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# Characterization of mammalian translocase of inner mitochondrial membrane (Tim44) isolated from diabetic newborn mouse kidney

**(transport machinery protein/diabetes mellitus)**

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**ABSTRACT** **Mammalian translocase of mitochondrial inner membrane (mTim44) was isolated during representational difference analysis of cDNA from diabetic mouse kidney. Streptozotocin-induced diabetic mouse kidney cDNA was prepared and subtracted by normal mouse kidney cDNA. By using one of the isolated cDNA fragments as a screening probe, full-length cDNA of mTim44 was isolated from λZAP kidney cDNA library. At the nucleotide level, mTim44 did not exhibit significant homology with any known genes; however, at the amino acid level, it had 50% similarity and 29% identity with yeast Tim44. C-terminal FLAG epitope-tagged mTim44 fusion protein was transiently expressed in COS7 cells. By using anti-FLAG epitope M2 monoclonal antibody, mTim44 was found to have its subcellular localization associated with mitochondria. By immunoelectron microscopy, mTim44 was seen in the paracrystalline structures within the mitochondria, as well as in their cristae. Mitochondrial import assay of *in vitro* translated mTim44 indicated that its precursor product (≈50 kDa) was imported and proteolytically processed to a mature ≈44-kDa protein, and its translocation was inner membrane potential (ΔΨ)-dependent. Imported mTim44 was protected from protease digestion in which outer membranes were selectively permeabilized with digitonin. The mature form of mTim44 could be recovered in the supernatant of sonicated mitochondrial membrane fraction treated with 0.1 M $Na_2CO_3$, pH 11.5. The data indicate that mTim44 is a mitochondrial inner membrane protein, one of the members of the mammalian TIM complex and up-regulated in hyperglycemic states.**

Gene regulation has received much attention in delineating various mechanisms involved in the pathogenesis of diabetic nephropathy, and most of the studies have focused on the extracellular matrix proteins, extracellular matrix-degrading enzymes (1, 2), integrins (3), and growth factors (4, 5). Interest in molecules unrelated to the extracellular matrix pathobiology in diabetic nephropathy has been limited. Recently, an interest to study mitochondrial pathobiology has arisen because glucose entry into the cell stimulates oxidative phosphorylation via the activation of glucokinase at the contact sites between outer and inner mitochondrial membranes (6). It is interesting to note that in hyperglycemia there is an up-regulation of mitochondrial-encoded genes, which include genes encoding for various oxidative phosphorylation enzymes, i.e., cytochrome oxidase I and III, NADH dehydrogenase IV, and 12S and 16S ribosomal RNA (7, 8). In addition, mitochondrial DNA length- and point-mutations have been described in hereditary diseases associated with diabetes. The mutations are confined to mitochondrial $tRNA^{Leu(UUR)}$, $tRNA^{Lys}$, and $tRNA^{Glu}$ genes (6). However, expression of some of the nuclear-encoded mitochondrial enzyme proteins, e.g., cytochrome oxidase 7a, remains unaltered. Because there are a number of enzymes that influence oxidative phosphorylation, it is conceivable that other nuclear-encoded mitochondrial proteins, e.g., those related to mitochondrial ATP-dependent transport, may be up- or down-regulated in the hyperglycemic state. In view of these considerations, we used representational difference analysis of cDNA (cDNA–RDA) (9–11) to detect the differentially expressed genes in kidneys of streptozotocin (STZ)-induced diabetes in newborn mice. A number of cDNA fragments (clones 1–9) were isolated, and seven of them had up-regulated expression. In this communication, we present the entire coding sequence of clone 6 and its characterization.

## MATERIALS AND METHODS

**Induction of Diabetes in Mice.** Hyperglycemia was induced in CD1 newborn mice ($n = 20$) (Charles River Breeding Laboratories) by an intraperitoneal injection of STZ (200 mg/kg of weight) in citrate buffer at pH 4.6. Control mice ($n = 20$) received buffer only. After 3 weeks, mice with plasma glucose levels of >250 mg/dl were selected. Their kidneys were harvested, snap-frozen in liquid nitrogen, and subjected to total RNA extraction.

**cDNA–RDA and Oligonucleotides.** The method of cDNA–RDA (9, 12) was adapted to isolate glucose-up-regulated genes in the postnatal kidney. Various deoxyoligonucleotides, i.e., R-Bgl-12 and -24, J-Bgl-12 and -24, and N-Bgl-12 and -24, were synthesized as described (9, 12).

**Generation of Representative Amplicons (R-Amplicons).** Total RNA from normal mouse (NM) and diabetic mouse (DM) kidneys was isolated (13), poly(A)$^+$ RNA selected, and first and second strand cDNAs were synthesized (14). Double-stranded cDNA (2 μg) was digested with *Dpn*II ( ↓ GATC) (New England Biolabs), purified by phenol/chloroform extraction and ethanol precipitation, and suspended in 20 μl of TE (10 mM Tris/1 mM EDTA, pH 8.0) buffer. Twenty microliters of *Dpn*II-digested cDNA was added to the ligation mixture containing 8 μl of R-Bgl-24 (1 mg/ml), 4 μl of R-Bgl-12 (1 mg/ml), 6 μl of 10× ligation buffer (Boehringer Mannheim), and 19 μl of water. Annealing was carried out at 50°C for 1 min, followed by cooling down to 10°C for 1 h in a PCR thermal cycler (Perkin–Elmer). Representative adaptor [R-adaptor (R-Bgl-12/R-Bgl-24)] ligation was initiated by adding 3 μl of T4 DNA ligase (400 units/μl) (Boehringer Mannheim), and the mixture was incubated at 16°C for overnight. The ligation mixture was diluted to 200 μl by adding 140 μl of TE, and then PCR mixture was prepared in four

Abbreviations: cDNA–RDA, representational difference analysis of cDNA; STZ, streptozotocin; yTim44, yeast Tim44; mTim44, mouse Tim44; NM, normal mouse; DM, diabetic mouse; R-adaptor, representative adaptor; R-amplicon, representative amplicon; HB, homogenization buffer.

