a novel gene located between the glucocerebrosidase and thrombospondin 3 genes in the mouse, is a mitochondrial protein that extends into the cytosol while anchored into the outer membrane at its COOH-terminus (Armstrong *et al*, 1997). In its NH_2-terminal region, metaxin shows significant sequence identity to yeast Tom37. However, important structural differences, apparently as the result of different mechanisms of targeting to membranes, also exist between the two proteins. Antibodies against metaxin, when preincubated with mitochondria, partially inhibited the uptake of

adrenodoxin precursor into mitochondria. Mice homozygous for disruption of the metaxin have an embryonic lethal phenotype, indicating that this protein is necessary for the early development of the mouse embryo. It remains to be elucidated whether metaxin is the mammalian counterpart of Tom37.

A 34 kDa protein Tom34 that functions as a subunit of the protein import apparatus on the outer membrane was identified (Nuttal *et al.* 1997). Tom34 was located on the surface of the mitochondria and was resistant to extraction under alkaline conditions. It has a large COOH-terminal domain exposed to the cytosol. Antibodies raised against this protein specifically inhibited *in vitro* import of pOTC into isolated rat liver mitochondria. In addition, the recombinant soluble domain competed with human Tom34 for pOTC import (Chewawiwat *et al.* 1999). This novel component of the protein import machinery has a 62 residue motif conserved with the Tom70 family of mitochondrial receptors, but otherwise appears to have no counterpart so far characterized in the mitochondria of any other species.

5-3. The Tim Complex

Since the positively charged presequence portions have moved across the outer membrane, they are pulled across the inner membrane by electrostatic potential across that membrane.

In yeast, the precursor proteins are imported across the inner membrane through a transmembrane channel formed by the Tim (translocase of the inner membrane of mitochondria) complex. The Tim complex is composed of at least five proteins (Tim54,

Tim44, Tim23, Tim22 and Tim17) (Pfanner *et al*, 1994, Schatz *et al*, 1996, Sirrenberg *et al*, 1996, Haucke and Schatz, 1997, Kerscher *et al*, 1997). Most of all (except Tim44) are firmly embedded within the inner membrane and appear to form protein-conducting channels. One is Tim23-Tim17 subcomplex and the other is Tim54-Tim22 subcomplex. Tim44 is exposed to the matrix side and is in contact with Tim23-Tim17 channel. Tim44 appears to function in cooperation with mitochondrial Hsp70 (Ssc1p) and GrpE as an ATP-driven 'import motor' that pulls the precursor chain across the Tim23-Tim17 channel into the matrix space. Tim23 shows dynamic dimer-monomer formation and constitute a part of a membrane potential ($\Delta\Psi$)-dependent protein-conducting channel (Bauer *et al*, 1996). Recently, the other subcomplex Tim54-Tim22 channel was identified and appears to be specific for the insertion of proteins into the inner membrane (Sirrenberg *et al*, 1996, Kerscher *et al*, 1997).

Human and *Drosophila melanogaster* Tim17s were isolated from animals (Bomer *et al*, 1996) (Fig 1). A comparison of amino acid sequence from human, *D. melanogaster* and *S. cerevisiae* revealed a similarity of 70 to 82%, including 46 to 62% identical amino acid residues. Tim17 has four hydrophobic segments that are predicted to function as transmembrane sequences. The targeting and assembly of human and *D. melanogaster* Tim17s as well as yeast Tim17 were characterized with yeast isolated mitochondria. Targeting signals in the mature proteins directed the Tim17 precursors to Tom70 present on the mitochondrial surface. The precursor was inserted into the inner membrane in a membrane potential ($\Delta\Psi$)-dependent manner, adopted a characteristic topology and assembled with Tim23. The mechanism of targeting and assembly were

indistinguishable between the Tim17s from distinct organisms, thereby indicating a high conservation during evolution. Besides Tim17, mammalian counterparts of Tim23 and Tim44 have been identified in data banks.

In animals, most studies have been performed in an *in vitro* system in which the preproteins synthesized in reticulocyte lysate were imported into isolated mitochondria. It is important to test the *in vitro* results *in vivo* and there is a need for procedures that will enable protein import to be investigated in intact cells. We developed a procedure of cotransfecting cDNAs for preproteins and those for import factors in cultured cells and of analyzing their effects by immunoblotting or pulse-chase experiments (Kanazawa *et al*, 1997). Coexpression of Tom20 retarded mitochondrial import and processing of pre-ornithine transcarbamylase (pOTC), whereas it stimulated the import of a fusion protein, pOTC-GFP consisting of the presequence of human pOTC fused to green fluorescent protein (GFP) (Yano *et al*. 1997). Coexpression of Tom34 with pOTC enhanced the processing of pOTC to the mature form, and coexpression of Tom34 antisense RNA retarded preprotein processing (Chewawiwat *et al*, 1999).

We report here that coexpression of metaxin in cultured cells inhibits the import of several preproteins into the mitochondria and results in their accumulation. Coexpression of the cytosolic domain of metaxin inhibited preprotein import, although the inhibition was much less than that observed with the intact metaxin. Different tissue distribution of metaxin from that of Tom20 and its separation from the Tom complex in blue native PAGE suggests that metaxin is not involved in the Tom complex.

6. MATERIALS AND METHODS

6-1. Plasmid Construction

Polymerase chain reaction (PCR) was employed for construction of the plasmid expressing the full-length human metaxin of 317 amino acid residues. Human cDNA library (Multi choice cDNA, OriGene, Rockville, MD, USA) was used as template. The upstream primer used was 5'-AGAGGGTGGGCAAGATGGCG-3' and the downstream primer was 5'CTTGG-GAGCGTGAGGACAAATC-3'. The PCR product was cloned into the *Hin*cII site of pGEM-3zf(+) (Promega, Madison, WI, USA) to yield pGEM–hMTX. The sequence was confirmed by sequencing. The *Nco*I fragment encoding the cytosolic domain of human metaxin (ΔhMTX), lacking the C-terminal hydrophobic region (40 amino acid residues) was excised from pGEM–hMTX and cloned into the same site of pET-30a(+) (Novagen, Madison, WI, USA) to yield pET–ΔhMTX. A mammalian expression vector pCAGGS (Niwa *et al* 1991) was provided by Dr. J. Miyazaki (Osaka University, Osaka). To construct pCAGGS-hMTX encoding full-length metaxin, the *Bam*HI/*Hin*dIII fragment was excised from pGEM–hMTX, blunted with T4 DNA polymerase and cloned into the blunted *Xho*I site of pCAGGS. PCR was performed for the construction of an expression plasmid for ΔhMTX using pGEM–hMTX as template. The upstream primer was 5'-AAAAAACTCGAGATGGCGGCGCCCATG-3' and the downstream primer was 5'-TTTTTTCTCGAGTCAGTTCCGGCGCCG-3'. The PCR fragment was digested with *Xho*I, and cloned into the same site of pCAGGS yielding pCAGGS-ΔhMTX.

Construction of pCAGGS-hTom20 (Terada *et al*, 1997), pCAGGS-pOTC (Yano *et al*, 1997), pCAGGS-pOTC-GFP (Yano *et al*, 1997) and pCAGGS-hAII (Gotoh *et al*, 1996) were described previously.

6-2. Preparation of ΔhMTX and Antibody Production

The recombinant plasmid pET–ΔhMTX was transformed into BL21 (DE3) cells (Stratagene, LA Jolla, CA, USA) and His-tagged ΔhMTX was expressed with 1 mM IPTG at 37°C for 4 h. ΔhMTX was recovered in the inclusion bodies, washed with 1% Triton X-100, solubilized in buffer A (20 mM Tris-HCl, pH 7.9, 0.5 M NaCl, 8 M urea) and subjected to Ni^{2+}-NTA Sepharose (Pharmacia Biotech, Buckinghamshire, UK) column chromatography. The column was first washed with buffer A containing 16 mM imidazole, and then ΔhMTX was eluted with buffer A containing 1 M imidazole. The eluate was dialyzed against 50 mM Tris-HCl, pH 7.8 containing 0.5 M NaCl and concentrated with Centricon-10 (Amicon, Beverly, MA, USA). The purified ΔhMTX was used to raise antibody in a rabbit.

6-3. RNA Blot Analysis

Human multiple tissue Northern blot was obtained from Clontech (Palo Alto, CA, USA). Multi-primed ^{32}P-labeled cDNAs for human Tom20 and hMTX were used as hybridization probes.

6-4. Cell Culture and DNA Transfection

COS-7 cells were cultured in 10 cm dishes in Dulbecco's modified Eagle's medium supplimented with 10% fetal calf serum at 37°C under an atmosphere of 5% CO_2 and 95% air. The cells were transfected with plasmids at 37°C for 4 h by the use of TransIT LT1 polyamine (Pan Vera, Medison, WI, USA) and cultured at 37°C for 24 h to allow expression.

6-5. Cell Fractionation

The cells were harvested with trypsinization, washed twice with phosphate buffered saline, and then suspended in ice-cold hypotonic buffer (10 mM Tris-HCl, pH 7.4, containing 5 mM magnesium chloride, 1 mM dithiothreitol and 1 mM phenylmethylsulfonyl fluoride). After sonication, the homogenate was used as whole cell extract. The homogenate was separated into the soluble and membrane fractions by centrifugation at 100,000 X *g* for 10 min in a refrigerated ultracentrifuge. The membrane fraction was extracted with 0.1 M sodium carbonate, pH 11.5, as described (Fujiki *et al*, 1982).

6-6. Preparation of Mitochondria from COS-7 Cells

COS-7 cells were harvested with PBS plus 1 mM EDTA, and washed twice with PBS. The cells were suspended in the mitochondria isolation buffer (3 mM Hepes-KOH, pH 7.4, 0.21 M mannitol, 0.07 M sucrose, 0.2 mM EGTA), homogenized with a Dounce homogenizer (Wheaton, Milville, NJ, USA), and then centrifuged at 500 X *g* for 5 min

at 4 °C. The supernatant was further centrifuged at 8,000 X *g* for 5 min at 4°C. and the precipitated mitochondria were washed twice with the same buffer.

6-7. Immunoblot Analysis

Proteins were separated by SDS-PAGE and electrotransferred onto nitrocellulose membranes. Antisera against human OTC (Kanazawa *et al.* 1997), gelly fish GFP (Yano *et al.* 1997), human arginase II (Ozaki *et al.* 1999), hTom20 (Yano *et al.* 1997), human Tom22 (Yano *et al.* 2000), Tom40 and human metaxin were used as primary antibodies. Monoclonal antibody against porin (Calbiochem. La Jolla, CA. USA) and Bcl-2 (Santa Cruz Biotechnology. Santa Cruz. CA. USA) were parchased commercially. Immunodetection was performed by the use of the chemiluminescence kit (ECL kit. Amersham. Buckinghamshire, UK).

6-8. Blue Native PAGE

Blue native PAGE was performed essentially as described (Schagger and Von Jagow. 1991. Dekker *et al*, 1998. Ryan *et al.* 1999). Briefly, COS-7 mitochondrial pellets (50-100 μg of protein) were lysed in 50 μl of ice-cold digitonin buffer (1% digitonin, 20 mM Tris-HCl, pH 7.4. 0.1 mM EDTA, 50 mM NaCl, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride). After a clarifying spin. 5 μl of sample buffer (5% Coomassie brilliant blue G, 100 mM Bis-Tris. pH 7.0, 500 mM 6-amino-*n*-caproic acid) was added to the supernatant. and electrophoresis was performed for about 6 h in a 6-16.5% polyacrylamide gradient gel in a cold room (4-6°C). For two dimensional gel

analysis, individual lanes were excised from the first-dimension blue native gel and layered on top of the stacking gel of a second dimension SDS-PAGE.

6-9. Other Methods

Protein was determined with the protein assay reagent (Bio Rad, Hercules, CA, USA) using bovine serum albumin as standard.

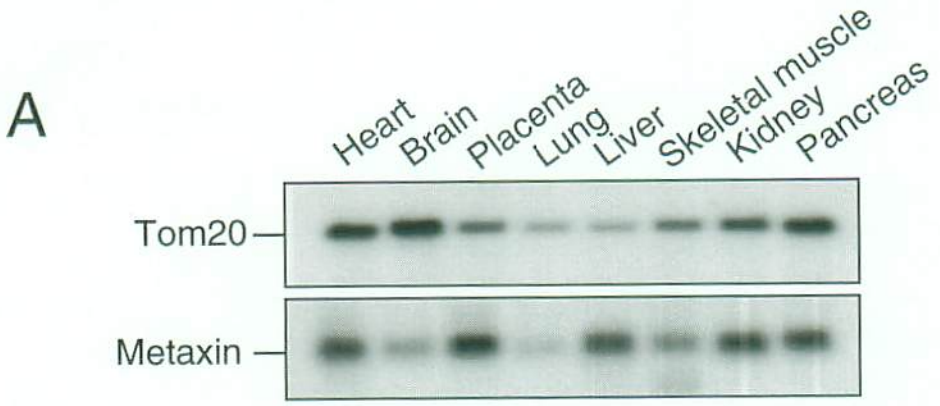
7. RESULTS

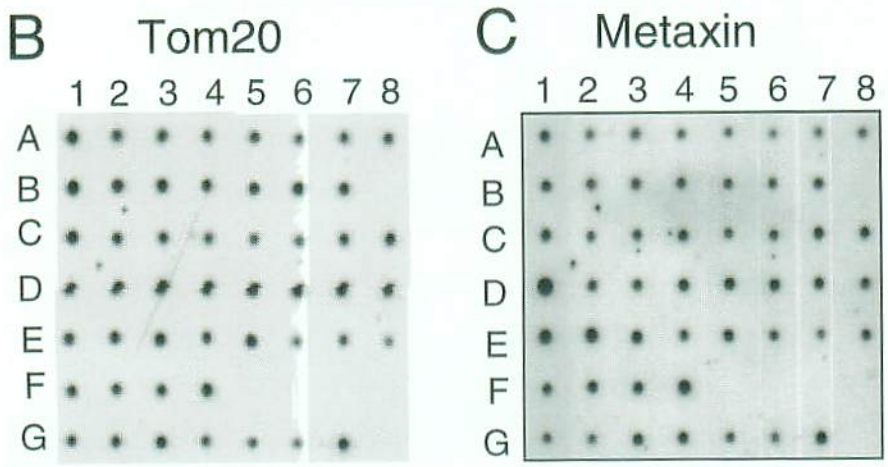
7-1. Distribution of Metaxin and Tom20 mRNAs in Human Tissues

The tissue distribution of metaxin was examined by RNA blot analysis and compared with that of Tom20, which is a core mitochondrial import receptor (Fig. 2). Tom20 mRNA of about 4.1 kb was expressed ubiquitously in various tissues, but not uniformly. Metaxin mRNA of about 1.3 kb was also found to be expressed ubiquitously, but not uniformly. However, there were a number of significant differences in the distribution of metaxin mRNA from that of Tom20 mRNA. For example, Tom20 mRNA but not metaxin mRNA, was abundant in the brain, whereas metaxin mRNA but not Tom20 mRNA, was abundant in the liver.

