

Research article

**THE IDENTIFICATION AND CHARACTERIZATION OF A NEW GTP-BINDING PROTEIN (Gbp45) INVOLVED IN CELL PROLIFERATION AND DEATH RELATED TO MITOCHONDRIAL FUNCTION**

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**Abstract:** We describe the identification and characterization of a GTP-binding protein with a molecular weight of 45 kD (Gbp45). Gbp45 cDNA was found to overlap with a hypothetical human protein, PTD004, the sequence of which was previously deposited in the databases. The gene for PTD004 was recently found to be one of the ATPases, hOLA1 (human Obg-like ATPase 1). The Gbp45 gene encodes a protein of 396 amino acid residues. Immunocytochemical analysis and examination with GFP-tagged protein revealed that Gbp45 is primarily located in the cytosolic compartment. Immunoblot analysis showed that the Gbp45 protein is strongly expressed in the neuronal tissues and pancreas. T43N and T56N mutations resulted in a loss of Gbp45's ability to bind to GTP and a loss of GTPase activity. In cultured cells, the transfection of wild-type Gbp45 accelerated cell proliferation, though T43N and T56N mutations induced cell death. Down-regulating Gbp45 expression decreased the cell proliferation rate and increased the rate of cell death induced by the inhibition of mitochondrial electron transport. These findings indicate that Gbp45 plays important roles in cell proliferation and death related to mitochondrial function.

**Key words:** Gbp45, GTPase, Small GTP-binding protein, Mutant, Cell growth, Mitochondria

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Abbreviations used: Gbp45 – GTP-binding protein with a molecular weight of 45 kD; SSH – subtraction suppression hybridization; GST – glutathione-S-transferase protein

## INTRODUCTION

Small monomeric GTP-binding proteins have been found to play important roles in the regulation of basic cellular processes in all organisms [1]. Approximately one percent of the human genome encodes proteins that either regulate or are regulated by direct interaction with this type of protein [2]. Through a series of complex biochemical networks, these highly conserved molecular switches control some of the most fundamental cellular processes that are common to all eukaryotes, including morphogenesis, polarity, movement, and cell division [2]. All small GTPases work as binary switches existing in GTP-bound or GDP-bound conformations [1]. The GTP-bound state functions as the “on”-state based on its interaction with downstream effectors or target proteins. GTP binding induces a conformational change mainly in two regions, switch 1 and switch 2, which relay the signal by interacting with and modifying the activity of effector proteins. These target proteins function as the molecular links between a given GTP-binding protein and the regulated cellular process.

Any event that decreases oxidative phosphorylation, issuing either from genetic damage or from the cell environment, can provide a proliferative advantage to transformed or tumor cells [3]. The mitochondria of rapidly growing tumors tend to be fewer in number and have smaller numbers of cristae than those of slowly growing tumors [4-6].

We report on the results of a cDNA subtraction screen performed with the aim of identifying GTP-binding proteins in human liver cancer cells treated with a mitochondrial respiratory inhibitor, and on the characterization of a human ORF, which was found to encode a novel GTP-binding protein. We also report on the identification and characterization of a newly identified GTP-binding protein, Gbp45, which does not exhibit sequence similarity with other GTP-binding proteins, although it contains GTP-binding motifs. An immunohistochemical analysis was performed in mouse tissues to determine its distribution in the body. A cytochemical analysis was also carried out to determine the intracellular location of this protein. The GTP-binding and GTPase activities were examined in mutants of this protein. The expression of this protein was also enhanced or reduced in cultured cells in order to examine its characteristics. We also discuss the possible functions of this protein in cells.

## MATERIALS AND METHODS

### Materials

The pEGFP-c1 and pGEX-4T-3 vectors were respectively purchased from BD Bioscience and GE Healthcare. The other reagents used were of the highest grade commercially available. Human hepatoblastoma (HepG2) cells and human embryonic kidney (HEK-293) cells were provided by the Riken Cell Bank (Japan) and cultured in Dulbecco's modified Eagle's medium (Sigma) supplemented with 10% fetal bovine serum and antibiotics.