The sequence reported in this paper has been deposited in the GenBank database (accession no. U69898).

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separate Eppendorf tubes, each containing 20 μl of PCR 10× buffer (Perkin–Elmer), 24 μl of nucleotide mix (2.5 mM dNTP each), 2 μl of R-Bgl-24, and 2 μl of diluted ligation mixture. The R-Bgl-12 was dissociated from R-adaptor-ligated double-stranded cDNA by heating at 72°C for 3 min, followed by a 3′ end filling-in reaction catalyzed by addition of 5 units of *Taq* polymerase at 72°C for 5 min. Twenty thermal cycles, 95°C for 1 min and 72°C for 3 min, were carried out. The PCR products of four tubes were combined, purified, and suspended in 100 μl of TE to yield R-amplicon (amplified PCR product) with a DNA concentration of ≈0.5 μg/μl. 300 μg of DM kidney and 100 μg of NM kidney R-amplicons were prepared. The R-amplicon prepared from DM kidney was used as a tester, whereas that from NM kidney was used as a driver. R-adaptors were removed from NM kidney R-amplicon by digesting with *Dpn*II, purified, ethanol-precipitated, and suspended in TE to yield a concentration of 0.5 μg/μl. This is the cut driver and was used for subtraction. To remove the digested R-adaptors, 20 μg of *Dpn*II-digested DM kidney R-amplicon was gel-purified by Qiaex II (Qiagen, Chatsworth, CA), and 2 μg of purified amplicon was ligated to J-Bgl-24/12 adaptors as described above. This is the J-ligated tester. Finally, J-ligated tester (DM kidney) and cut driver (NM kidney) were used for subtractive hybridization to generate first difference product (DP1), followed by the generation of second (DP2) and third difference products (DP3).

**Cloning and Sequence Analyses of Difference Products.** R-amplicon of DM kidney, DP1, DP2, and DP3 were analyzed by 2% agarose gel electrophoresis. The DP3 was digested with *Dpn*II to remove the linkers, and the whole population of DNAs in DP3 solution was ligated into *Bam*HI-digested pBluescript KS(+) (Stratagene). Bacterial colonies were picked to prepare plasmid DNAs for nucleotide sequencing (15). Homology search was performed by the BLAST program via National Center for Biotechnology Information on-line service.

**Northern Blot Analyses.** Thirty μg of total RNAs extracted from NM and DM kidneys were glyoxylated, subjected to 1% agarose gel electrophoresis, and capillary-transferred to nylon membranes. The membrane blot was hybridized with [$\alpha$-$^{32}$P]dCTP-labeled up-regulated cDNAs isolated from DM kidneys and washed at high stringency conditions with 0.1× SSC (1× SSC = 0.15 M sodium chloride/0.015 M sodium citrate, pH 7) and 0.1% SDS at 60°C, and autoradiograms were prepared.

**cDNA Library Screening and Sequencing.** The λZAP mouse newborn kidney library was prepared and screened as detailed previously (16, 17). Briefly, about $0.5 \times 10^6$ phage recombinants were plated, and nitrocellulose filter lifts of phage plaques were made and hybridized with the [$\alpha$-$^{32}$P]dCTP random radiolabeled 380-bp cDNA clone 6, the expression of which was up-regulated in DM kidney, as revealed by cDNA–RDA (see *Results*). Positive plaques were picked and purified by dilutional secondary and tertiary screenings. Phage cDNAs were amplified, agarose gel-purified, and ligated into pBluescript phagemid KS(+), by using XL1-Blue MRF′ *Escherichia coli* (Stratagene). Single-stranded DNAs were prepared by VCSM13 helper phage and sequenced. Hydropathic analyses (18), sequence homology (19), and protein structural analyses (20) were performed with Genetics Computer Group package 8.0.1.

**Generation of Eukaryotic Expression Constructs.** PCR was used to generate a eukaryotic expression construct by using full-length cDNA of mTim44, isolated by λZAP library screening with clone 6. 5′ *Bam*HI site (GGATCC), Kozak's sequence (ACCATGG) (21), C-terminal FLAG epitope, and 3′ *Xho*I site (CTCGAG) were introduced by using sense primer 5′-GGGGGGGGATCCACCATGGCGGCGGCACGTCTG-3′ and antisense primer 5′-GGGGGGCTCGAGTCA***CTTGTCATCGTCGTCCTTGTAGTCAG***GATCTGCTCTGTGCT-3′. GC clamps (GGGGGG) were also included into the primer sequences. The restriction enzyme sites are underlined, and the nucleotide sequence of FLAG epitope (N-Asp-Try-Lys-Asp-Asp-Asp-Asp-Lys-C) is given in italic bold letters. To prepare a construct lacking FLAG epitope, antisense primer 5′-GGGGGGCTCGAGTCAGAGGATCTGCTCTGTGCT-3′ was used. The PCR-amplified products were digested with *Bam*HI–*Xho*I-digested PCR, agarose gel-purified, subcloned into the *Bam*HI–*Xho*I-digested pBluescript KS(+), and designated as BS KS(+)/TIM-FLAG and BS KS(+)/TIM, containing and lacking the FLAG epitope. Both constructs, BS KS(+)/TIM-FLAG and BS KS(+)/TIM, were sequenced to ensure proper in-frame ligation and *Taq* polymerase fidelity. The cDNA insert of BS KS(+)/TIM-FLAG was subcloned into *Bam*HI-*Xho*I-digested pcDNA3.1 (Invitrogen) and designated as pcDNA3.1/TIM-FLAG. The plasmid DNAs were purified by CsCl gradient centrifugation.