7-2. Intracellular Concentration of Metaxin

The concentrations of metaxin in monkey kidney-derived COS-7 cells and human cervical carcinoma HeLa cells were measured by immunoblot analysis using purified his-tagged ΔhMTX as standard (Fig. 3). The purified his-tagged ΔhMTX (molecular size calculated from the sequence, 39.7 kDa) always gave two polypeptides; the faster-migrating one was presumbly a degradation product. By using an antibody against ΔhMTX, a metaxin polypeptide of 35 kDa was detected in COS-7 and HeLa cells. The concentration of metaxin in COS-7 cells was 0.25 μg per mg of total protein, assuming that the antibody cross-reacted equally with the monkey protein, and that in HeLa cells was 0.30 μg per mg of total protein.





D

Tissues

	1	2	3	4	5	6	7	8
A	whole brain	amygdala	caudate nucleus	cere-bellum	cerebral cortex	frontal lobe	hippo-campus	medulla oblongata
B	occipital lobe	putamen	substantia nigra	temporal lobe	thalamus	sub-thelamic nucleus	spinal cord	
C	heart	aorta	skeletal muscle	colon	bladder	uterus	prostate	stomach
D	testis	ovary	pancreas	pituitary gland	adrenal gland	thyroid gland	salivary gland	mammary gland
E	kidney	liver	small intestine	spleen	thymus	peripheral leukocyte	lymph node	bone marrow
F	appendix	lung	trachea	placenta				
G	fetal brain	fetal heart	fetal kidney	fetal liver	fetal spleen	fetal thymus	fetal lung	

Fig. 2. **Distribution of Tom20 and metaxin mRNAs in human tissues.** (A) Human multiple tissue Northern blot (2 μg of polyA$^+$ RNA) was probed with ^{32}P-labeled cDNAs for Tom20 or metaxin. The same blot was used for Tom20 and metaxin. (B) and (C) A human RNA master blot was probed with amounts of polyA$^+$ RNA samples that were normalized to the mRNA expression levels of seven different housekeeping genes. The same blot was used in B and C. (D) Tissue diagram of the human RNA master blot is shown.

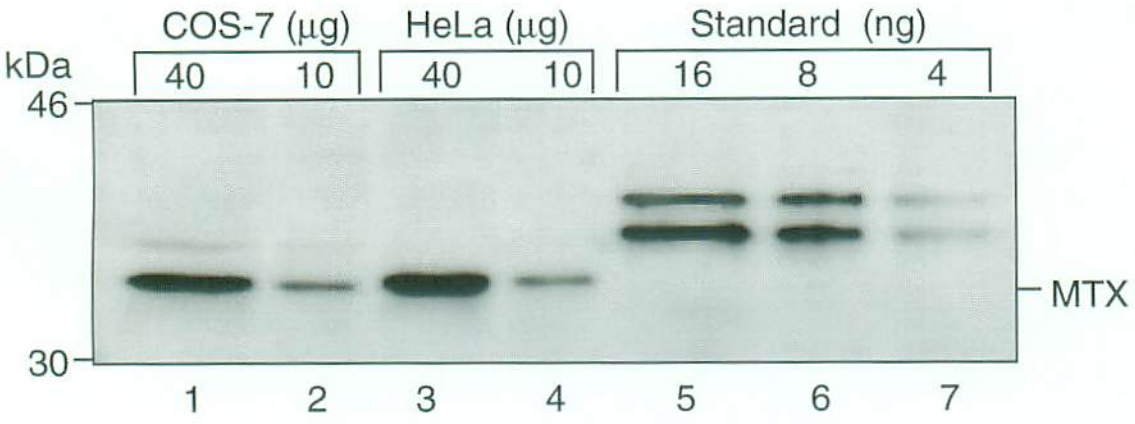


Fig. 3. **Content of metaxin in cultured cells.** 40 μg (lanes 1 and 3) or 10 μg of protein (lanes 2 and 4) from monkey COS-7 or human HeLa cell extracts were subjected to immunoblot analysis for metaxin using anti-human metaxin serum (1:1000 dilution) as a primary antibody. Purified ΔhMTX (lanes 5-7; 16, 8 and 4 ng) were used as standard.

7-3. Intracellular Localization of Metaxin

When COS-7 cells were fractionated into the soluble and membrane fractions, endogenous metaxin was recovered exclusively in the membrane fraction and was not extracted with 0.1 M Na_2CO_2 (Fig. 4A). When human metaxin cDNA was transfected in COS-7 cells, human metaxin was highly expressed (Fig. 4B). When the transfected COS-7 cells were fractionated, most metaxin (about 90%) was recovered in the membrane fraction and was not alkali-extractable. These results together with a previous report (Armstrong *et al.* 1997) show that exogenously-expressed human metaxin is integrated into the mitochondrial membrane, where the endogenous protein is localized.

7-4. Overexpression of Metaxin Resulted in Impaired Import of Mitochondrial Preproteins and Their Accumulation in Cultured Cells

Effects of overexpression of human metaxin on mitochondrial import and processing of preproteins in COS-7 cells were studied by immunoblot analysis (Fig. 5). When pOTC alone was expressed, it was imported and processed to the mature form almost completely and only a small amount of unprocessed pOTC was detected (Fig. 4A). When increasing amounts of metaxin was coexpressed, pOTC import was progressively inhibited and increasing amounts of pOTC accumulated. Similar results were obtained for another natural preprotein, pre-arginase II, and a chimeric preprotein pOTC-GFP (Fig. 5, B and C).

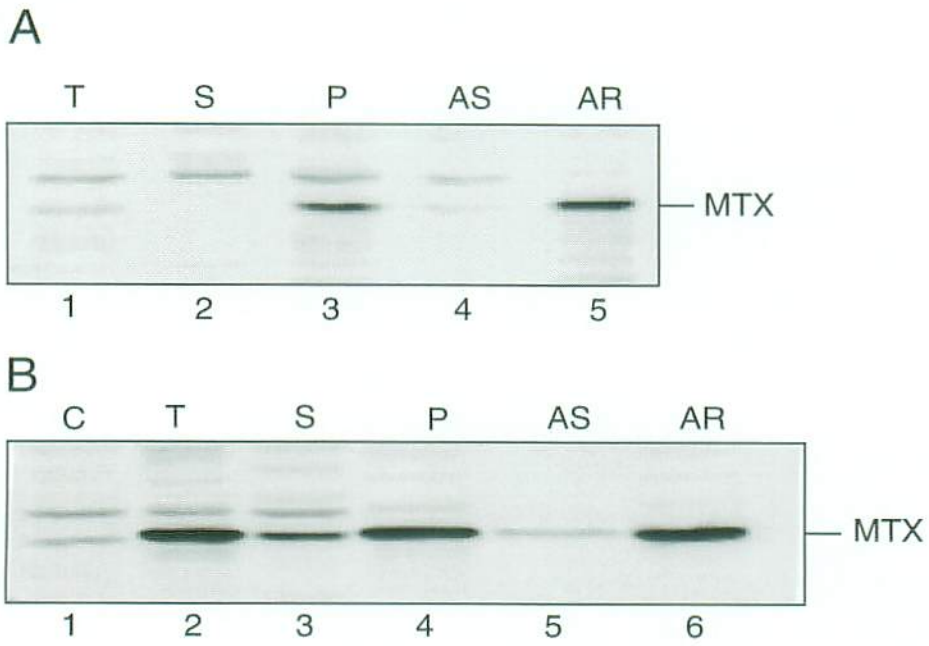
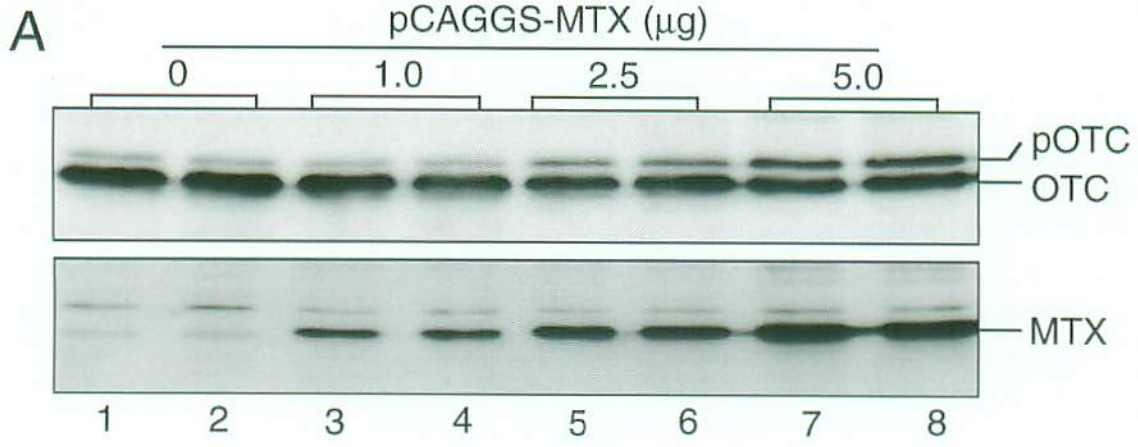


Fig. 4. **Subcellular localization of endogenous metaxin and expressed human metaxin.** COS-7 cells (A) or COS-7 cells transfected with pCAGGS-hMTX (B) were fractionated into the soluble (S) and membrane (P) fractions. Membrane fractions were further extracted with 0.1 M sodium carbonate and separated into alkali-soluble (AS) and alkali-resistant (AR) fractions. Whole cell extract (T) and fractionated samples were subjected to immunoblot analysis for metaxin using anti-human metaxin serum (1:1000 dilution) as primary antibody. Panel A: 30 μg of protein were applied in each lane. Panel B: Protein applied was 30 μg (lane 2), 24 μg (lane 3), 6 μg (lane 4), 2.4 μg (lane 5), and 3.6 μg (lane 6). Protein was distributed in these fractions at the same ratios. Lane 1, extract of non-transfected COS-7 cells (30 μg of protein).


A
pCAGGS-MTX (μg)
0
1.0
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pOTC
OTC
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2
3
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5
6
7
8


B
pCAGGS-MTX (μg)
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pAII
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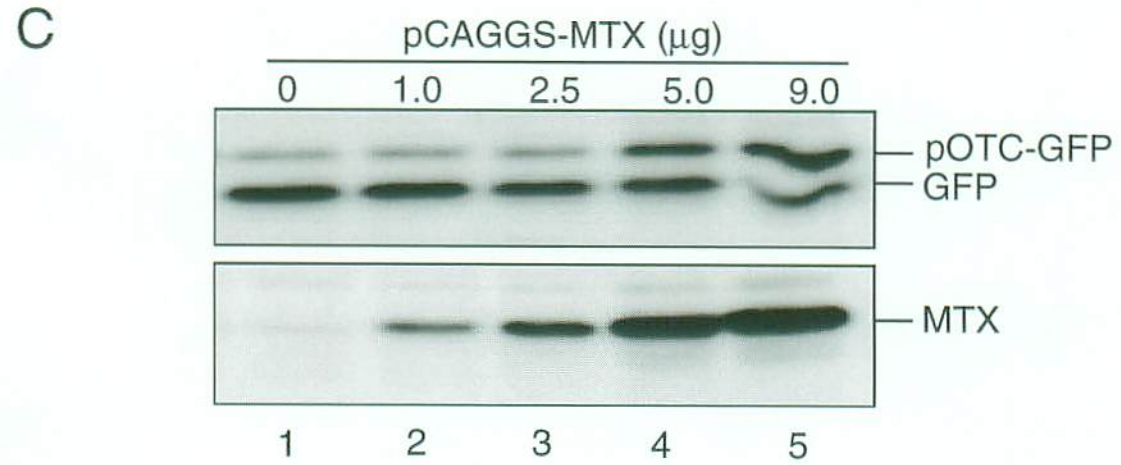
C
pCAGGS-MTX (μg)
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pOTC-GFP
GFP
MTX
1
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5

Fig. 5. **Effect of overexpression of hMTX on mitochondrial import of pOTC, pre-arginase II and pOTC-GFP in COS-7 cells.** COS-7 cells in 10-cm culture dishes were transfected with 5 μg of pCAGGS-pOTC (panel A), 1 μg of pCAGGS-hAII (panel B) or 1 μg of pCAGGS-pOTC-GFP (panel C) with indicated amounts of pCAGGS-hMTX. Total amount of plasmids was adjusted to 10 μg with pCAGGS. Twenty four hours after transfection whole cell extracts (5 μg of protein) were subjected to immunoblot analysis for metaxin (MTX), pOTC/OTC, pre-arginase II (pAII)/arginase II (AII) and pOTC-GFP/GFP by using antisera against metaxin, OTC, arginase II and GFP, respectively, as primary antibodies. All antisera were used at 1:1000 dilution.

7-5. Effect of Coexpression of Tom20 and Metaxin on Preprotein Processing

In order to gain insight into the relationship between Tom20 and metaxin, two outer membrane components, we examined the effect of coexpression of these two proteins on pOTC import (Fig. 6). Overexpression of Tom20 caused impaired import of pOTC and its accumulation, as we reported previously (Terada *et al.* 1997). Overexpression of metaxin also resulted in impaired import of pOTC (also see above). When Tom20 and metaxin were coexpressed, pOTC import was inhibited to an extent similar to that by Tom20 alone.