### **Constructing and sequencing the subtraction suppression hybridization (SSH) cDNA libraries**

SSH libraries were generated using the reagents and protocols provided by BD Biosciences. To find the genes connected with mitochondrial electron transport, we selected HepG2 cells, because of their relatively high resistance to mitochondrial respiratory inhibition. In the SSH library, the RNA from HepG2 treated for 24 h with 0.1  $\mu$ M antimycin A, a specific mitochondrial electron transport inhibitor, was used as a “tester” and the RNA from untreated HepG2 as a “driver”. A reverse transcriptase-polymerase chain reaction analysis of the SSH products showed that the level of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase decreased more than 1000-fold in the SSH library compared with unsubtracted cDNA, suggesting that the subtraction procedure was effective. Clones from each SSH library were sequenced with M13 primers using an ABI BigDye Terminator v2.0 Cycle Sequencing kit (Applied Biosystems) and ABI 3700 DNA Analyzers (Applied Biosystems), according to the manufacturers' protocols.

### **Annotation of the sequencing results**

The sequences generated through deep sequencing of clones from the SSH library were submitted to BLAST searches of various online databases, including the NCBI, GenBank, EMBL, DDBJ, and PDB, to determine the identity of the clones. The sequences extended *in silico* were used to search for corresponding qualifiers.

### **cDNA cloning**

According to the EST sequence of the selected clone, 30-mers and 27-mers were synthesized (5'-GGA ATT CCT TCT CTC GCT GCC GCT GGG ACC-3' and 5'-TCC CCC GGG GGG GGG GGG GGT ACA CAG-3') for PCR (94°C, 30 s; 65°C, 30 s; and 72°C, 2 min, for 35 cycles) using 5  $\mu$ l of a human liver cDNA library as a template. The 1153-base PCR product was cloned using a TA-cloning kit (BD Biosciences). Approximately 1,000 plaques were screened. Single positive clones were isolated after two more rounds of rescreening. The size of the inserts was determined by PCR using two primers from the pT-Adv vector, and the inserts were subcloned. DNA sequencing was performed on an Applied Biosystems automated DNA sequencer.

### **Animal experiments**

All the experiments were approved by the Animal Care and Use Committee of Osaka City University Medical School. Male and female C57BL/6J mice (10-12 weeks old) were obtained from Jackson Laboratories. The mice were allowed free access to laboratory chow and tap water, and used for analysis. Immediately after perfusion of the mice with ice-cold 0.9% NaCl solution, tissue specimens were obtained and used for Western blotting.

**Antibodies**

Polyclonal anti-Gbp45 serum to Gbp45 was generated by immunizing rabbits with a peptide corresponding to 14 residues (WTIRKGTKAPQAAG) of the protein coupled via an N-terminal cysteine. The antiserum was subsequently affinity-purified against the antigenic peptide.

**Gel electrophoresis and Western blotting**

All the samples for Western blotting were first separated on 12% SDS-polyacrylamide minigels prior to transfer onto a polyvinylidene difluoride membrane. The blots were probed with the affinity-purified anti-Gbp45 antibody or anti- $\beta$ -actin antibody at a dilution of 1:1000 as the primary antibody, followed by the addition of a horseradish peroxidase-coupled goat antirabbit IgG secondary antibody (PIERCE) used at a dilution of 1:1000. The amount of bound antibody was detected with the ECL Western Blotting Detection System (GE Healthcare). The expression of the Gbp45 protein was standardized with reference to that of  $\beta$ -actin.

**Construction of the GFP-tagged Gbp45-encoding vector**

pEGFP-Gbp45 encodes a fusion protein consisting of the GFP tag at the N-terminus and the amino acids of the human Gbp45 protein. To form this plasmid, a fragment encoding the EcoRI and SmaI restriction sites and amino acids was amplified by PCR as described above in cDNA cloning. pEGFP-Gbp45 was synthesized by ligating the EcoRI/SmaI-treated PCR products into the EcoRI/SmaI sites of the pEGFP-c1 vector. GFP-tagged mutant <sup>T43N</sup>Gbp45 and <sup>T56N</sup>Gbp45-encoding vectors were also constructed with Stratagene's QuikChange II site-directed mutagenesis kit, according to the manufacturer's instructions.