**Expression and Analysis of Eukaryotic Construct.** COS7 (ATCC) cells were cultured in DMEM supplemented with 10% fetal calf serum, penicillin, and streptomycin. The cells were plated into two 35-mm tissue culture Petri dishes ($10^5$ cells in each), grown overnight, and transfected transiently with the pcDNA3.1/TIM-FLAG construct and pcDNA3.1 by using a PerFect transfection kit (Invitrogen). Lipofection was performed with a lipid mixture of Pfx-2 or Pfx-6. After 24 h, the cells were radiolabeled with [$^{35}$S]methionine (0.2 mCi/ml) and incubated for 16 h. Media were removed, and cells were washed with ice-cold PBS three times. They were lysed by adding 1 ml of immunoprecipitation buffer (10 mM Tris·HCl, pH 7.4/150 mM NaCl/2 mM EDTA/1.25% Triton X-100/10 mM benzamidine/10 mM ε-amino-*n*-caproic acid/2 mM phenylmethylsulfonyl fluoride) and vigorously shaken for 4 h at 4°C. The extracts were centrifuged for 10 min at 14,000 rpm in a Microfuge (Beckman) at 4°C. Immunoprecipitation was performed by adding 10 μg of the M2 FLAG epitope monoclonal antibody (Kodak/IBI) to 0.5 ml of lysate ($\approx 0.5 \times 10^{-6}$ dpm), and the mixtures were swirled in an orbital shaker for 18 h. Protein A-Sepharose was added, and the mixtures were swirled for another 2 h. Pellets were then prepared in a Microfuge and washed 4 times with immunoprecipitation buffer. Immunoprecipitated complexes were dissolved in 2× sample buffer and subjected to SDS/10% PAGE under reducing conditions. Gels were fixed in 10% acetic acid/10% methanol, treated with 1 M salicylic acid, and dried, and autoradiograms were prepared.

For immunofluorescence microscopy, $5 \times 10^4$ cells/well were plated in a Lab-Tek chamber slide system (Nunc), grown overnight, and transiently transfected with pcDNA3.1/TIM-FLAG plasmid construct as described above. Immunofluorescence microscopy was performed as previously described (22–24). After 24 h, the cells were washed 3 times with PBS, fixed with 4% paraformaldehyde in PBS for 10 min at 20°C, and then permeabilized with 0.1% Triton X-100 in PBS for 3 min. The cells were then incubated with anti-FLAG M2 monoclonal antibody (8 μg/ml) (Eastman Kodak) for 2 h, followed by another incubation with rhodamine-conjugated goat F(ab′)$_2$ anti-mouse IgG (25 μg/ml) (Cappel) for 2 h at 20°C. Cells were examined by a Zeiss fluorescence microscope equipped with rhodamine filters.

Immunoelectron microscopy was performed on the transfected cells (25). Cells were fixed in a mixture of 4% paraformaldehyde and 0.1% glutaraldehyde at 4°C for 1 h and then scraped from the dish, and a pellet was prepared in a Microfuge. The pellet was dehydrated in a graded series of ethanols and embedded in LR white resin (Polysciences). 600-Å thick sections were prepared and mounted on Formvar/carbon-coated nickel grids. Sections were then incubated with anti-FLAG M2 monoclonal antibody (100 μg/ml) for 6 h at 22°C. Control sections were incubated with mouse IgG only. After 3 washes with 50 mM Tris/BSA buffer, pH 7.5, the sections were incubated with 1:5 diluted goat anti-mouse IgG conjugated with colloidal gold (Research Diagnostics, Flanders, NJ) for 60 min at 22°C. The sections were washed with Tris/BSA, stained with 3% uranyl acetate, and 0.1% lead citrate and examined with an electron microscope.

**Isolation of Mouse Mitochondria and Protein Import Assay.** Mitochondria were prepared from mice livers (26), and their protein concentration was adjusted to 30 mg/ml. Ten microliters of mitochondrial preparation was added to 38 $\mu$l of import buffer (0.22 M mannitol/0.07 M sucrose/10 mM Hepes-KOH, pH 7.6/1 mM $MgCl_2$/1 mM DTT/1 mM EDTA), supplemented with 0.5 mM ATP/0.1 mM GTP/5 mM NADH/1.5 mM creatine phosphate/15 $\mu$g/ml creatine kinase/20 mM sodium succinate. The import assay was initiated by adding 2 $\mu$l of *in vitro* synthesized [$^{35}$S]methionine-labeled Tim44 protein, prepared by using TNT-coupled reticulocyte lysate systems (Promega) and the BS KS(+)/TIM construct. The identity of *in vitro* synthesized Tim44 was confirmed by SDS/PAGE autoradiogram before the import assay. The import reaction mixture was incubated at 30°C for 1 h under conditions with intact inner membrane potential ($\Delta\Psi$) or $\Delta\Psi$ disrupted by the addition of 1.0 $\mu$M carbonyl cyanide *m*-chlorophenylhydrazone. The reaction was terminated with the addition of 2× sample gel-loading buffer, and the products were analyzed by SDS/10% PAGE. Second, proteinase resistance was assessed by incubating postimport reaction products with 200 $\mu$g/ml proteinase K at 4°C for 30 min, in the presence or absence of varying concentrations of digitonin (0.1–0.5 mg/dl). The proteinase was inactivated by adding 2 mM phenylmethylsulfonyl fluoride and incubated at 4°C for 10 min. The reaction mixtures were then analyzed by SDS/PAGE. Third, for membrane protein analyses, mitochondrial pellets were prepared after the import reaction, resuspended in 150 $\mu$l of $Na_2CO_3$, pH 11.5, sonicated, and incubated on ice for 30 min. The insoluble membrane fractions were sedimented by centrifugation at 100,000 × *g* for 10 min, and the supernatant was subjected to SDS/PAGE analysis.