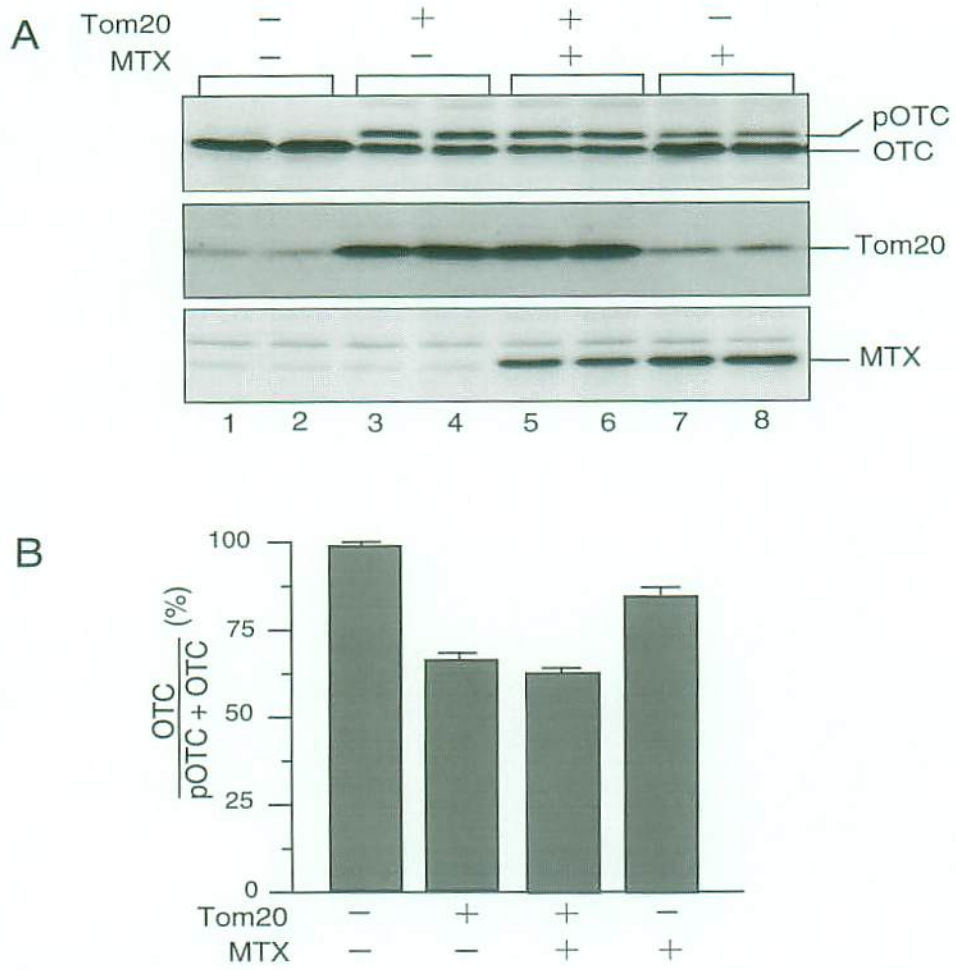

Fig. 6. **Effect of coexpression of human metaxin and hTom20 on pOTC import in COS-7 cells.** A: pCAGGS-pOTC (5 μg) was cotransfected with indicated combinations of pCAGGS-hMTX (5 μg), pCAGGS-hTom20 (5 μg) in COS-7 cells cultured on 10-cm dishes. The total amount of transfected plasmids was adjusted to 15 μg with pCAGGS. Whole cell extracts (5 μg of protein) were subjected to immunoblot analysis for metaxin (MTX), pOTC/OTC and Tom20 by using antisera against metaxin, OTC and Tom20, respectively. All antisera were used at 1:1000 dilution. B: The results in (A) were quantified and percent processing of pOTC is shown as means ± ranges (n = 2).

7-6. Effect of Overexpression of ΔhMTX on pOTC Processing

The cytosolic domain of human metaxin (ΔhMTX) was overexpressed and its effect on pOTC processing was studied. When COS-7 cells expressing ΔhMTX were fractionated into the soluble and membrane fractions, about 90% of ΔhMTX was recovered in the soluble fraction and the remaining 10% was recovered in the membrane fraction (Fig. 7). ΔhMTX associated with the membrane fraction was largely extracted with alkali.

When ΔhMTX was coexpressed with pOTC, the preprotein import was inhibited and its accumulation was evident (Fig. 8). However, the inhibition by overexpression of ΔhMTX was weaker than that by overexpression of full-length metaxin; despite the similar level of expression of full-length metaxin and that of the truncated metaxin.

7-7. Digitonin Concentration is Critical for Membrane Protein Extraction

To achieve the maximum solubilization of Tom proteins and other outer membrane proteins, COS-7 mitochondria were isolated and solubilized with different concentrations of digitonin (Fig. 9). We found that proteins are mostly extracted with 1% or 2% digitonin. Little extraction was seen with 0.1% digitonin and moderate extraction could be achieved with 0.5% digitonin. More than 90% extraction was achieved for Tom proteins with 1% or 2% digitonin. Therefore, in the further experiments (Blue native PAGE), COS-7 mitochondria were solubilized with 1% digitonin-containing buffer.

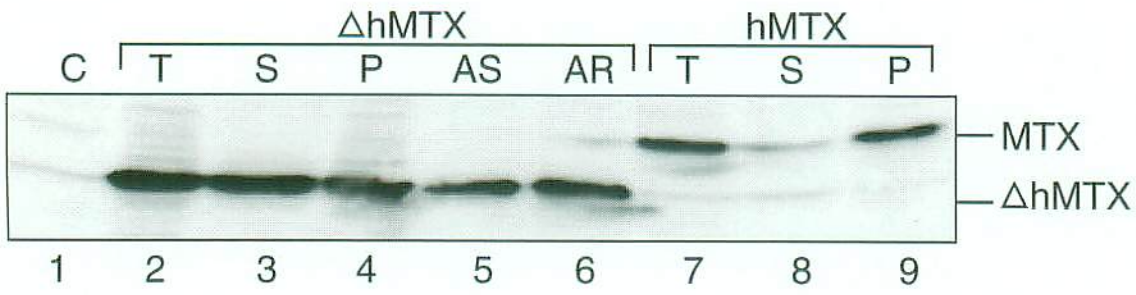

Fig. 7. **Subcellular localization of ΔhMTX.**

COS-7 cells in 10-cm culture dishes were transfected with 5 μg pCAGGS-hMTX or pCAGGS-ΔhMTX and total cell extracts (T) were fractionated into the soluble (S) and membrane (P) fractions. The membrane fraction was further extracted with 0.1 M sodium carbonate and separated into the alkali-soluble (AS) and alkali-resistant (AR) fractions. These fractions were subjected to immunoblot analysis using an antiserum against metaxin (MTX) (1:1000 dilution). Protein applied was 30 μg (lanes 2 and 7), 24 μg (lanes 3 and 8), 6 μg (lanes 4 and 9), 2.4 μg (lane 5) and 3.6 μg (lane 6). Protein was distributed in these fractions at the same ratios. Lane 1, total extract of non-transfected cells (30 μg of protein).

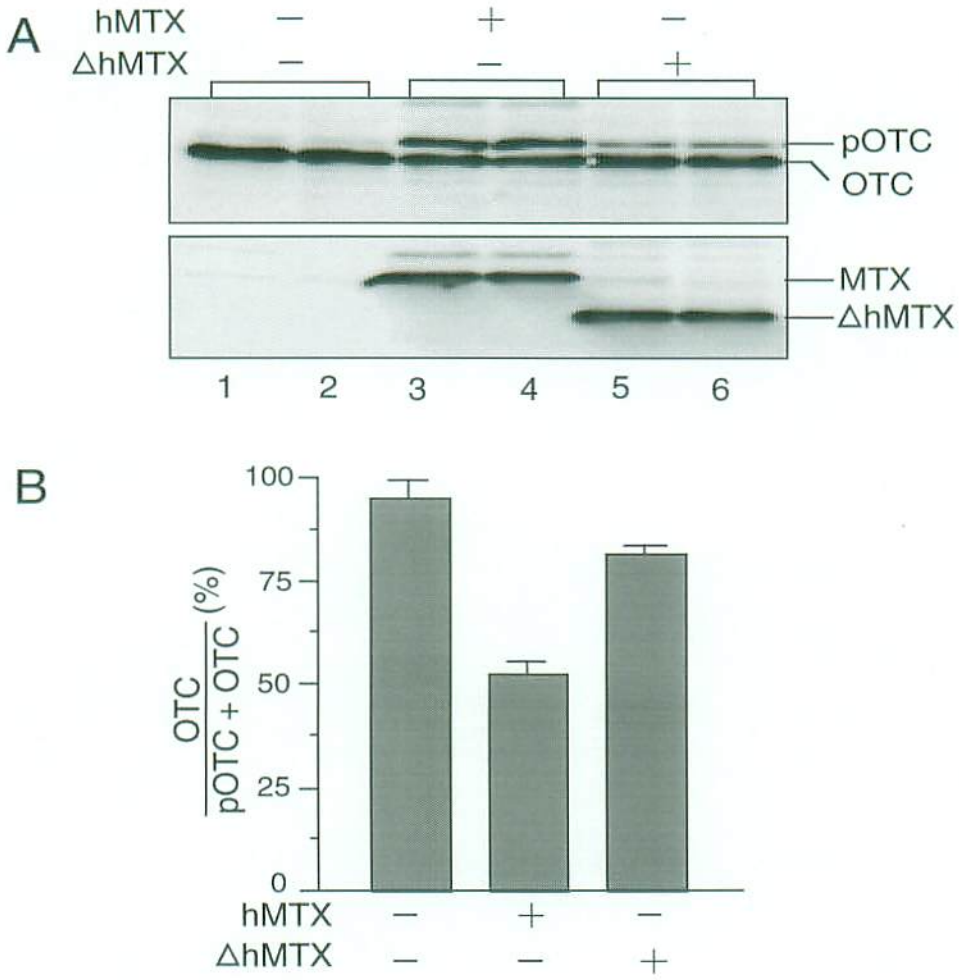

Fig. 8. **Effect of overexpression of ΔhMTX on pOTC processing in COS-7 cells.** A: 5 μg of pCAGGS-pOTC was cotransfected with 5 μg of pCAGGS-hMTX or pCAGGS-ΔhMTX in COS-7 cells cultured on 10-cm dishes. Total amount of transfected plasmids was adjusted to 15 μg with pCAGGS. Whole cell extracts (5 μg of protein) were subjected to immunoblot analysis using anti-OTC antiserum (1:1000 dilution) or anti-metaxin antiserum (1:1000 dilution). B: The results in (A) were quantified and percent processing is shown as means ± ranges (n = 2).

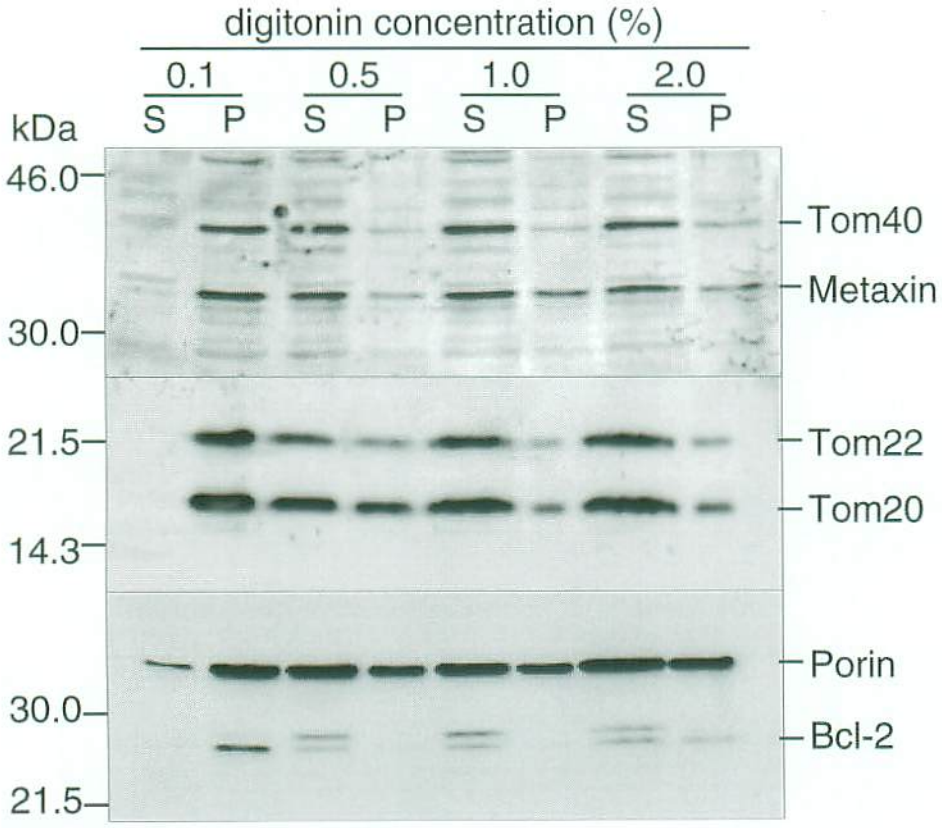

Fig. 9. **Effect of digitonin concentration on extraction of Tom proteins.** COS-7 mitochondrial pellet (50-100 μg of protein) was lysed in 50 μl of ice-cold digitonin buffer (0.1% digitonin for lanes 1 and 2, 0.5% digitonin for lanes 3 and 4, 1% digitonin for lanes 5 and 6, and 2% digitonin for lanes 7 and 8) and same portions of supernatant (S) and pellet (P) were loaded for immunoblot analysis using antisera against Tom40, Tom22, Tom20 and metaxin (all X 1000) and monoclonal anibodies against porin and Bcl-2.

7-8. Separation of the Tom Complex, Metaxin and Porin in Blue Native PAGE

COS-7 mitochondria were isolated, solubilized with digitonin, and subjected to blue native PAGE followed by second dimension SDS-PAGE. Tom40 and Tom22 migrated as a large complex of ~400 kDa. Tom20 migrated partly as a complex of ~400 kDa and partly in smaller forms of ~40 kDa and ~100 kDa with a smear up to 200 kDa (Fig. 10). On the other hand, metaxin was found solely in a form of ~50 kDa. Thus, metaxin is apparently not associated with the Tom complex, and appears to exist in the mitochondrial outer membrane as a monomer or dimer. Porin also migrated as a complex of ~400K. It could be a part of the Tom complex or a different complex.