**Fluorescence imaging and vector transfection**

HEK-293 cells were grown on coverslips, stained with anti-Gbp45 antibody followed by FITC-conjugated anti-rabbit IgG antibody, and used for fluorescence imaging. In another experiment, pEGFP-Gbp45 was transiently transfected into HEK-293 cells with Lipofectamine<sup>TM</sup> 2000 (Invitrogen) according to the manufacturer's instructions. The cells were then used for fluorescence imaging. Conventional fluorescence images were obtained with a Zeiss Axiovert 100 microscope equipped with a standard fluorescein filter and a Zeiss Plan-Neofluoar oil immersion lens.

**Construction of the glutathione-S-transferase protein-tagged (GST-tagged) wild-type Gbp45 and mutant Gbp45-encoding vectors**

pGEX-Gbp45 encodes a fusion protein consisting of the GST tag at the N-terminus and amino acids of the human wild-type Gbp45 protein. To form this plasmid, a fragment encoding the EcoRI and SmaI restriction sites and amino acids was amplified by PCR as described above for cDNA cloning. pGEX-Gbp45 was synthesized by ligating the EcoRI/SmaI-treated PCR products into

the EcoRI/SmaI sites of the pGEX-4T-3 vector. GST-tagged mutant <sup>T43N</sup>Gbp45 and <sup>T56N</sup>Gbp45-encoding vectors were also constructed.

#### **Construction of the hairpin siRNA expression vector**

Gene silencing was performed via a plasmid-based siRNA method [7]. The sequence of the human Gbp45 mRNA from bp 169 to 187 was selected to construct the hairpin siRNA expression vector. The selected sequences were compared with the human genome database and EST database to ensure that only the Gbp45 gene was targeted. To design the hairpin siRNA insert, we specified a 19-nt sequence derived from the transcript, separated by a 9-nt spacer from the reverse complement of the same 19-nt sequence. The hairpin siRNA sequences were synthesized as two complementary DNA oligonucleotides, annealed and ligated into the BamI/HindIII sites of the pRNA-U6.1/Neo vector (Genscript, Piscataway, NJ) to form the vector for silencing the Gbp45 gene, pRNA-U6.1/Neo-Gbp45. The resulting transcript was predicted to form a 19-bp stem-loop structure.

#### **Protein purification**

GST-tagged wild-type Gbp45 and mutant <sup>T43N</sup>Gbp45 and <sup>T56N</sup>Gbp45 proteins were purified as described below. The proteins were expressed as a fusion protein with GST in *E. coli* DE21. They were purified on a glutathione-Sepharose 4B affinity chromatography column by elution with 10 mM glutathione, and subsequently released from GST by cleavage with thrombin. The proteins were separated from GST by filtration through a Microcon Ultracel-YM 5000 membrane (Millipore).

#### **GTP<sub>γ</sub>S binding assay**

The degree of binding of [<sup>35</sup>S]GTP<sub>γ</sub>S to Gbp45 and its mutants <sup>T43N</sup>Gbp45 and <sup>T56N</sup>Gbp45 was assessed with the modified rapid-filtration technique described previously [8]. 120 ng of protein was diluted in 40 μl of 1 μM [<sup>35</sup>S]GTP<sub>γ</sub>S-containing (3.0 × 10<sup>3</sup> cpm/pmol) reaction buffer consisting of 20 mM Tris-HCl (pH 8.0), 5 mM MgCl<sub>2</sub>, 1 mM EDTA, 1 mM DTT, and 3 mM dimyristoyl phosphatidylcholine. The incubations were carried out at 30°C for 2 h, and the samples were added to 2 ml of ice-cold stop solution (20 mM Tris-HCl, pH 8.0, 25 mM MgCl<sub>2</sub>, and 100 mM NaCl<sub>2</sub>) and then applied to 25-mm nitrocellulose filters (Schleicher & Shunell, BA-85). The filters were rapidly washed (under suction) with four successive 2-ml volumes of stopping solution, oven-dried, dissolved, and counted in scintillation fluid.