## RESULTS

**Isolation of Differentially Expressed Genes in STZ-Induced DM Kidney.** Three weeks after the injection of STZ, blood glucose levels in DM were 287 ± 25 mg/dl, whereas levels in the control NM were 105 ± 13 mg/dl. The cDNA of DM kidneys was subtracted from NM kidney cDNA by using the cDNA–RDA method. The third difference product (DP3) was ligated into *Bam*HI-digested pBluescript KS(+), and plasmid preparations were made from randomly picked bacterial colonies. Nine clones were isolated and sequenced. A GenBank search indicated that 4 clones had homologous sequences with known genes, and the other 5 clones did not exhibit significant homology with known nucleotide sequences. The clones with known sequences were a cDNA fragment of $Na^+$,$K^+$-ATPase (clones 1 and 2), a fusion protein of ubiquitin and ribosomal protein L40 (clone 3), and ferritin L-subunit gene (clone 4). To ascertain the up-regulated gene expressions of the isolated cDNAs in DM kidney, Northern blot analyses were performed. Seven of 9 clones showed single transcripts: clones 1 and 2 (≈4 kb), clone 3 (≈0.7 kb), clone 4 (≈1.2 kb), clone 5 (≈1 kb), clone 6 (≈2 kb), and clone 9 (≈1.5 kb). Densitometric analysis revealed 2- to 5-fold up-regulated expressions in DM kidney compared with NM kidney (Fig. 1). Two clones (clones 7 and 8) did not hybridize with the mRNA of both DM and NM kidneys.

**Isolation and Structural Analysis of mTim44 cDNA.** Northern blot analysis of clone 6 revealed a 5-fold higher expression in DM kidney (Fig. 1). A 1764-bp cDNA clone (GenBank accession no. U69898) was isolated from λZAP mouse kidney cDNA library by using clone 6 as a screening probe. It had an ORF (9–1364 bp) and a potential initiation codon with Kozak consensus sequence. The ORF encoded 452 amino acids with a predicted ≈50-kDa size protein and no N-linked glycosylation sites (Fig. 2). Although a GenBank search revealed no significant homology of the 1764-bp cDNA clone with known available nucleotide sequences, the BLASTX protein sequence search indicated that it has 29% identity and 50% similarity with yTim44 gene for mitochondrial inner membrane transport machinery protein (Fig. 2). Because of the homology characteristics and the results obtained in mitochondrial import assays (vide infra), this 1762-bp cDNA clone was designated mammalian translocase of inner mitochondrial membrane (mTim44), a homologue of yTim44 (27). Kyte–Doolitle hydrophobicity analyses indicated the protein to be rather hydrophilic with no apparent hydrophobic transmembrane domains. Among the N-terminal 64 amino acids, only one acidic residue (glutamic acid), in contrast to 12 basic residues (8 arginines) and 18 polar residues (11 serines), was present. Such a composite of amino acids is believed to be characteristic of mitochondrial targeting peptide (28). These target peptide sequences are rich in arginine and hydroxylated amino acids and are devoid of extended hydrophobic stretches. Plotting of 1–18 N-terminal amino acids, which include those with predicted $\alpha$-helices, on a "helical wheel" (29) seems to indicate that the positively charged residues (circles) are clustered on one side of the helix whereas nonpolar residues (squares) are on the opposite side. Such an amphiphilic configuration of the N-terminal amino acid residues can potentially be formed by the mitochondrial signal peptide sequences that are thought to be important for aiding the protein translocation across the mitochondrial membranes.

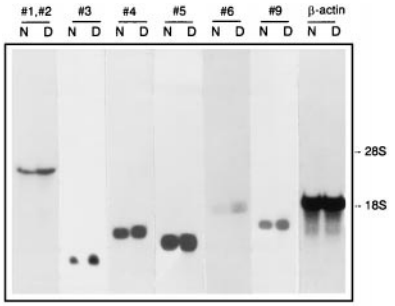


FIG. 1. Northern blot analyses of differentially expressed genes in normal and DM kidneys. Total RNA (30 $\mu$g) from normal (N) and diabetic (D) mouse kidneys was denatured with glyoxal, subjected to 1% agarose gel electrophoresis, transferred to nylon membranes, and hybridized with [$\alpha$-$^{32}$P]dCTP-radiolabeled cDNA fragments of differentially expressed clones (clones 1–9) and $\beta$-actin. Seven genes had up-regulated expression. The transcripts of clones 7 and 8 were rarely expressed and did not yield any hybridization signal.