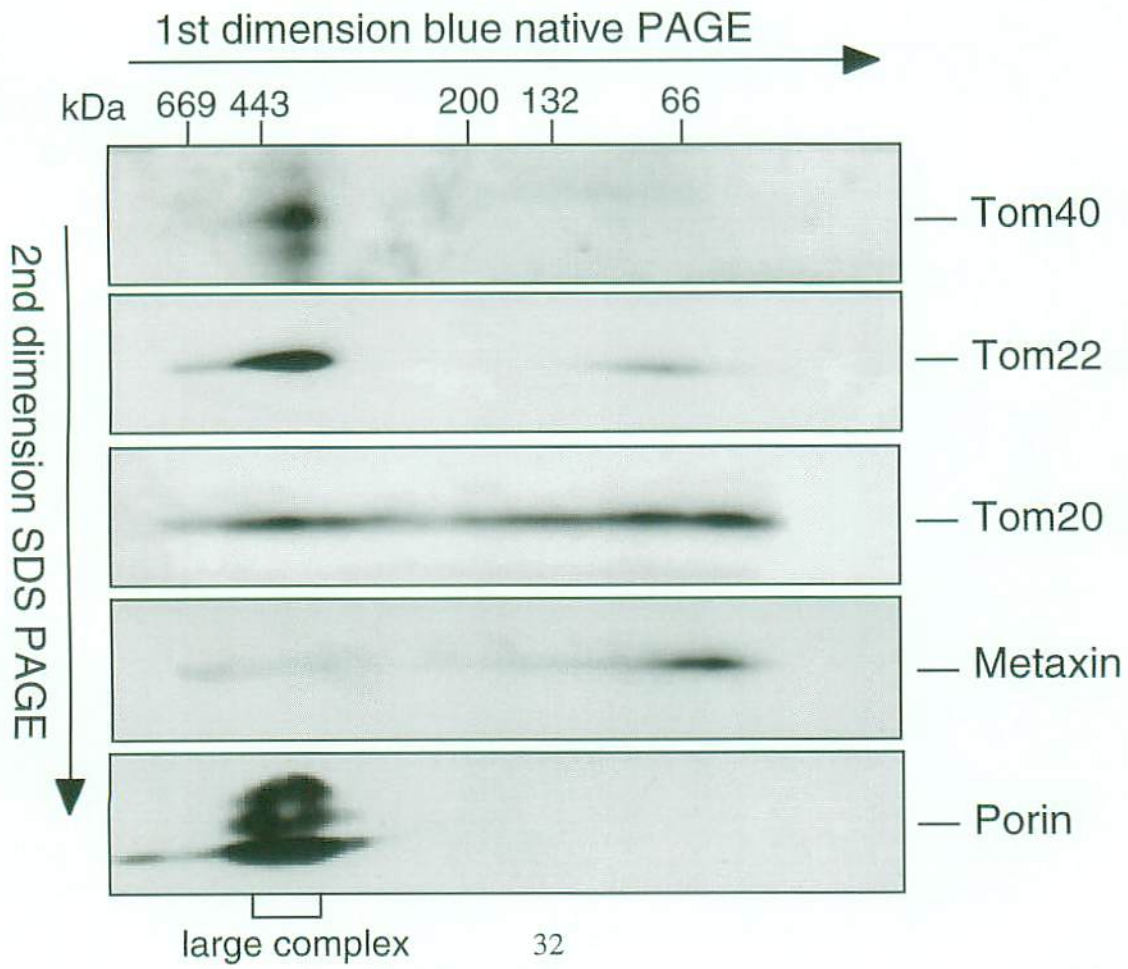

Fig. 10. **Blue native PAGE of the Tom components, metaxin and porin.** Isolated COS-7 mitochondria (50-100 μg of protein) were lysed in digitonin buffer and subjected to blue native PAGE in the first dimension and SDS-PAGE in the second dimension as described in Materials and Methods. After electrophoresis, proteins were blotted and then immunodecorated with antisera (1:1000 dilution) against Tom40, Tom22, Tom20 or metaxin and monoclonal antibody against porin. The position of large complex (~400 kDa) is indicated. Molecular size markers in blue native PAGE are thyroglobulin (669 kDa), apoferritin (443 kDa), β-amylase (200 kDa) and albumin (132 and 66 kDa). Estimated molecular sizes of Toms and metaxin in blue native PAGE in the text are approximate.

7–9. Separation of Bcl-2 in Blue Native PAGE

Bcl-2 is an antiapoptotic protein which is located in mitochondrial outer membrane. Mitochondria from COS-7 cells and those from Bcl-2-overexpressed COS-7 cells were isolated, solubilized, and subjected to blue native PAGE followed by SDS-PAGE (Fig. 11, A and B). Endogenous Bcl-2 migrated as a monomer or dimer in blue native PAGE (Fig. 11A). Bcl-2 overexpression was confirmed by SDS-PAGE (left lanes of Fig. 11), and overexpressed Bcl-2 also migrated as a monomer or dimer in blue native PAGE. These results indicate that Bcl-2 is not involved in the Tom complex.

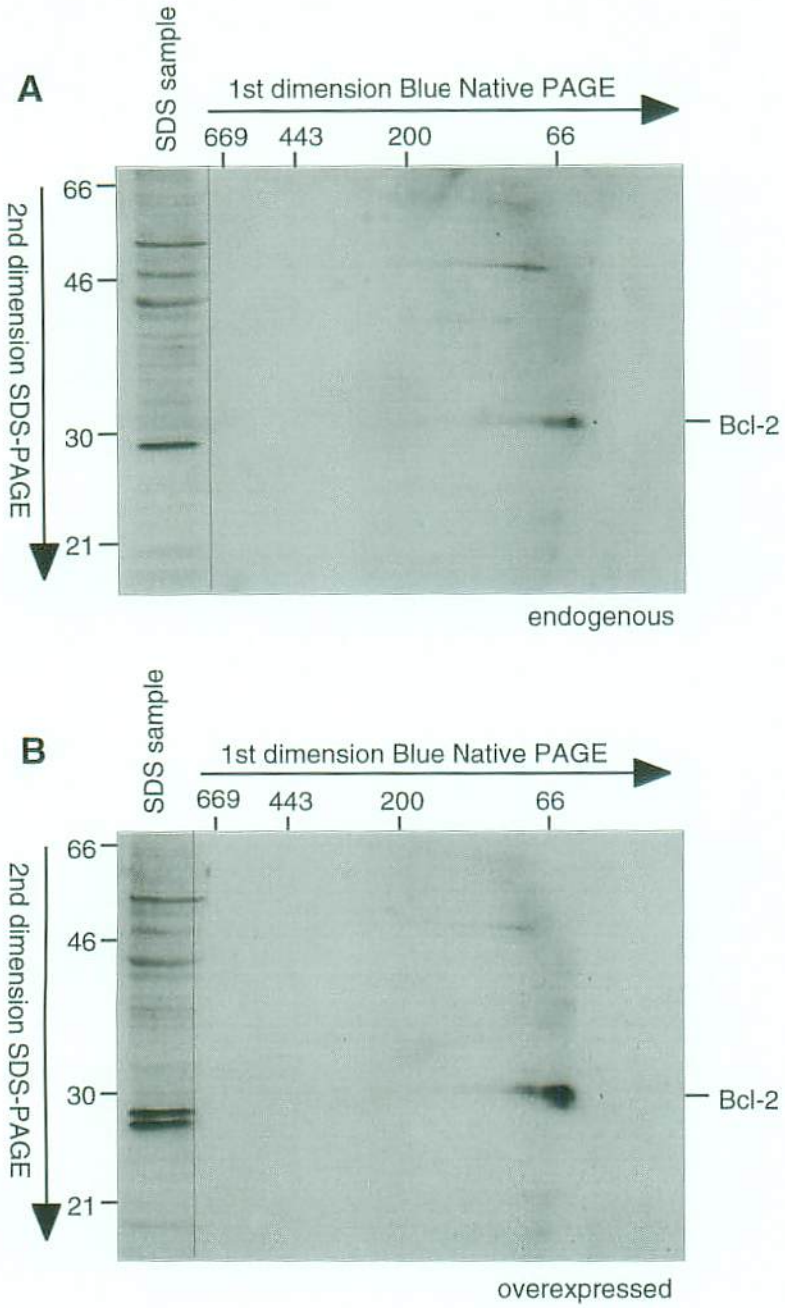

Fig. 11. **Blue native PAGE for endogenous and overexpressed Bcl-2.** Normal COS-7 mitochondria (A) or Bcl-2-overexpressed COS-7 mitochondria (B), each 50-100 μg

protein, were lysed in 1% digitonin buffer. Blue native PAGE was run in the 1st dimension and SDS-PAGE in the 2nd dimension. After electrophoresis proteins were blotted and immunodecorated with a monoclonal antibody against Bcl-2.

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8. DISCUSSION

The outer mitochondrial membrane contains components of the translocation machinery for the import of proteins. These consist of receptor components, which ensure the fidelity of protein import and restrict import to proteins with appropriate targeting information, and components comprising the general insertion pore that allows access to the inside of the mitochondrion (Neupert *et al*, 1997). Recently, Kunkele *et al* (1998) reconstituted the Tom components of *Neurospora* into liposomes and showed that this complex forms a cation-selective, high conductance ion channel that can translocate preproteins across the lipid bilayer. This functional complex consists of Tom70, Tom40, Tom22, and Tom20 in the ratio of 1.5:8:3.1:2, and the small Toms 6 and 7. Tom7 and Tom6 are required to promote the association of the functional complex (Dekker *et al*, 1998). The Tom complex from *S. cerevisiae* also contains Tom5 (Dietmeier *et al*, 1997), which functionally involved in preprotein transfer from receptors to Tom40.

The only essential components of the translocase in fungi are Tom40 and Tom22 (for reviews, see Ryan *et al*, 2000), raising the question as to the specific roles of each subunit in the complex. Tom40 is tightly associated with receptor protein Tom22 along with Tom7, Tom6 and Tom5. The receptor proteins Tom20 and Tom70 associate with this complex in a weaker manner where they are involved in the initial recognition of preproteins. Additionally, the finding of an apparently novel component metaxin (Bornstein *et al*, 1995), with no obvious homolog in fungi, raises further question about the role of this component in mammalian cells. Metaxin contains a putative

mitochondrial outer membrane signal anchor domain at its C-terminus, and a truncated form of metaxin lacking this signal anchor domain had a reduced association with mitochondria. In addition, metaxin was highly susceptible to proteases in intact mitochondria. Therefore, metaxin is a mitochondrial protein that extends into the cytosol while anchored into the outer membrane at its C-terminus. In its N-terminal region, metaxin shows significant sequence identity to Tom37, a component of the outer membrane portion of the mitochondrial preprotein translocation apparatus in *S. cerevisiae*, but important structural differences, including apparently different mechanisms of targeting to membranes, also exist between the two proteins. Antibodies against metaxin inhibited the import of pre-adrenodoxin into isolated mitochondria (Armstrong *et al*, 1997). The early embryonic lethal phenotype of mice lacking metaxin suggests that efficient import of proteins into mitochondria is crucial for cellular survival. In the present study, I showed that overexpression of human metaxin in COS-7 cells results in inhibition of mitochondrial import and processing of preproteins including pOTC, pre-arginase II and a fusion preprotein pOTC-GFP, and in their accumulation in the cell. The cytosolic domain of human metaxin had similar but less marked effects. As shown previously, overexpression of Tom20 in COS-7 cells also inhibited mitochondrial import of pOTC, whereas the cytosolic domain of Tom20 had little effect (Terada *et al*, 1997). The effect of Tom20 overexpression on pOTC-GFP import is complicated. It inhibits the import when pOTC-GFP is expressed moderately, whereas it stimulated the import when the preprotein was expressed very strongly. Among the other mammalian transport factors, overexpression of Tom34 stimulated the

import of pOTC (Chewawiwat *et al*, 1999), and that of HSDJ (hdj-2/dj2), a cytosolic DnaJ homologue which participates in mitochondrial protein import, inhibited the pOTC import (Kanazawa *et al*, 1997). Overexpression of an unrelated cytosolic protein, phenylalanine hydroxylase, had no effect (Kanazawa *et al*, 1997). I speculate that if a factor is limiting the preprotein import, its overexpression may result in stimulation of the import. In contrast, when a factor is not limiting the import, its overexpression may result in inhibition due to an imbalance of the import factors or disruption of complexes containing these factors which are required in the import pathway.

The functional role of metaxin in mitochondrial protein import is enigmatic. Metaxin is expressed ubiquitously, but its tissue distribution differs from that of Tom20, which is a core component of the transport machinery. The preprotein translocase of the outer mitochondrial membrane is a multisubunit machinery containing receptors and a general import pore (GIP). Blue native PAGE analysis showed that, the yeast receptor Tom22 stably associates with Tom40, the main component of the GIP, in a complex with a molecular weight of ~400 kDa, while the other receptors, Tom20 and Tom70 are more loosely associated with this GIP complex and can be found in distinct subcomplexes (Dekker *et al*, 1998). Tom20 exists mainly in a dissociated form of about 40 to 100 kDa and partly with the 400 kDa complex. A yeast mutant lacking both Tom20 and Tom70 can still form the GIP complex when sufficient amounts of Tom22 are synthesized, suggesting that Tom20 and Tom70 are not essential for the generation of the GIP complex. Here, I performed blue native PAGE of mammalian Tom components and showed that Tom40 and Tom22 migrate as a complex of ~400 kDa, like fungal Toms.

Tom20 was partly associated with this Tom complex and migrated partly in smaller dissociated forms. These results show that the mammalian Tom complex is similar in structure to the yeast complex. On the other hand, metaxin migrated separately in a form of ~50 kDa. These results indicate that metaxin is not a component of the Tom complex. The fact that the cytosolic domain of metaxin inhibits the preprotein import, may suggest that it binds with the preprotein and inhibits the productive transport.

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Part-2

Characterization and Functional Analysis of a Novel Hsp70 Cochaperone dj4

10. INTRODUCTION

Several physical and chemical conditions favor inappropriate folding of proteins and are thus hazardous to cells. Among these proteotoxic conditions are elevated temperature, anoxia and exposure to ethanol, heavy metals, or other chemical denaturants. It has been recognized for >30 years that mild temperature elevation can induce a so-called heat shock response in all cells (reviewed by Lindquist and Craig, 1988). This response is characterized by a rapid shutdown of the synthesis of most proteins, with a dramatic transient increase in the synthesis of a small set of proteins, accordingly called heat shock proteins (Hsps). A similar response is observed after other prototoxic insults, so the more general terms stress response and stress proteins are sometimes used. For years the function of Hsps was unknown, but studies in the past 15 years have defined the major Hsps as central components of the molecular chaperone/protein folding machinery. Thus stress responses are evolutionary adaptations to quickly resolve misfolded proteins and restore the normal protein-folding environment of cells. In addition, an initial sublethal exposure to heat or other stresses can conditions cells for enhanced survival during exposure to subsequent, even more severe, stresses (Gerner and Schneider, 1975); this is commonly termed thermotolerance and is largely attributable to induction of Hsps (reviewed by Parsell *et al*, 1993).

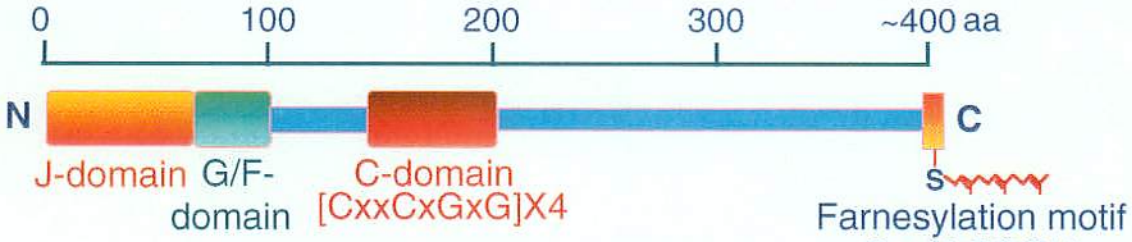
Many Hsps and their associated cochaperones are constitutively expressed in all cells. There are several multigene Hsp families, and individual genes within families differ to varying degrees with respect to sequence and expression patternes, as well as function

and subcellular localization of the respective gene products. Major Hsp families named to reflect the approximate molecular size (in kilodaltons) of family members, are Hsp100, Hsp90, Hsp70, Hsp60, Hsp40 and the small Hsp family (typically 20 to 25 kDa). Hsp70, Hsp60 and Hsp40 family members play important roles in nascent chain folding (Hartl, 1996). Hsp70 members are also major components in membrane translocation processes. The small Hsps have important functions in disaggregation or degradation of misfolded complexes (Gething, 1997), but it is not clear how important they are for nascent chain folding. Hsp90 also may have a role in nascent chain folding, but it is most notable for its numerous associations with important regulatory proteins (Pratt and Toft, 1997). Activities of Hsp70 family members are regulated by partner chaperones including DnaJ/Hsp40 cochaperone.