#### **The GTPase activity assay**

The GTPase activity was assayed by measuring the radioactivity of [<sup>32</sup>P]Pi released from [γ-<sup>32</sup>P]GTP [8]. Purified Gbp45 and its mutants <sup>T43N</sup>Gbp45 and <sup>T56N</sup>Gbp45 were incubated for 30 min at 30°C in a reaction mixture (100 μl) containing 1 μM [γ-<sup>32</sup>P]GTP (3.0 × 10<sup>3</sup> cpm/pmol), 20 mM Tris-HCl at pH 7.5, 1 mM EDTA, 6 mM MgCl<sub>2</sub>, 1 mM DTT, 800 mM NaCl, and 3 mM DMPC.

After the incubation, a 50- $\mu$ l aliquot was added to 0.75 ml of an ice-cold stopping solution containing 5% (w/v) charcoal and 50 mM  $\text{NaH}_2\text{PO}_4$ . The mixture was centrifuged at 1,000 x g for 10 min at room temperature. The amount of [ $^{32}\text{P}$ ]Pi released from [ $^{\text{R}}\text{-}^{32}\text{P}$ ]GTP was then estimated by counting the radioactivity of 0.5 ml of the clear supernatant.

#### **Transfection of Gbp45 and its mutant vectors and cell death assays**

The pEGFP-Gbp45, pEGFP- $^{143\text{N}}$ Gbp45 and pEGFP- $^{156\text{N}}$ Gbp45 vectors were transiently transfected into HEK-293 cells with Lipofectamine<sup>TM</sup> 2000. After 24 h, the cells were used for fluorescence imaging. Conventional fluorescence images were obtained with a Zeiss Axiovert 100 microscope equipped with a standard fluorescein filter and a Zeiss Plan-Neofluoar oil immersion lens. The degree of cell mortality was also determined using the trypan blue exclusion assay, performed as follows. Cells were suspended by gentle pipetting, and 50  $\mu$ l of 0.4% trypan blue solution was mixed with 200  $\mu$ l of the cell suspension (final concentration 0.08%) at room temperature. The stained cells were counted within 3 min of mixing with the trypan blue solution. 100 cells were counted for each trypan blue exclusion assay. The cell mortality was then determined as the percentage of trypan blue-stained cells, representing the population of dead cells in the whole cell population, including both adherent and floating cells, at the termination of the experiments.

#### **Establishing a cell line with stably reduced expression of Gbp45**

HEK-293 cells were transfected with pRNA-U6.1/Neo-Gbp45 and cultured in DMEM medium supplemented with 10% FBS, antibiotics, and 0.1 mg/ml of G418 sulfate. After a 2-week culture, G418-resistant colonies were collected, and single cell clones (HEK-293-siRNA-Gbp45) were obtained by limited dilution. For the experimental controls, the empty vector (pRNA-U6.1/Neo) was also transfected into HEK-293 cells, followed by G418 selection to obtain HEK-293-siRNA.

#### **Statistical analysis**

All the experiments were repeated at least three times with independent treatments, each of which yielded essentially the same results. All the cell-death and cell-viability experiments were performed with  $n = 7$ . All the values in the figures are the means  $\pm$  SD. The statistical examination was performed by the analysis of variance followed by the post hoc test, with findings of  $p < 0.05$  considered significant.

## **RESULTS**

#### **Identification and sequence characterization**

We picked one of the spots that was strongly expressed in the antimycin A-treated cells (indicated by the arrow in Fig. 1) and cloned it completely. Based on the possible presence of a GTP-binding region and sequence with GTPase activity, we named the protein Gbp45 (GTP-binding protein with a molecular weight of 45 kD).

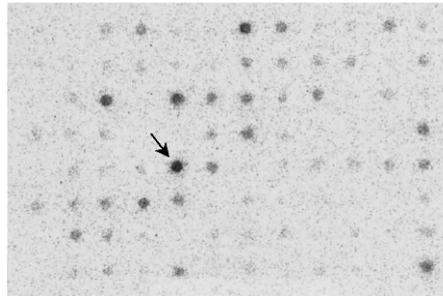


Fig. 1. Subtraction suppression hybridization. The spots expressed in antimycin A-treated cells are presented as described in the text. One of the strongly expressed spots (arrow) was cloned.