**Immunolocalization and Immunoprecipitation of C-Terminal FLAG Epitope-Tagged mTim44.** A chimeric construct of mTim44 and FLAG epitope was prepared. FLAG epitope was

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mTim44      1 MAAARLRGGWCRCPRRCLGSGIQFLSSHNLPHGSSYQISRPGRELTLTKS 50
yTim44      1 ....MHRSTPIRTS....GTSSRTLTARYRSQYTGLLVARVLFSTSTTRA 42

mTim44     51 YSSGSRKGFLSGLLDNIKQELAKNKEMKESIKKFRDEAKKLEESDALQEA 100
yTim44     43 QGGNPRSP.LQIFRDTFKKEWEKSQELQENIKTLQDASGKLGESEAYKKA 91

mTim44    101 RRKYKSIESETVRTSRAIKKKLGELTGTVKESLDEVSKSDLGRKIKEGVE 150
yTim44     92 REAYLKAQ....RGSTIVGKTLKKTGETMEHIATKAWESELGKNTRKA.. 135

mTim44    151 EAARTAKQSAESVSKSGEKLGKTAAFKAISQGVESVKKELDESVLGQTGP 200
yTim44    136 .AAATAKKLDESF....EPVRQTKIYKEVSEVI.......DDGESSRYGG 173

mTim44    201 YRRPERLRKRTEFAGAKFKESKVFEANEEALGVVLHKDSKWYQQW....K 246
yTim44    174 FITKEQRRLKRERDLASGKRHRAVKSNEDAGTAVVATNIESKESPGKKVE 223

mTim44    247 DFKDNMVVFNRFFEMQMK.YDESDNVLIRASRALTDKVTDLLGGLFSKTE 295
yTim44    224 DFKEKTVVGRSIQSLKWKLWDESSENPLIVVMRKITNKV....GGFFAETE 269

mTim44    296 MSEVLTEILRVDPTFDKDHFLHQCEYDIIPNILRAMISGELDILKDMCYE 345
yTim44    270 SSRVYSQFKLMDPTFSNESFTRHLREYIVPEILEAVVKGDVKVLKKWFSE 319

mTim44    346 ATYSQLAHPIQQAKALGFQFHSRILDISNVDLAMGKMM..EQGPVLIVTF 393
yTim44    320 APFNVYAAQQKIFKEQDVYADGRILDIRGVEIVSAKLLAPQDIPVLVVGC 369

mTim44    394 QAQVVMVIKNSK.GEVYDGDPDKVQRMLYVWALCRDQEEL..NPYAAWRL 440
yTim44    370 RAQEINLYRKKKTGEIAAGDEANILMSSYAMVFTRDPEQIDDDETEGWKI 419

mTim44    441 LDISASSTEQIL 452
yTim44    420 LEFVRGGSRQFT 431
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FIG. 2. Sequence alignment comparison of mTim44 and yTim44. Amino acid sequences of mouse mTim44 and yTim44 were aligned by the GAP program in the Genetics Computer Group package. The analyses revealed 29% identity and 50% similarity between mTim44 and yTim44. The sequence data of mTim44 are available in GenBank/EBL/DDBJ (accession no. U69898).

incorporated in the C-terminal end of mTim44 to avoid interference with the recognition of N-terminal mitochondrial signal sequence, and COS7 cells were transiently transfected with the eukaryotic expression vector, pcDNA3.1/TIM-FLAG. The incorporated FLAG epitope is used to immunoprecipitate and localize mTim44 at the subcellular level with the anti-FLAG M2 monoclonal antibody. Immunoprecipitation of [$^{35}$S]methionine-labeled cellular lysate with anti-FLAG M2 antibody revealed a band of the size of ≈44 kDa, only in COS7 cells transfected with pcDNA3.1/TIM-FLAG (Fig. 3). The ≈44-kDa protein probably represents the mature form of mTim44 in which the ≈6-kDa segment has been proteolytically cleaved. No distinct bands of 44 kDa were visualized in the lysates of nontransfected cells or cells transfected with pcDNA3.1. Immunofluorescence microscopy with M2 monoclonal antibody revealed intracellular distribution of mTim44 in ≈10% cells transfected with pcDNA3.1/TIM-FLAG, i.e., FLAG positive cells (Fig. 4). The pattern of localization was identical with that observed for various mitochondrial proteins (22–24). In some of the transfected cells, the immunofluorescence was seen in the form of rodlike elements, suggesting the localization of mTim44 to paracrystalline structures of mitochondria (Fig. 4*B*, arrows). Specific immunofluorescence staining was not observed in cells transfected with pcDNA3.1 or in nontransfected cells (Fig. 4*A*, arrowhead).

The localization of mTim44 to mitochondrial cristae was confirmed by immunoelectron microscopy (Fig. 5). Control sections revealed background staining only (Fig. 5 *A* and *B*). Interestingly, in transfected cells, some mitochondria had a normal structure of the cristae (Fig. 5*A*), whereas in others the cristae had a paracrystalline- or honeycomb-like appearance (Fig. 5 *B*, *D*, and *F*). The latter appearance is reminiscent of "zig-zag out of phase cristae" seen in tissues with very early stages of ischemia (30). Moreover, such an appearance of the mitochondrial cristae is readily visualized in tissues embedded under conditions of low protein denaturation (31) and is usually not seen in tissues processed by conventional methods (30, 32). There was a heavy localization of ImmunoGold particles in these honeycomb-like structures (Fig. 5 *C*, *D*, *F*, and *G*), although the mitochondria with normal appearing cristae also had a reasonable degree of immunolocalization of mTim44 (Fig. 5*E*). The immunolocalization of mTim44 could be readily seen in the paracrystalline arrays and cristae in the same mitochondria and