10–1. DnaJ/Hsp40 Cochaperone

Molecular chaperones of the DnaJ/Hsp40 family are found ubiquitously in prokaryotic and eukaryotic cells and play diverse roles in many cellular processes such as translation, translocation and protein folding (Cyr *et al*, 1994; Kelley *et al*, 1998). In addition to its intrinsic chaperone activity, DnaJ is able to interact directly with and stimulate the ATPase activity of the *Escherichia coli* Hsp70 homolog DnaK (Langer *et al*, 1992; Szabo *et al*, 1994). DnaJ thus cooperates with DnaK in protein folding and also in mediating assembly of macromolecule complexes. Nearly 30 DnaJ homologs have been identified in mammals (Ohtsuka and Hata, 2000). DnaJ homologs are highly diverse at the primary sequence level but tend to share various combinations of at least

three domains (Cyr *et al*, 1994). These three domains appear to correlate with various biochemical functions of the protein (Szabo *et al*, 1996). The most highly conserved domain, the J-domain of approximately 70 amino acid residues that is often found near the amino terminus and interacts with Hsp70 members. The other two domains, a glycine and phenylalanine-rich region (G/F) domain possibly acting as a flexible linker, and a cysteine-rich region (C-domain) containing 4 [CxxCxGxG] motifs resembling a zinc-finger domain (Bork *et al* 1992, Szabo *et al*, 1996) (Fig. 1). Major DnaJ homologs



Types	Domains		
	J	G	C
Type I	+	+	+
Type II	+	+	−
Type III	+	−	−

Fig. 1. **Structure of DnaJs.** Major mammalian DnaJ homologs possess at least three distinct domains; N-terminal J-domain of ~70 amino acid residues, an adjacent G/F-domain that is rich in glycine and phenylalanine residues and a cysteine-rich C-domain which contain 4[CxxCxGxG] motifs resembling a zinc-finger domain. Some mammalian DnaJ homologs have the "CaaX" farnesylation motif at their C-termini. J, J-domain; G, G/F-domain and C, C-domain.

in mammalian cytosol are dj1 (Hsp40/hdj-1/DjB1) (Raabe and Manley, 1991; Ohtsuka *et al*, 1993), dj2 (HSDJ/hdj-2/rdj-1/DjA1) (Chellaia *et al*, 1993; Oh *et al*, 1993), dj3 (cpr3/DNAJ3/rdj2/DjA2) (Andres *et al*, 1997) and recently identified dj4 (DjA4) (Hata and Ohtsuka, 2000). These members of the DnaJ family are classified into three groups according to their domain structures (Cheetham and Caplan, 1998; Ohtsuka and Hata 2000). Type I members have all three domains (J-, G/F- and C-), type II members have J- and G/F-domain but no C-domain, and type III members have J-domain alone. Type I contains four members, three in the cytosol and one in the mitochondria (Ohtsuka and Hata, 2000). dj2, dj3 and dj4 correspond to the three cytosolic members and have the "CAAX" prenylation motif at their COOH termini. In fact, dj2 and dj3 have been shown to be farnesylated (Andres *et al*, 1997; Kanazawa *et al*, 1997). On the other hand, dj1 belongs to type II and has no farnesylation motif. Previously, we found that dj2 and dj3, not dj1, in combination with Hsc70, facilitate mitochondrial protein import and luciferase refolding (Terada *et al*, 1997; Terada and Mori, 2000).

10–2. Hsp70 Chaperone Family

Members of the Hsp70 family are the most widely studied and abundant group in eukaryotic cells. In the cytosol, Hsp70 binds to nascent polypeptides before their release from the ribosome (Beckmann *et al*, 1990). All members of the Hsp70 chaperone class possess two distinct domains: a highly conserved N-terminal ATPase domain which binds ADP and ATP very tightly (in the presence of Mg^+ and K^+) and hydrolyzes ATP, and a more divergent C-terminal domain, which binds short hydrophobic peptides of the

target substrates (Flynn *et al*, 1991). Cooperation of both domains is needed for protein folding.

The Hsp70 family is very large, with most organisms having multiple members; most eukaryotes have at least a dozen or more different Hsp70, found in a variety of cellular compartments. Some of the better known mammalian members are Hsc70 (or Hsp73), the constitutive cytosolic member; Hsp70 (or Hsp72), the stress-induced cytosolic form; BiP (or Grp78), the ER form; and mHsp70 (or mito-Hsp70, or Grp75), the mitochondrial form. In yeast the homologs of Hsp70 and BiP are known as Ssa1-4, Ssb1-2 in cytosol, Ssc in mitochondria and Kar2 in ER. In *E. coli*, the major form of Hsp70 is DnaK.

The Hsp70 preferentially bind unfolded or partially folded proteins and do not bind normal native proteins. It is likely that only some newly synthesized proteins require the assistance of chaperones. In coimmunoprecipitation studies with anti-Hsp70 antibodies and pulse-chase labeling, it was observed that smaller proteins were disproportionately absent, suggesting that they may fold more rapidly, either with or without the assistance of Hsp70 (Beckmann *et al*, 1990).

10–3. Chaperones and Thermotolerance

The induction of Hsps has been shown to coincide with the development of thermotolerance. This refers to the adaptive response of cells expose to elevated temperatures. All conditions or agents capable of inducing Hsps do so by activating HSF (Morimoto *et al*, 1997). Upon activation, HSF translocate into the nucleus where

they bind to the heat shock responsive elements (HSE) and activate the transcription by *hsp* genes (for review see Wu *et al*. 1995). The induction of Hsps in response to stress and the subsequent thermotolerance is transient. When the stress element is removed, these cells continue to function normally and the level of Hsps drop back to basal levels with time (Samali and Orrenius, 1998).

Cells expose to nonlethal, elevated temperatures become transiently resistant to subsequent heat shock. Thermotolerance development is paralleled by expression of heat shock proteins, which include members of the Hsp70 family (Li *et al*, 1992). Hsp70 family are found to contribute to the heat protection of several substrate proteins. Hsp70 alone can protect topoisomerase I and DNA polymerase against heat inactivation and facilitate the reactivation of heat-inactivated topoisomerase I in a concentration dependent manner (Ciavarra *et al*, 1994; Ziemienowicz *et al*, 1995). For reactivation of DNA polymerase, ATP is required (Ziemienowicz *et al*. 1995). For the recovery of heat-denatured firefly luciferase and chemically denatured β-galactosidase, Hsp70 alone is not sufficient. Addition of DnaJ homologs plus ATP is required. DnaJ homolog is believed to stabilize binding of Hsp70 to the substrate by stimulating hydrolysis of Hsp70-bound ATP (Minami *et al*. 1996). Subsequent incubation at normal temperature leads to spontaneous refolding of β-galactosidase (Freeman and Morimoto, 1996), while refolding of luciferase needs additional factors that are present in reticulocyte lysate (Minami *et al*, 1996).

In vivo, a chaperone function of Hsp70 is suggested from studies on heat effects on endogenous and exogenous proteins. Protection against heat-induced nuclear protein

aggregation in thermotolerant HeLa S3 cells correlates with the expression level of Hsp70 (Stege *et al.* 1995). Overexpression of transfected Hsp70 in Rat-1 cells protects against nuclear protein aggregation, independent of ATP binding domain (Stege *et al.* 1994). Little is known about the specific contribution of Hsp70 and its chaperone activity to thermotolerance development. Constitutive overexpression of Hsp70 has been shown to increase heat resistance, and the level of this resistance correlates to the level of Hsp70 expression in individual clonal cell lines (Li *et al.* 1992). Furthermore, constitutive overexpression of Hsp70 was shown to reduce the rate of *Drosophila* cell growth (Feder *et al.* 1992). This might directly lead to an altered response to heat and therefore influence clonal thermoresistance.

In the present study, we investigated properties of newly found dj4 and its function in comparison with those of dj1, dj2 and dj3, including tissue distribution, intracellular localization and heat inducibility. dj4 was increased when H9c2 heart muscle cells were differentiated. Overexpression of Hsp70 protected H9c2 cells from severe heat shock-induced cell death, and coexpression of dj4 or dj2 with Hsp70 gave better protection.

11. MATERIALS AND METHODS

11–1. Plasmid Construction

Mouse dj4 cDNA (GenBank, accession number AB032401) was amplified by polymerase chain reaction (PCR) using mouse heart cDNA and cloned into the *Hin*cII site of pGEM-3zf(+) (Promega, Madison, WI, USA) to generate pGEM-mdj4. The PCR oligonucleotides were 5'-AGAGGAGCAGACTTCAGAAG-3' (sense) and 5'-CATTCATCATGTACTAGAGTCC-3' (antisense), giving a fragment of 1275 base pairs. The nucleotide sequence was verified by sequencing. A second PCR was done to construct the expression plasmid using 5'-ATGGTGAAGGAGACCCAGTACTATG-3' (sense) and 5'-GTCATAGCTGTTTCCTG-3' (antisense) as primers and pGEM-mdj4 as template. A PCR product of 1321 base pairs containing full length dj4 cDNA was directly inserted into the *Bam*HI site of pQE32 (Qiagen, Chatworth, CA, USA) to generate an expression plasmid pQE-mdj4. pCAGGS-dj4, a mammalian expression plasmid for mouse dj4, was constructed by inserting the full-length cDNA fragment for mouse dj4 used above into the *Xho*I site of the plasmid pCAGGS (Niwa *et al*, 1991). pCAGGS-Hsp70 (Gotoh *et al*, 2001) and pCAGGS-dj2 (Kanazawa *et al*, 1997), human Hsp70 and human dj2 expression plasmids were described. cDNA for rat dj1 was isolated by PCR using mRNA from lung of a male Wistar rat. PCR was carried out using dj1 primers corresponding to nucleotides 226-775 (GenBank, accession number AB028272, mouse). The PCR product was inserted into the *Hin*cII site of pGEM-3zf(+), yielding pGEM-rdj1. cDNA for rat dj2 was isolated by PCR using mRNA from rat liver.

PCR was carried out using dj2 primers corresponding to nucleotides 390-989 (GenBank, accession number U53922). The PCR product was inserted into the *Hin*cII site of pGEM-3zf(+), yielding pGEM-rdj2. cDNA for rat dj3 was isolated by PCR using mRNA from rat liver. PCR was carried out using dj3 primers corresponding to nucleotides 336-991 (GenBank, accession number U95727). The PCR product was inserted into the *Hin*cII site of pGEM-3zf(+), yielding pGEM-rdj3. cDNA for rat Hsc70 was isolated by PCR using mRNA from rat liver. PCR was carried out using Hsc70 primers corresponding to nucleotides 1630-1979 (GenBank, accession number M19141). The PCR product was inserted into the *Hin*cII site of pGEM-3zf(+), yielding pGEM-rHsc70. cDNA for rat Hsp70 was isolated by PCR using mRNA from rat liver. PCR was carried out using Hsp70 primers corresponding to nucleotides 2894-3911 (GenBank, accession number X74271). The PCR product was inserted into the *Bam*HI site of pGEM-3zf(+), yielding pGEM-rHsp70.

11–2. Protein Purification and Antibody Production

The recombinant plasmid pQE-mdj4 was transformed into *Escherichia coli* M15[pREP4] (Qiagen, Chatworth, CA, USA) and hexahistidine-tagged mdj4 (H_6mdj4) was induced with 1 mM isopropyl-1-thio-β-*D*-galactopyranoside. H_6mdj4 was recovered in inclusion body and purified by Ni^{2+}-NTA-Sepharose (Amersham Pharmacia Biotech, Buckinghamshire, UK) column chromatography under denaturing conditions. Briefly, insoluble H_6mdj4 protein was solubilized in buffer A (20 mM Tris-HCl, pH 7.9, 0.5 M NaCl, 6 M guanidine hydrochloride) containing 5 mM imidazole and subjected to a

Ni^{2+}-NTA-Sepharose column. The column was washed with buffer A containing 30 mM imidazole, and then H_6mdj4 was eluted with buffer A containing 1 M imidazole. The eluted protein was dialyzed against 50 mM Tris-HCl, pH 7.9 containing 0.75 M NaCl and was further purified by using the same column under native conditions. Finally, the eluate was again dialyzed and concentrated with Centricon-30 (Amicon, Beverly, MA, USA).

11–3. Antibodies

Rabbit antisera against human dj1(Terada *et al.* 1997), human dj2 (Kanazawa *et al.* 1997) and gelly fish green fluorescent protein (GFP) (Yano *et al.* 1997), and rat 1B5 monoclonal antibody specific to Hsc70 (Terada *et al*, 1995) were used. Anti-mouse dj4 antiserum was raised in a rabbit by injecting the purified dj4. Other antibodies were obtained from the following sources; monoclonal antibodies against Hsp70 (C92), Hsp60 and BiP/Grp78 from StressGen Biotechnologies Corp (Victoria, Canada), monoclonal antibody against glyceraldehyde 3-phosphate dehydrogenase (GAPDH) from Chemicon International (Temecula, CA, USA), monoclonal antibody against nucleoporin from Transduction Laboratories (Lexington, KY, USA), monoclonal antibody against dj2 from NeoMarkers (Fremont, CA, USA), Cy3-labeled goat anti-rabbit IgG from Amersham Pharmacia Biotech, and Alexa fluor 488-labeled goat anti-rat IgG from Molecular Probes (Eugene, OR, USA).

11–4. Cell Culture

H9c2 cells, a clonal line derived from rat heart (ATCC CRL-1446: ATCC, Rockville, MD. USA), were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 25 mM glucose, 44 mM $NaHCO_3$, 4 mM L-glutamine and 1 mM sodium pyruvate, supplemented with 10% fetal calf serum at 37°C under an atmosphere of 5% CO_2 and 95% air to 70-80% confluency. Medium was changed every 3 days. For heat shock treatment, culture dishes were sealed with parafilm and floated on a water bath at 43°C for 30 min. The cells were then returned to a CO_2 incubator at 37°C and recovered for various time periods. Severe heat treatment was done by transferring cells to DMEM containing 20 mM HEPES-NaOH, pH 7.4, preheated to 47°C. Sealed dishes were transferred to a water bath at 47°C and maintained for 90 min or 120 min. To promote differentiation of H9c2 cells, serum concentration was reduced to 1% and cells were cultured up to day 28 (Kimes and Brandt, 1976).