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1 CAGCCGTTGAGGGGACGGGCTGCGTTCTCTCTCTCTCTCCCGCCTCCAGCTGCCGG
61 CAGGACCTTTCTCTCGTGCCTGGGACCCCGTGTGATGCCCCAGGCOGAGCAOGATGC
M P 2
121 CCCCTAAAAAGGGAGGTGATGGAATTAACCACCCCAATCAITGGAAGATTGGAACT
P K K G G D G I K P P P I I G R F G T S 22
181 CACTGAAAATGGTATTGGTGGATTGCCAAATGTGGGAAATCTACTTTCTCAATGTT
L K I G I V G L P N V G K S T F F N V L 42
241 TAACCAATAGTCAGGCTTCAGCAGAAAATCCCGTTCTGCACTATTGATCCTAATGAGA
T N S Q A S A E N F P F C T I D P N E S 62
301 GCAGAGTACCTGTGCCAGATGAAAGTTGACTTTCTTTGTCAATACCACAAACGACAA
R V P V P D E R F D F L C Q Y H K P A S 82
361 GCAAAATTCCTGCCTTTCTAAATGTGGTGGATATTGCTGGCCTGTGAAAAGGAGCTCACA
K I P A F L N V V D I A G L V K G A H N 102
421 ATGGGCAGGGCCTGGGGAATGCTTTTTATCTCATATTAGTGCCTGTGATGGCATCTTC
G Q G L G N A F L S H I S A C D G I F H 122
481 ATCTAACACGTCTTTGAAGATGATATCACGCACGTGAAGGAAGTGTAGATCTIA
L T R A F E D D D I T H V E G S V D P I 142
541 TTCGAGATATAGAAAATAACATGAAGAGCTTCAGCTTAAAGATGAGGAAATGATTGGGC
R D I E I I H E E L Q L K D E E M I G P 162
601 CCATTATAGATAAACTAGAAAAGGTGGCTGTGAGAGGAGAGATAAAAACTAAAACCTG
I I D K L E K V A V R G G D K K L K P E 182
661 AATATGATATAATGTCAAAGTAAAATCCTGGGTTATAGATCAAAGAAAACCTGTTCGCT
Y D I M C K V K S W V I D Q K K P V R F 202
721 TCTATCATGATTGGAATGACAAAGAGATTGAAGTGTGAATAAACACTTATTTTGACTT
Y H D W N D K E I E V L N K H L F L T S 222
781 CAAAACCAATGGTCTACTTGGTTAATCTTTCTGAAAAGACTACATTAGAAAAGAAAACA
K P M V Y L V N L S E K D Y I R K K N K 242
841 AATGGTTGATAAAAAATTAAGAGTGGGTGGACAAGTATGAOCCAGTGCCTTGGTCATTC
W L I K I K E W V D K Y D P G A L V I P 262
901 CTTTATGTTGGGCTTGAACTCAAGTTCGAAGAATTGAGTGCCTGAGGAGAGACAGAAGT
F S G A L E L K L Q E L S A E E R Q K Y 282
961 ATCTGGAAGOGAACATGACACAAAGTCTTTGCCAAGATCAITTAAGGCTGGGTTGCAG
L E A N M T Q S A L P K I I K A G F A A 302
1021 CACTCCAAC TAGAATACTTTTCTACTGCAGGCCAGATGAAGTGCCTGCATGGACCATCA
L Q L E Y F F T A G P D E V R A W T I R 322
1081 GGAAAGGGACTAAGGCTCCTCAGGCTGCAGGAAAGATTACACAGATTTTGAAGGGGAT
K G T K A P Q A A G K I H T D F E K G F 342
1141 TCATTATGGCTGAAGTAATGAAATACGAAGATTTAAAGAGGAAGGTTCTGAAAATGCAG
I M A E V M K Y E D F K E E G S E N A V 362
1201 TCAAGGCTGCTGGAAAAGTACAGACAACAAGGCAGAAAATATAATTGTGAAGATGGAGATA
K A A G K Y R Q Q G R N Y I V E D G D I 382
1261 TTATCTCTTCAAATTTAACACACCTCAACAACCGAAGAAGAAAATAAAATTTAGTTATTG
I F F K F N T P Q Q P K K K 402
1321 CTCAGATAAACATACAACCTCCAAGGCATCTGATTTTAAAAAATAAAAATTTCTGAA
1381 AACCAATGGCACAATAAAGTGGGGAGATGGGAATCTTTGACAAAATAATATTTTAT
1441 TTGTTTTAAAAATTAATACTGTGTACCCCCCCCCCATGAAATGCAGGTTCACTAAA
1501 TGTGAACAGCTTTGCTTTTCACTGATTAAGACCTACTCCAATTTGAGAAGCTTTTCA
1561 GGAACCAATTTACTCTCATGATCTTCAATTAATCTCCATCATGTATGCCAAGCTGCAC
1621 ATTTGACAGTGAAGACAATGTGGCTTGTCTCTTTTGAATCTACAGATAATGCATGTTTT
1681 ACAGTACTCCAGATGCTCACTCAATAAACATTTGACAAAACAAAAAATAAAAAA
1741 AAAAAAAAAAAAAAAAAAAAA