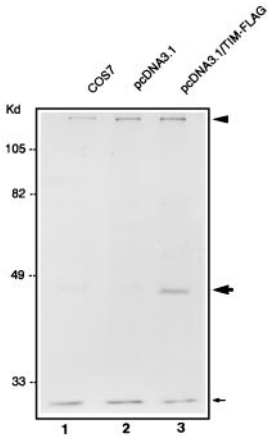


FIG. 3. Immunoprecipitation analysis mTim44-FLAG fusion protein expressed in COS7 cells. COS7 cells were transfected with pcDNA3.1 or with pcDNA3.1/TIM-FLAG construct, and the cells were radiolabeled with [$^{35}$S]methionine. Immunoprecipitation was performed by employing M2 anti-FLAG monoclonal antibody. Lane 1, COS7 cells only; lane 2, COS7 cells transfected with pcDNA3.1; lane 3, COS7 cells transfected with pcDNA3.1/TIM-FLAG construct. In lane 3, an autoradiographic band of ≈44 kDa is visualized (arrow). The arrowhead indicates the radioactivity at the interface of the stacking and running gel. The small arrow indicates free radioactivity.

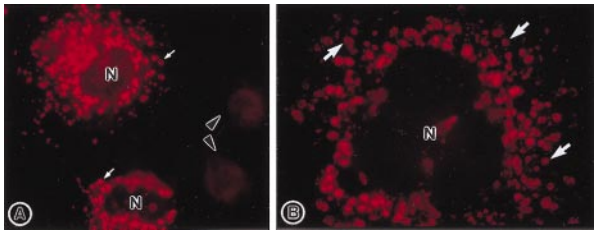


FIG. 4. Immunofluorescence micrographs showing subcellular expression of Tim44-FLAG fusion protein in COS7 cells. The COS7 cells were grown on Lab-Tek chamber slides and transfected with the pcDNA3.1/TIM-FLAG construct. The cells were washed with PBS, fixed in 4% paraformaldehyde, and permeabilized with 0.1% Triton X-100. Localization of mTim44-FLAG fusion protein was delineated by the successive application of M2 anti-FLAG antibody and rhodamine-conjugated goat F(ab′)2 anti-mouse IgG to the permeabilized COS7 cells. About 10% of COS7-transfected cells were FLAG positive. The transfected cells show intracellular distribution of mTim44 associated with mitochondria (arrows in *A*). No immunoreactivity is seen in the nontransfected cell (arrowhead in *A*). In *B*, the immunofluorescence of rod-shaped elements (arrows) suggests the presence of mTim44 in the mitochondrial paracrystalline structures. (*A*, ×1,250; and *B*, ×2,500.)

was not restricted to the zones of contact between the two mitochondrial membranes (Fig. 5*H*).

**mTIM44 Is a Mitochondrial Inner Membrane Protein and Proteolytically Processed by Mitochondrial Import.** [$^{35}$S]Methionine-labeled mTim44 was synthesized *in vitro* by employing the reticulocyte lysate system (26). Addition of mitochondria into the import reaction, containing radiolabeled mTim44, resulted in the visualization of two SDS/PAGE autoradiographic bands, i.e., a premature form (≈50 kDa) and a proteolytically processed mature form (≈44 kDa) (Fig. 6*A*, lane 2). In the absence of mitochondria in the reaction mixture, only one band, corresponding to the premature form, was observed (Fig. 6*A*, lane 1). To determine whether mitochondrial import and the proteolytic conversion was membrane potential ($\Delta\Psi$)-dependent, carbonyl cyanide *m*-chlorophenylhydrazone, an effective uncoupler of $\Delta\Psi$ in the mitochondrial system, was added into the import reaction mixture (33–35). With carbonyl cyanide *m*-chlorophenylhydrazone treatment, only the premature form was observed, indicating that mitochondrial import of mTim44 is $\Delta\Psi$-dependent (Fig. 6*A*, lane 4). The imported mature form of mTim44 in intact mitochondria was protected from proteinase K enzymatic digestion (Fig. 6*A*, lane 3). During concomitant treatment of proteinase K and digitonin (0.2 mg/ml), the latter selectively permeabilized the outer mitochondrial membrane and allowed protease access to the intermembrane space (36–38), resulting in the visualization of a single band of mTim44 with molecular size of ≈44 kDa, indicating that the mature form is protected from proteinase K digestion (Fig. 6*B*, lane 3). At higher concentrations of digitonin (0.3–0.5 mg/ml), which disrupts the inner mitochondrial membrane, the SDS/PAGE autoradiographic band of the mature form of mTim44 was not visualized (Fig. 6*B*, lanes 4 and 5). To determine the association of mTim44 with the mitochondrial membranes, mitochondria from the reaction mixtures were pelleted, resuspended in 0.1 M $Na_2CO_3$, pH 11.5, and then sonicated to release the proteins of intermembrane space and matrix (39). SDS/PAGE analyses of the supernatant of sonicated and pelleted membranes revealed a band of ≈44-kDa, indicating that the mTim44 is associated with membrane (Fig. 6*A*, lane 5).