11–5. RNA Blot Analysis

Total RNA was isolated from mouse tissues and cultured H9c2 cells using the acid guanidium thiocyanate-phenol-chloroform extraction procedure (Chomczynski and Sacchi, 1987). RNA (2 μg per lane) was elctrophoresed in denaturing formaldehyde-agarose (1%) gels and was blotted onto nylon membranes (Schleicher & Schuell, Dassel, Germany). Hybridization was performed with the digoxigenin-labeled antisense RNA probes, synthesized from cDNAs under the control of T7 or SP6 promoter, using a transcription kit (Roche Diagnostic GmbH, Mannheim, Germany). cDNAs for the

following proteins were used for synthesis of probes: pGEM-mdj4 for mouse dj4, pGEM-rdj1 for rat dj1, pGEM-rdj2 for rat dj2, pGEM-rdj3 for rat dj3, pGEM-rHsc70 for rat Hsc70 and pGEM-rHsp70 for rat Hsp70. Chemiluminescence signals derived from hybridized probes were detected using a DIG luminescence detection kit (Roche Diagnostic GmbH) and the intensity of chemiluminescence was quantitated using a LAS1000plus chemiluminescence imager (Fuji Photo Film Co., Ltd., Tokyo).

11–6. Immunoblot Analysis

Mouse tissues were homogenized in 9 vol. of 20 mM HEPES-KOH, pH 7.4, containing 0.5% Triton X-100, 20% glycerol, 1 mM dithiothreitol, 50 μM antipain, 50 μM leupeptin, 50 μM chymostatin, and 50 μM pepstatin. The homogenates were centrifuged at 25,000 x*g* for 30 min at 4°C, and the supernatants were used as tissue extracts. H9c2 cells were trypsinized, washed twice with cold phosphate buffered saline (PBS) and homogenized in 10 mM Tris-HCl, pH 7.4, containing 5 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 5 μM antipain, 5 μM leupeptin, 5 μM chymostatin, and 5 μM pepstatin. After sonication and centrifugation at 15,000 x*g* for 10 min, the supernatant served as cell extracts. The tissue or cell extracts were subjected to SDS-10% polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes using a semi-dry transfer apparatus (Bio-Rad Laboratories, Hercules, CA, USA). Immunodetection was performed by using an ECL detection kit (Amersham Pharmacia Biotech). Intensity of chemiluminescence was quantitated using a LAS1000plus chemiluminescence imager.

11–7. Heart Fractionation

Fractionation procedure of mouse heart was performed essentially as described (Green *et al.* 1948: Schneider *et al.* 1948). Briefly, mouse heart was homogenized in 9 vol. of homogenization buffer (0.25 M sucrose containing 50 mM HEPES-KOH, pH 7.4) using a glass-Teflon homogenizer (5-6 strokes). The homogenate was centrifuged at 600 x*g* for 10 min at 4°C, and the precipitate was washed three times with 0.25 M sucrose to obtain the nuclear fraction. The supernatant was centrifuged at 5,000 x*g* for 15 min at 4°C, and the precipitate was washed three times with 0.25 M sucrose to obtain the mitochondrial fraction. The second supernatant was then centrifuged at 100,000 x*g* for 1 h at 4°C, and the supernatant and the precipitate were used as the cytosolic and microsomal fractions.

11–8. Immunofluorescent Staining

H9c2 cells were grown on coverslips. After appropriate treatment, cells were kept on ice, washed three times with PBS and twice with acetone-methanol (1 : 1) and fixed in acetone-methanol (1 : 1) for 3 min at -20°C. Fixed cells were blocked with 10% fetal calf serum in PBS for 1 h at 4°C. After washing three times with PBS, cells were incubated with primary antibodies for 1 h at 4°C. After washing four times with PBS, cells were reincubated with fluorescent-conjugated secondary antibodies for 1 h at 4°C. Finally, cells were washed four times with PBS. Immunostained cells were immediately analyzed using an Olympus BX50 fluorescent microscope equipped with appropriate filters, and images were taken using a C5810 color-chilled 3CCD video camera system

(Hamamatsu Photonics, Hamamatsu).

Immunodetection of dj4 in mouse heart was performed essentially as described (Koshiyama *et al.* 2000). Briefly, mouse was deeply anesthesized and perfused with ice-cold PBS followed by 4% paraformaldehyde. Heart was desected, soaked in ice-cold PBS for a few min and in 4% paraformaldehyde for 4 h. Then the tissue was kept overnight in PBS at 4°C. Tissue was dehydrated through a graded series of ethanol and xylene, and embedded into a paraffin block. Sections (8 μm) were cut, deparaffinized and digested with 0.05% trypsin at 37°C for 1 h to improve penetration of antibodies. The section was incubated for 1 h with anti-dj4 (1:200 dilution) and then washed 3 times with PBS for 10 min. Nonimmune rabbit serum was used as negative control. The sections were incubated with fluorescent conjugated Cy3-labeled goat anti-rabbit IgG (1:200 dilution) for 1 h. After washing three times with PBS, immunostained tissues were embedded in PBS and immediately analyzed as described above.

11–9. Other Methods

Survival or death of H9c2 cells was quantified by a trypan blue exclusion method. Protein concentration was determined with the protein assay reagent (Bio-Rad Laboratories, Hercules, CA, USA) using bovine serum albumin as standard.

12. RESULTS

12–1. Expression of dj4 mRNA in Mouse Tissues

We studied the distribution of dj4 mRNA in mouse tissues (Fig. 2). dj4 mRNA of about 3.3 kb was expressed most strongly in heart and testis, moderately in brain and ovary and weakly in other tissues. In testis, a smaller form of about 1.8 kb was expressed very strongly.

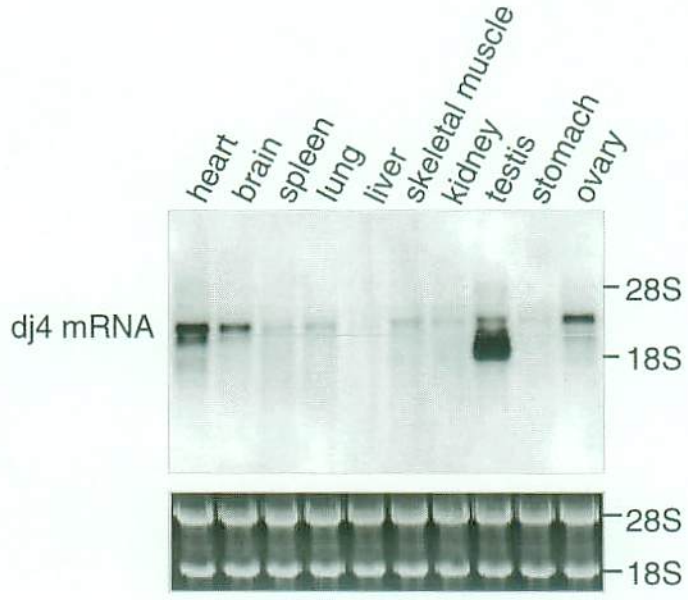


Fig. 2. **RNA blot analysis for dj4 in mouse tissues.** Total RNAs (2.0 μg) from mouse tissues were subjected to RNA blot analysis, using digoxigenin-labeled anti-sense RNAs as probes. The positions of 28S and 18S rRNAs are shown on the right. Lower panel shows ethidium bromide staining of 28S and 18S rRNAs.

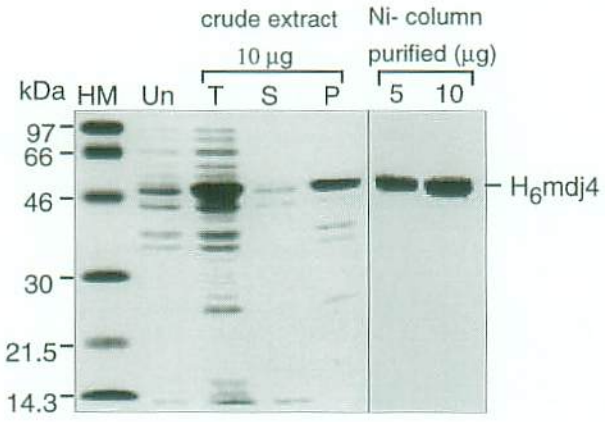


Fig. 3. **Expression and purification of hexahistidine-tagged dj4.** M15[pREP4]/pQE32-dj4 overnight culture (0.5 ml) was mixed with 1.5 ml (pre-warmed at 37˚C) of LB media containing 100 μg/ml kanamycin. After 2.5 h 1 mM IPTG was added and cells were cultured for another 3.5 h at 37˚C. Then cells were harvested by centrifugation and resuspended in binding buffer (50 mM Na-phosphate, pH 7.8, 300 mM NaCl), sonicated and fractionated into soluble and insoluble fractions. Uninduced whole cell extract (Un), induced whole cell extract (T), soluble (S) and insoluble (P) fractions were subjected to CBB staining. H_6mdj4 was recovered in the insoluble fraction and was purified by Ni-NTA-Sepharose column as described in "Materials and Methods". Purified dj4 (5 μg and 10 μg) was applied to see the purity.

12–2. Purification of hexahistidine-tagged dj4

E. coli expressed hexahistidine-tagged dj4 was recovered in the inclusion body and purification of the recombinant protein was done by Ni^{2+}-NTA-Sepharose column chromatography under denaturing conditions (see "Materials and Methods" for detail) (Fig. 3). To obtain a better purification, eluate obtained from the first purification was further purified using the same column under native conditions. Purity obtained was >90%.

12–3. Tissue Distribution and Intracellular Concentration of dj4 Protein

We prepared a polyclonal antibody against dj4 and checked its specificity and cross-reactivity with other DnaJ homologs. In immunoblot analysis, this antibody gave a major single band of 46 kDa in many tissues except testis (Fig. 4B). In cross-reactivity test, anti-dj4 and anti-dj3 were specific to each chaperones, whereas anti-dj2 cross-reacted weakly with dj3 and dj4 (Fig. 4A). dj4 protein of 46 kDa was expressed most strongly in heart and testis, moderately in brain and uterus and weakly in other tissues (Fig. 4B). Testis gave additional two larger species. The tissue distribution of dj4 protein was similar to that of dj4 mRNA. Extraspecies of dj4 mRNA and protein in testis remain to be characterized.

Distribution of dj4 was compared with those of other cytosolic chaperones. Hsc70 was expressed uniformly in all tissues, whereas Hsp70 was expressed in many tissues to different degrees. dj1, dj2 and dj3 were expressed ubiquitously, but their tissue distribution was not uniform and differed among DnaJ homologs. Taking these results

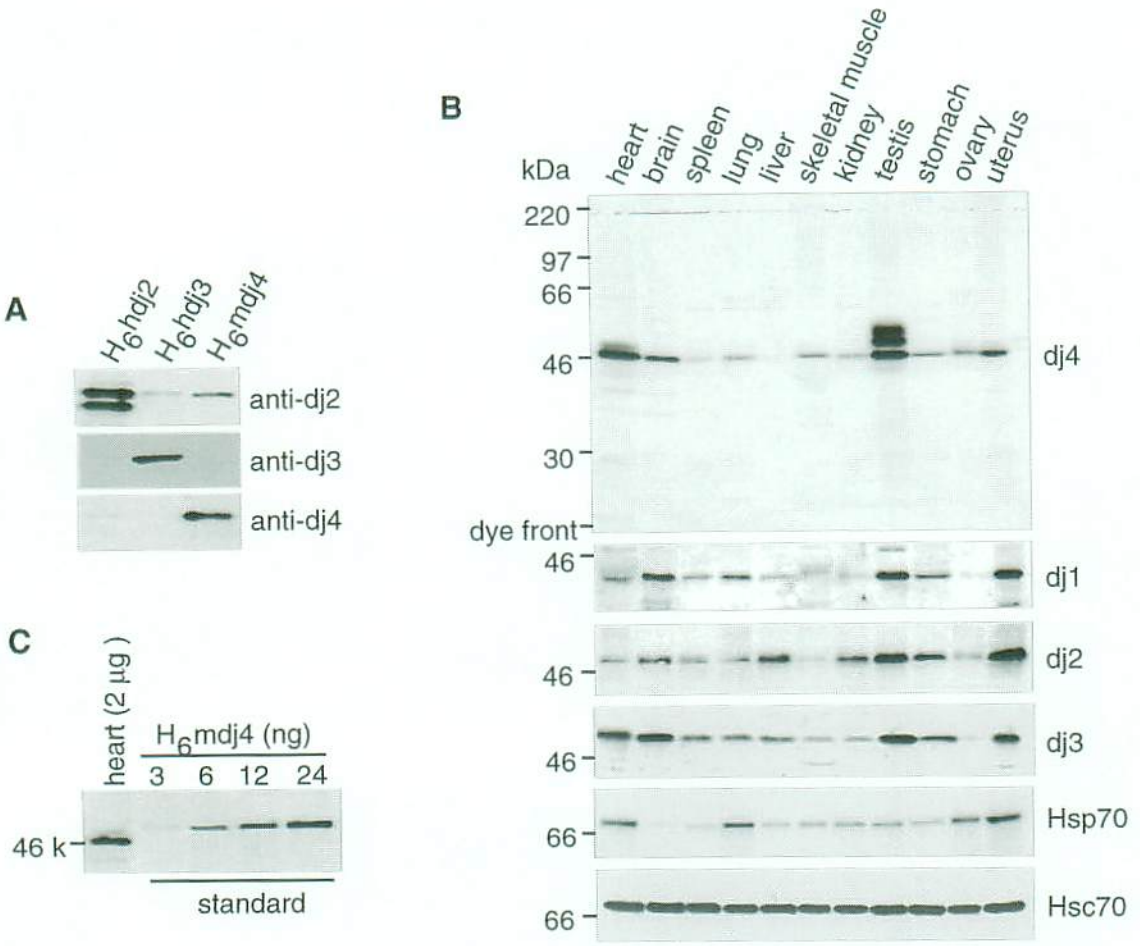


Fig. 4. **Tissue distribution and intracellular concentration of dj4 protein.** A. Cross reactivity among the DnaJ antibodies. Purified histidine-tagged human dj2 (H_6hdj2), human dj3 (H_6hdj3) and mouse dj4 (H_6mdj4) (each 30 ng) were subjected to immunoblot analysis using antisera against human dj2, human dj3 or mouse dj4. Amino acid sequence identities between human and mouse dj2 and dj3 are 99% (from database). Those between mouse DnaJ homologs are, 55% between dj2 and dj3, 51% between dj3 and dj4, and 67% between dj2 and dj4 (Hata *et al*, 2000; Ohtsuka and Hata,

2000). B. Tissue distribution of dj4 and other chaperones. Tissue extracts (2.0 μg of protein for dj2 and 5.0 μg of protein for dj4, dj1, dj3, Hsp70 and Hsc70) were subjected to immunoblot analysis using rabbit antisera against dj4, dj1, dj2 (1:1000) and dj3 (1:200), monoclonal anti-hsp70 (0.2 μg IgG/ml) and monoclonal anti-Hsc70 (0.5 μg IgG/ml) as primary antibodies. Protein molecular mass markers (Rainbow-colored markers; Amarsham Pharmacia Biotech) were myosin (220 kDa), phosphorylase *b* (97 kDa), serum albumin (66 kDa), ovalbumin (46 kDa) and carbonic anhydrase (30 kDa). C. Content of dj4 in mouse heart. Heart homogenate (2.0 μg of protein) and purified histidine-tagged mouse dj4 (H_6mdj4) (3, 6, 12 and 24 ng) were subjected to immunoblot analysis.