## DISCUSSION

In the analyses of differential regulation of various genes, DD-PCR has been employed with a certain degree of success. For instance, with the use of DD-PCR, some of the differentially expressed genes in the hyperglycemic state were isolated from aortic smooth muscle cell (40), retinal pericyte (41), and heart (8); however, no mRNA transcripts were detected. The

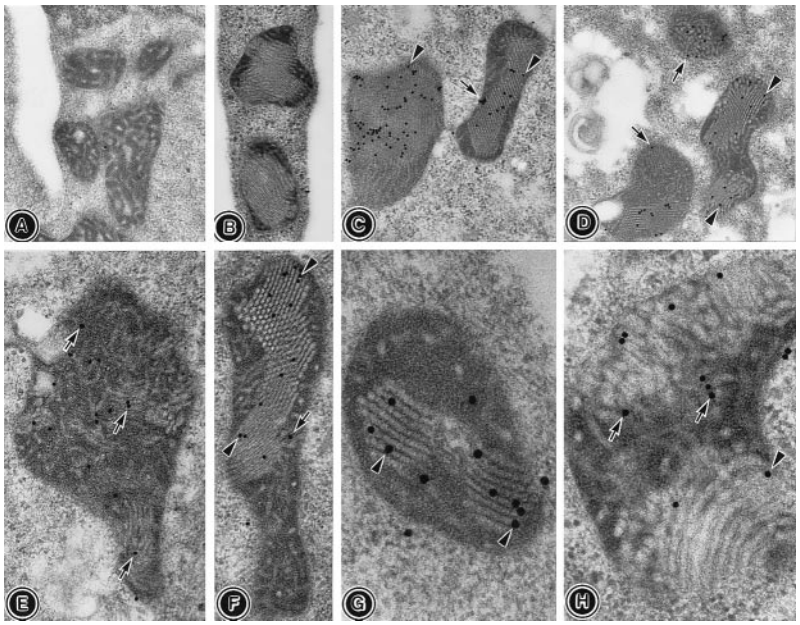

FIG. 5. Electron micrographs showing the immunolocalization of mTim44 in the mitochondrial cristae of COS7 cells transfected with the pcDNA3.1/TIM-FLAG construct. The cells were fixed and processed for immunoelectron microscopy. The electron microscopy sections were successively incubated with primary mouse anti-FLAG M2 monoclonal antibody and secondary goat anti-mouse IgG antibody conjugated with colloidal gold. The mitochondrial cristae of the transfected cells had paracrystalline arrays or a honeycomb-like appearance besides their normal configuration as internal ridges or finger-like protrusions of the inner membrane into the mitochondrial matrix (*C*, *D*, *G*, and *F*). A relatively high concentration of ImmunoGold particles is seen in the honeycomb-like structures or paracrystalline arrays (arrowheads in *C*, *D*, *F*, *G*, and *H*) compared with normal appearing mitochondrial cristae (arrows in *C*, *D*, *E*, *F*, and *H*). Conceivably, the paracrystalline arrays or honeycomb-like structures are derived from the mitochondrial cristae under stress conditions (30). *A* and *B* are mitochondria from control tissue sections incubated with mouse IgG only. (*A–C*, ×27,000; *D*, ×20,000; *E* and *F*, ×40,000; *G*, ×100,000; and *H*, ×70,000.)

isolation of such false positive clones is mainly because of the use of a relatively short 5′ random primer and a nonspecific oligo(dT) primer. In contrast, the cDNA–RDA yields highly reproducible and nonbiased results in terms of selective detection of differentially expressed genes. In the present study, nine subtracted genes were found in the STZ-induced diabetic state with seven exhibiting differential up-regulation in the mouse kidney. Like the up-regulation of clones 1 and 2 ($Na^+$,$K^+$-ATPase), the $\alpha$1 and $\beta$1 subunits of this enzyme have been previously reported with increased expression in diabetic rat kidney (42). Similarly, the up-regulated expression of clone 4 (L-ferritin) may be because of a common effect of glucose in which it induces differential expression of ferritin in certain organ systems, e.g., pancreas (43), and a net increase in its serum level (44) in the diabetic state. Clone 3 seems to be a mouse homologue of recently reported rat ribosomal protein L40, which contains the N-terminal conserved 76 amino acids of ubiquitin (45). The functional significance of L40 is unknown. The full coding sequence of clone 6 had no significant homology at the nucleotide level; however, its deduced amino acid sequence revealed 29% identity and 50% similarity with yeast translocase of the inner mitochondrial membrane 44, yTim44.

In general, mitochondria of yeast, insect, or mammalian cell and plant chloroplasts import their nuclear-encoded proteins from the cytoplasm in a precursor form of preproteins with N-terminal extensions; the latter serve as targeting/signal peptides (28, 46–48). Recently, several proteins involved in mitochondrial protein import have been identified, and they include: (*i*) cytosolic cofactors; (*ii*) mitochondrial outer membrane proteins, including import receptors and the general insertion pore; (*iii*) mitochondrial inner membrane proteins; (*iv*) matrix-processing peptidase; (*v*) matrix heat shock proteins Hsp70 and Hsp60, and their related macromolecules (49). The cofactors prevent the misfolding and aggregation of the positively charged N terminus of the preproteins in an ATP-dependent manner in the cytosol. Interestingly, the positively charged amino acids at the N terminus influence the uptake of prepeptides into the mitochondria (50). The preproteins bind to the receptors at the surface of the outer mitochondrial membrane. They are then translocated across the mitochondrial outer membrane by the general insertion pore complex. The matrix-targeted preproteins are then received by the mitochondrial inner membrane transport machinery, which further translocates them across the inner membrane; the latter process is believed to be membrane potential-dependent. In the matrix, Hsp70 binds to the preproteins (51, 52) after which the presequence is cleaved by the mitochondrial processing peptidase. Finally, the mature mitochondrial proteins undergo folding with the aid of Hsp60.

The mitochondrial outer membrane complex has been described in both mammalian and yeast systems, and the components of the inner membrane complex so far have been cloned and characterized in the yeast system only. The yTim44 was

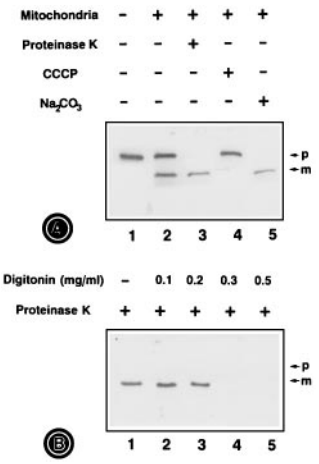


FIG. 6. Mitochondria import assay of mTim44. (*A*) [$^{35}$S]Methionine-labeled mTim44 was generated by *in vitro* coupled transcription–translation, using a rabbit reticulocyte lysate system. The reticulocyte lysates were incubated in the absence (lane 1) or presence of isolated mitochondria at 30°C for 30 min (lane 2). The experiments were also performed in the presence (lane 4) or absence of carbonyl cyanide *m*-chlorophenylhydrazone (CCCP, 1 $\mu$M) (lanes 1, 2, 3, and 5). The reactions, represented by lanes 1, 2, and 4, were terminated by adding the sample buffer and analyzed by SDS/PAGE. The import reaction, represented by lane 3, was extended for another 30 min by incubation with proteinase K (200 $\mu$g/ml) at 4°C and terminated by adding 2 mM phenylmethylsulfonyl fluoride. The import reaction mixture, represented in lane 5, was reconstituted with 150 $\mu$l of 0.1 M $Na_2CO_3$, pH 11.5, mitochondria-sonicated, and incubated on ice for 30 min; the insoluble membrane fraction was sedimented by centrifugation at 100,000 × *g* for 10 min, and the supernatant was recovered and analyzed. (*B*) Following the import reaction as described for *A*, incubation mixtures were treated with proteinase K (200 $\mu$g/ml) at 4°C for 30 min in the absence (lane 1) and presence (lanes 2–5) of various concentrations of digitonin (0.1–0.5 mg/ml). The precursor form (50 kDa) and processed mature form (44 kDa) of mTim44 are indicated by letters p and m, respectively.

originally cloned from yeast by using the positive selection procedure for the isolation of yeast mutants exhibiting perturbed mitochondrial protein import (53–56). Subsequently, other members of the yeast inner membrane complex were isolated and cloned, and they include Tim23 (57, 58) and Tim17 (58–60). Additional novel components—i.e., 33-kDa and 14-kDa proteins—of inner membrane complex have been also described in the yeast system (61).

The mTim44, described in this study, appears to be a mammalian homologue of yTim44 for the following reasons: the presence of N-terminal mitochondrial targeting sequence in the preprotein form, its immunolocalization to the mitochondria, import of *in vitro* synthesized mTim44 into mitochondria after cleavage of its signal sequence, and the import dependence on the inner membrane potential ($\Delta\Psi$). In addition, like yTim44, mTim44 seems to be a hydrophilic protein without any hydrophobic stretches. Most likely its major portion is associated with the matrix face of the inner mitochondrial membrane, because the mature form of mTim44 exhibited proteinase resistance under the conditions in which outer mitochondrial membranes were selectively permeabilized. The functional significance of yTim44, as an inner mitochondrial membrane import protein, was elucidated by import inhibition experiments with anti-yTim44 antibody (54, 56). Support for yTim44 import functions is also derived from experiments in which yTim44 could be specifically crosslinked to a preprotein in transit across the mitochondrial membranes (55). Finally, the strongest evidence comes from *in vivo* studies on mutant yeast where depletion of yTim44 resulting in the inhibition of mitochondrial transport was observed (53).

The *in vivo* functional evidence needs to be elucidated for mTim44 as well, because it seems to be conserved in mammalian cells. However, this may require elaborate gene knockout strategies in the mouse model. Alternatively, one may explore the genetic mutations in the mitochondrial inner membrane proteins in various disease processes—i.e., familial diabetes—where point mutations were found in the mitochondrial subunits of the respiratory chain complex involved in oxidative phosphorylation. In diabetes mellitus, various abnormalities have previously been reported, such as mitochondrial swelling (62, 63), mitochondrial dysfunction related to $Ca^{2+}$ transport (64), and point or length mutations in its DNA (6, 65, 66). In addition, increased expression of mitochondrial-encoded genes involved in oxidative phosphorylation has been described in diabetes (7). Although a net increase in oxidative phosphorylation in tissues exposed to elevated concentrations of glucose has been observed (6, 67, 68), the expression of other nuclear-encoded mitochondrial enzymes, e.g., cytochrome 7a, is unaltered (7). It is conceivable that up-regulation of certain mitochondrial import proteins—e.g., mTim44—may contribute to the enhanced transport of nuclear-encoded mitochondrial enzyme proteins, which would be expected to cause a net increase in oxidative phosphorylation in diabetes.

In summary, mammalian mTim44, an essential component of the TIM complex that is up-regulated in the hyperglycemic state, is described in this study. The availability of mTim44 along with Tim17 (69) should facilitate the isolation of other members of TIM complex by using systems that involve protein–protein interactions, e.g., yeast two-hybrid system.

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