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together, one specific feature of dj4 was that this protein, but not other DnaJ homologs, was expressed most strongly in heart.

The concentration of dj4 in mouse heart was measured by immunoblot analysis using purified H_6mdj4 as standard (Fig. 4C). H_6mdj4 migrated as a polypeptide of 47 kDa in a SDS-polyacrylamide gel. mdj4 was calculated to represent about 1% of total heart protein. Accordingly, the concentration of mdj4 in other tissues (except testis) were much lower.

12–4. Subcellular Localization of dj4

In immunofluorescent staining of dj4 in mouse heart, cytoplasm of muscle cells was

stained (Fig. 5A). Mouse heart was fractionated into nuclear, mitochondrial, microsomal and cytosol fractions (Fig. 5B). Fractionation was assessed with marker proteins. dj4 was recovered mostly in the cytosol fraction and partly in the nuclear fraction. On the other hand. Hsc70 was recovered in the nuclear and cytosol fractions, and slightly in the microsomal fraction. Taking distribution of total protein into consideration (see legend for Fig. 4B), amount of Hsc70 in the cytosol fraction was much higher than that in the nuclear fraction. Therefore, distribution of dj4 was similar, although not identical, with that of Hsc70.

We next examined localization of dj4 in H9c2 cells and compared it with that of Hsc70, using double immunofluorescence microscopy (Fig. 5C). Under normal conditions, dj4 was localized almost exclusively in the cytoplasm and colocalized with Hsc70. When cells were heat-shocked, dj4 migrated from the cytoplasm to the nucleus, and roughly colocalized again with Hsc70. All these results suggest that dj4 is able to work as a cochaperone of Hsc70 (and Hsp70, see Discussion) under both normal and stressed conditions.

12–5. Expression of dj4 in H9c2 Cells

Endogenous dj4 was detected by immunoblot in H9c2 cells, but the basal level was about 20-fold lower than that in mouse heart and about 3-fold lower than that in skeletal muscle (Fig. 6).

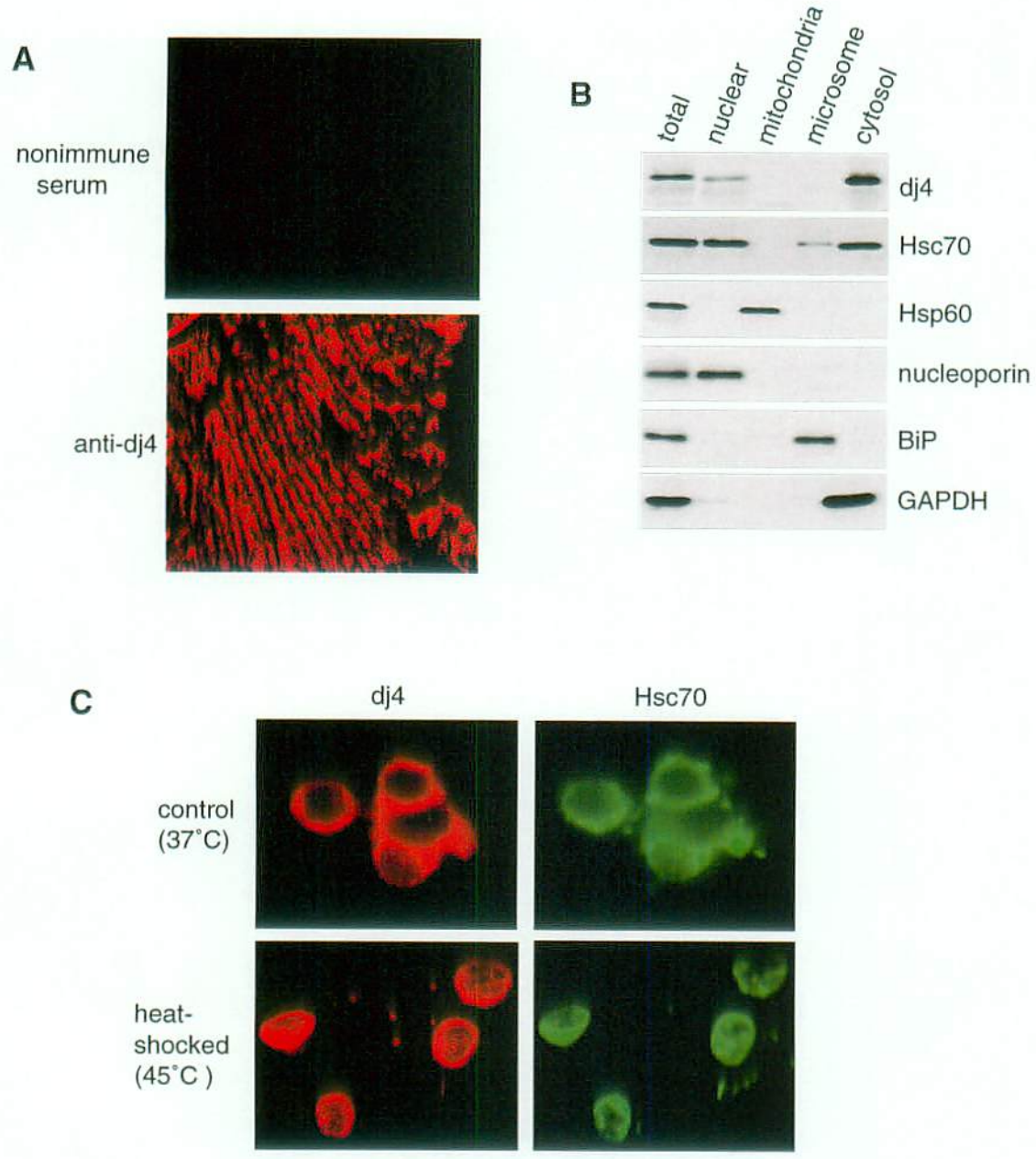

Fig. 5. **Immunofluorescence staining and subcellular distribution of dj4.** A. Immunostaining of mouse heart for dj4. Mouse heart paraffin sections were immunostained with anti-dj4 antiserum (1:200 dilution) or with nonimmune rabbit serum (1:200) as negative control. Secondary antibody used was Cy3-labeled goat anti-

rabbit IgG. Fluorescence images were taken under identical conditions. B. Subcellular distribution of dj4 in heart. Mouse heart was subfractionated as described under "Materials and Methods". Total extract, nuclear, mitochondrial, microsomal and cytosol fractions (5 µg of protein) were subjected to immunoblot analysis. Distribution of total protein in nuclear, mitochondrial, microsomal and cytosolic fractions was 9.1%, 8.4%, 32.1% and 50.4%, respectively. C. Distribution of dj4 in H9c2 cells before and after heat shock. Cells were grown on coverslips at 37°C (control) or heat shocked at 45°C for 15 min and allowed to recover at 37°C for 1 h. Cells were double stained with rabbit anti-dj4 antiserum and rat monoclonal antibody against Hsc70. Secondary antibodies used were Cy3-labeled goat anti-rabbit IgG and Alexa fluor 488-labeled goat anti-rat IgG.

Fig. 6. **Expression of dj4 in H9c2 cells.** H9c2 cell extract (lanes 1and 2, 20 µg and 5 µg respectively), and mouse heart (5 µg) or skeletal muscle (5 µg) tissue extract were loaded in SDS-polyacrylamide gel and subjected to immunoblot analysis for dj4 using anti-dj4 serum (1:1000 dilution) as primary antibody.

12–6. Changes of dj4 and Other Chaperones During Differentiation of H9c2 Cells

H9c2 cells were differentiated by culturing for up to 28 days with a decreased serum concentration. Under these conditions, extensive cell fusion into multinuclear tubular cells was observed (data not shown). dj4 appeared to decrease slightly at day 7 and then increased up to day 28 (Fig. 7). Induction at day 28 was about 3.2-fold over the level at day 3. In contrast, dj1 remained little changed up to day 21 and decreased at day 28, and dj2 remained little changed up to day 28. dj3 increased up to day 21 or day 28, but less markedly than dj4. Hsc70 remained unchanged during the period.

12–7. Heat Shock Induction of dj4 and Other Chaperones in H9c2 Cells

Effects of heat shock on mRNAs and proteins for dj4 and other chaperones in H9c2 cells are shown in Fig. 8. Cells were heat-shocked at 43°C for 30 min and returned for recovery to 37°C for 2 h and 4 h for mRNAs (Fig. 8A) and up to 6 h for proteins (Fig. 8B). dj4 mRNA was induced by about 11-fold at 2 h and remained high at 4 h. dj4 protein was somewhat decreased at 0 h and 1 h, and was then increased up to 6 h. Increase at 6 h over control was about 2.4-fold. dj1 mRNA was markedly increased at 2 h and decreased at 4 h. dj1 protein was increased gradually up to 4 - 6 h. dj2 mRNA and protein were markedly increased. In contrast, dj3 mRNA was decreased by heat shock and its protein remained little changed. Hsp70 mRNA and protein were not detected before heat treatment, and were markedly increased after treatment. Hsc70 mRNA was moderately increased, whereas its protein remained little changed.

12–8. Preheat Treatment Enhances Survival of H9c2 Cells Against Severe Heat Treatment

H9c2 cells were heat-treated at 43°C for 30 min and then subjected to severe heat treatment at 47°C for 90 min or 120 min. Without preheat treatment, cell survival decreased to 35% and 23% after severe heat treatment for 90 min and 120 min, respectively (Fig. 9). When cells were preheat-treated, survival increased to 63% and 42%, respectively, after severe heat treatment. These results along with results in Fig. 8 suggest that chaperone pair of Hsp70 and dj4 and/or dj2 is responsible for the increased cell survival.

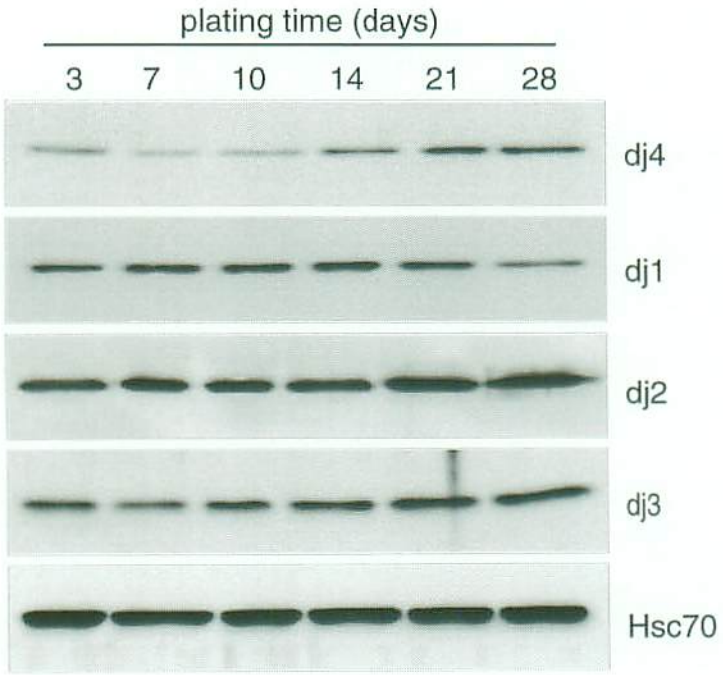

Fig. 7. **Changes in dj4 and other chaperones during differentiation of H9c2 cells.** Cells were cultured in DMEM containing 10% fetal calf serum. After 7 days of plating

(near 100% confluent), medium was changed to DMEM containing 1% serum to promote differentiation. Medium was changed in every 3 days and cells were collected at indicated times. Whole cell extracts (5.0 μg of protein) were subjected to immunoblot analysis for DnaJ proteins and Hsc70.

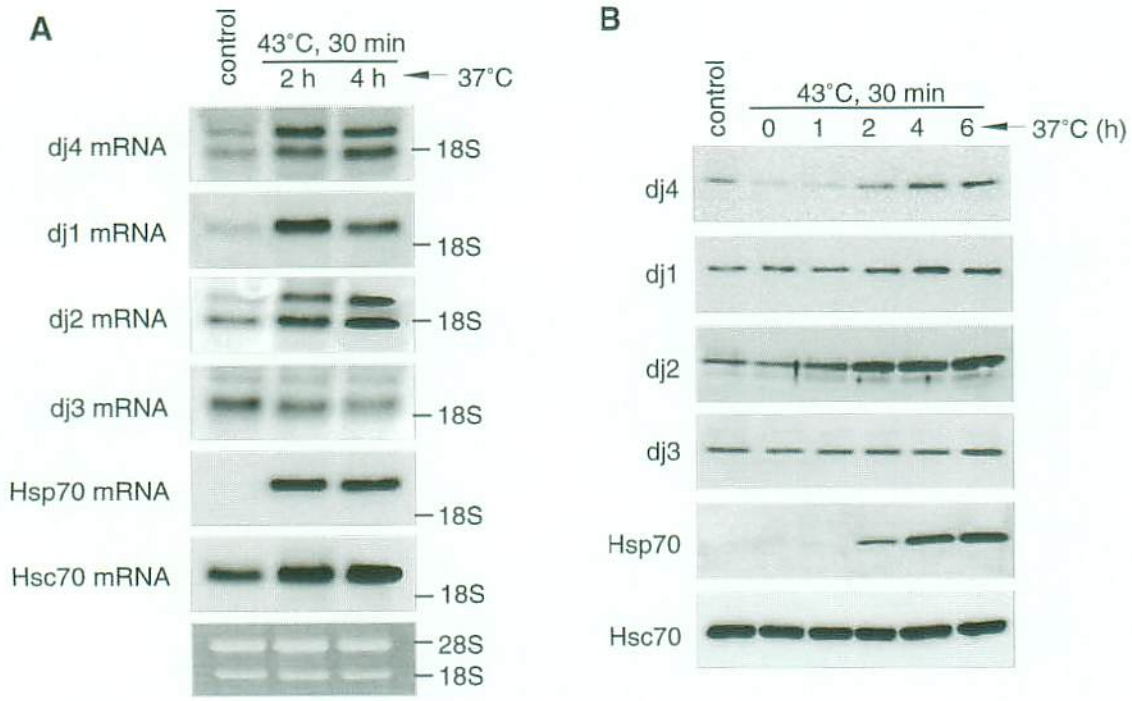

Fig. 8. **Heat shock induction of mRNAs and proteins for dj4 and other chaperones in H9c2 cells.** A. Cells were maintained at 37°C (control) or heated at 43°C for 30 min and allowed to recover at 37°C for 2 h or 4 h. Total RNAs (2.0 μg) were subjected to RNA blot analysis, using digoxigenin-labeled RNAs as probes. The

position of 18S rRNA is shown. Bottom panel shows ethidium bromide staining of 28S and 18S rRNAs. B. Cells were heat-shocked as in (A) and recoverd at 37°C for the indicated time periods. Whole cell extracts (5.0 μg of protein) were subjected to immunoblot analysis for Hsp70. Hsc70 and DnaJ proteins using their respective antibodies.

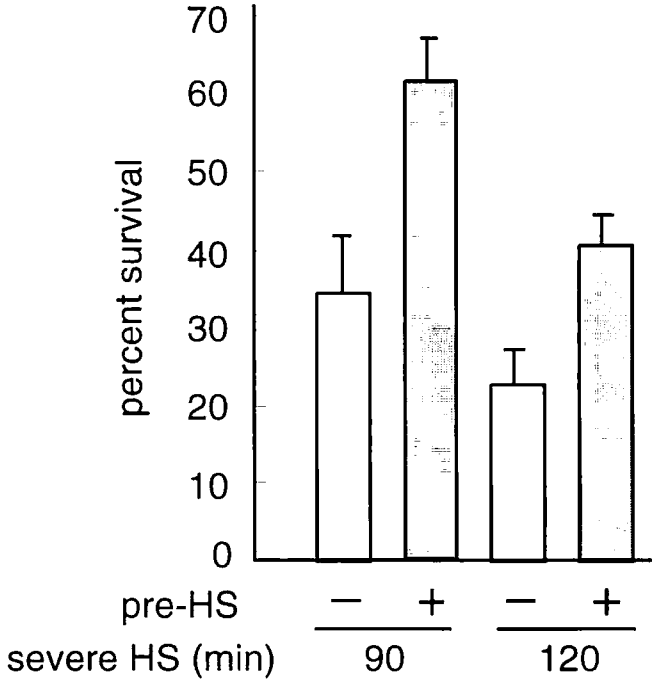

Fig. 9. **Effect of heat pretreatment on thermotolerance of H9c2 cells.** Control cells and heat-pretreated cells (43°C for 30 min and recovery at 37°C for 6 h: pre-HS) were subjected to severe heat treatment (severe HS) at 47°C for 90 min or 120 min. Cell survival was assessed as described in "Materials and Methods". Results from three independent experiments were expressed as means ± S. D.

12–9. Coexpression of Hsp70 and dj4 or dj2 Enhances Survival of H9c2 Cells Against Severe Heat Treatment

H9c2 cells were cotransfected with a GFP expression plasmid with an Hsp70 plasmid alone or in combination of a dj4 plasmid or a dj2 plasmid and cell survival after severe heat treatment was assessed by the number of GFP-positive cells (Fig. 10A) and by the level of GFP protein (Fig. 10, B and C). In control cells, GFP-positive cells were markedly decreased after severe heat treatment. When GFP and Hsp70 were coexpressed, the decrease was markedly prevented. When Hsp70 and dj4 along with GFP were coexpressed, a better prevention was obtained, although the effect of dj4 was slight. dj2 was also effective in enhancing cell survival. These results were confirmed by immunoblot analysis of GFP (Fig. 10, B and C). When cell survival was assessed by trypan blue exclusion, similar results were obtained (Fig. 10D). These results indicate that Hsp70 can enhance survival of H9c2 cells after severe heat treatment, and that dj4 and dj2 can further improve the survival.

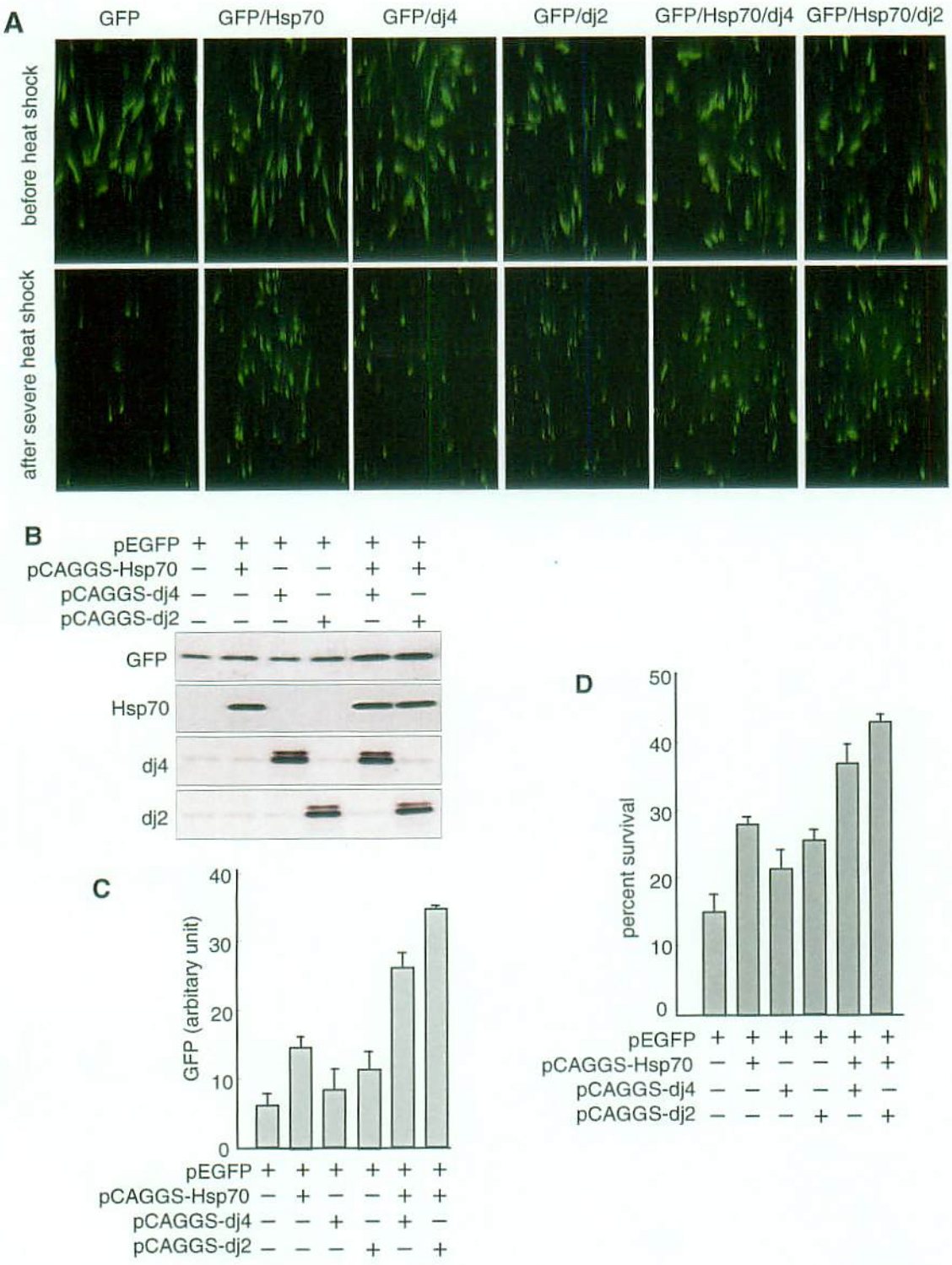

Fig. 10. **Effect of overexpression of Hsp70, dj4 or dj2 on thermotolerance of H9c2 cells.** A. Cells grown on 35-mm dishes were transfected with pEGFP (2.0 μg) alone or

with Hsp70 (0.2 μg), dj4 (0.67 μg) or dj2 (0.67 μg) chaperone plasmids in pCAGGS as indicated, using the GenePORTER transfection reagent (Gene Therapy Systems, San Diego, CA, USA). The amount of total plasmid was adjusted to 2.0 μg with pCAGGS. Transfection efficiency was 35%. 48 h after transfection, cells were subjected to severe heat shock by transferring sealed dishes to water bath at 47°C for 2 h. GFP fluorescence was observed before and after severe heat shock. B. Cell extracts (5.0 μg of protein) were subjected to immunoblot analysis for GFP, Hsp70, dj4, dj2 and Hsc70 proteins. The larger species of dj4 and dj2 are unfarnesylated precursor forms. C. The results for GFP in (B) and other two independent experiments were quantified and are shown as means ± S. D. (n = 3). D. Cells were transfected and subjected to severe heat shock as in (A). Cell viability was monitored by trypan blue exclusion. Results are expressed as means ± S. D. (n = 3).

13. DISCUSSION

dj4 cDNA was recently isolated based on a cDNA database which encodes 397 amino acid residues. It shows 67 and 51% identities with the previously identified murine Hsj2 and mdj3, respectively. The sequence of dj4 contains four repeats of CxxCxGxG motif which are characteristic of typeI Hsp40/DnaJ proteins, and a CaaX prenylation motif at the carboxy terminus. dj4 mRNA was reported to be highly expressed in heart and testis of mice (Hata and Ohtsuka, 2000). In the present study using mouse tissues, we confirmed this at both mRNA and protein levels, and further showed that dj4 is also expressed at lower levels in many other tissues. dj4 as well as dj2 and dj3 belongs to type I of DnaJ family and has C-terminal prenylation motif. In fact, overexpression of dj4 and dj2 gave larger species on SDS-polyacrylamide gel that apparently correspond to unprenylated precursor forms (Fig. 10B). Farnesylation of dj2 and dj3 were demonstrated (Andres *et al*, 1997; Kanazawa *et al*, 1997). In contrast, dj1 belongs to type II and has no prenylation motif. dj4 was calculated to represent about 1.0% of total protein in heart; this value is much higher than those of dj1, dj2 and dj3 in mouse heart. dj4 was also present in heart muscle cell-derived H9c2 cells, but was undetectable in COS-7 and neuronal PC12 cells (data not shown). dj4, but not other DnaJ homologs, was markedly induced in H9c2 cells during differentiation. All these results suggest that dj4 has a special role in heart muscle cells.

An initial sublethal exposure to heat or other stresses can condition cells for enhanced survival during exposure to subsequent, even more severe, stresses; this is commonly termed thermotolerance. The induction of Hsps has been shown to coincide with the

development of thermotolerance. This refers to an adaptive response of cells exposed to elevated temperatures. All conditions or agents capable of inducing Hsps do so by activating heat shock factors (HSFs) (Morimoto *et al.* 1997). Upon activation, HSFs translocate into the nucleus where they bind to the heat shock responsive elements (HSE) and activate the transcription by Hsp genes (Wu, 1995). The induction of Hsps in response to stress and the subsequent thermotolerance are transient. When the stress is removed, the cells continue to function normally and the levels of Hsps drop back to basal levels with time (Samali and Orrenius, 1998).

Members of the Hsp70 family are abundent in both prokaryotic eukaryotic cells and have been extensively studied. The major heat shock protein Hsp70 is induced in cells of wide variety of organisms in response to heat shock or other environmental stresses and is assumed to play an important role in protecting cells from thermal stress (Samali and Orrenius, 1998). It is thought to play a critical role in thermotolerance of mammalian cells, presumably due to its chaperone activity. Several studies have shown that overexpression of Hsp70 protects cells from severe heat treatment (Li *et al.* 1991: Heads *et al*, 1994; Nollen *et al.* 1999). They reported that Hsp70 has a protective function against thermal stress and that stable overexpression of an inducible isoform of Hsp70 can confer a significant degree of thermotolerance to heart-derived H9c2 muscle cells. Nolllen *et al* (1999) separately suggested that the inducible form of Hsp70 contributes to the stress-tolerant state by increasing the chaperone activity in the cytoplasm. Heat shock treatment induces expression of several heat shock proteins and subsequent post-ischemic myocardial protection. Overexpression of Hsp70 in a

transgenic mouse increases the resistance of heart ischemia and repurfusion injury (Marber *et al*, 1995; Plumier *et al*, 1995). Overexpression of Hsp70 in a transgenic mouse also decreases the infarct size *in vivo* and have a direct cardioprotective effect to enhance postischemic recovery of the intact heart (Hutter *et al*, 1996; Radford *et al*, 1996).

Cellular stress can trigger a process of self-destruction known as apoptosis. Cells can also respond to stress by adaptive changes that increase their ability to tolerate normally lethal conditions. Hsp70 has been reported to protect cells from apoptosis at the level of both cytochrome *c* release and initiator caspase activation (Mosser *et al*, 2000) or by preventing formation of active apoptosome (Beere *et al*, 2000; Saleh *et al*, 2000). Excess nitric oxide (NO) induces apoptosis in some cell types, including macrophages. Hsp70 has been reported to protect cells from various stresses, including apoptosis-inducing stimuli. However, effect of Hsp70 cochaperones has little been studied. Gotoh *et al* (2001) found that Hsp70/Hsc70-dj1 or dj2 chaperone pair prevents nitric oxide-mediated apoptosis in macrophages and that Hsp70-DnaJ chaperone pairs exerted their anti-apoptotic effects upstream of caspase 3 activation, and apparently upstream of cytochrome *c* release from mitochondria. Hsp70/Hsc70 alone was little effective. Here I showed that, in H9c2 cells, Hsc70 and dj4 are colocalized in the cytoplasm under normal conditions, and in the nucleus after heat shock. Hsp70 also migrates from the cytoplasm to the nucleus after heat shock (Nollen *et al*, 1999). When H9c2 cells were heat-pretreated, dj4 and dj2, in addition to Hsp70, were markedly induced and cells became resistant to severe heat shock. Transfection experiments showed that Hsp70

alone enhances survival of H9c2 cells after severe heat shock, and that coexpression of dj4 or dj2 further enhances survival, although not markedly. Because dj4 is much more abundant than dj2 and dj3 in heart muscle cells, it is likely that Hsp70/Hsc70-dj4 chaperone pair is mainly responsible for the thermotolerance. The effect of this chaperone pair on protection of heart muscle cells against other stresses such as ischemia remains to be studied.

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