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Fig. 2. The complete nucleotide and amino acid sequences of the cloned cDNA.

We cloned the cDNA based on the database using PCR and sequenced it completely. The cloned cDNA has 1759 bp and encodes a 396-amino acid polypeptide (Fig. 2). The cDNA was found to overlap with a human protein, PTD004, whose sequence was previously deposited in the databases (GenBank accession no. AF078859). When homology protein searches were performed against databases for other species, two conceptual translation products in mouse (NP\_080218.1) and rat (NP\_001029099.1) were found with extremely high identity scores (Fig. 3). The protein sequence identities with *Xenopus tropicalis* (NP\_001008044.1), *Drosophila melanogaster* (NP\_572580.1) and *Arabidopsis thaliana* (NP\_174346.1) were respectively 86%, 74% and 62%. Its theoretical pI is 7.64, and its predicted molecular weight is 44.7 kDa (as predicted by the Compute pI/Mw tool). The predicted functional domains of G1, G2-1, G2-2, G3, G4, and G5 are suggested in Fig. 3. There are no other remarkable domains in the protein.

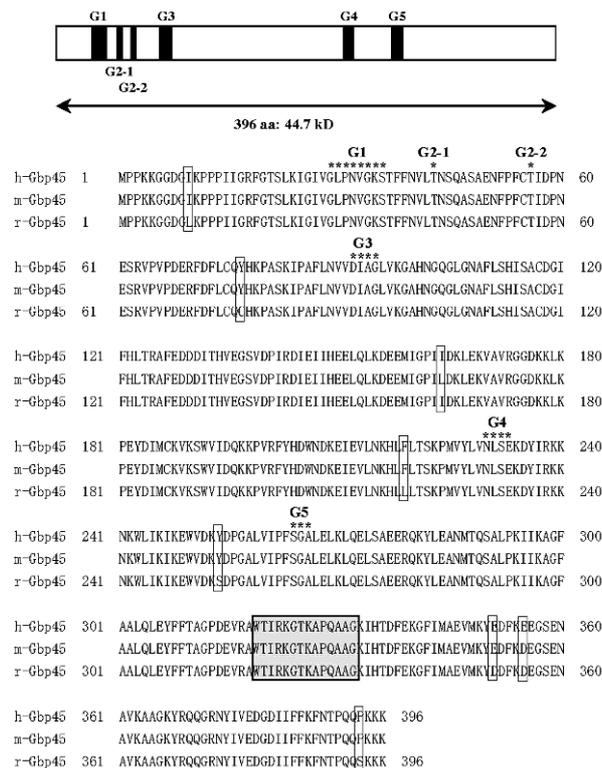


Fig. 3. The domain organization and evolutionary conservation of Gbp45. A schematic overview of Gbp45 protein is presented based on sequence analysis. A comparison of human (h-Gbp45), mouse (m-Gbp45), and rat ERp43 amino acid (r-Gbp45) sequences is also presented. The predicted functional domains of G1, G2-1, G2-2, G3, G4, and G5 are suggested as indicated by the \*. The white boxes show the amino acid differences. The grey box shows the target of the polyclonal anti-Gbp45 serum as described in the text.

### The expression of the Gbp45 protein in various tissues

The tissue distribution of the Gbp45 protein in male and female mice is summarized in Fig. 4. Gbp45 was found to have a relatively high expression in the neuronal tissues and pancreas, but only a very low expression was detected in other tissues.

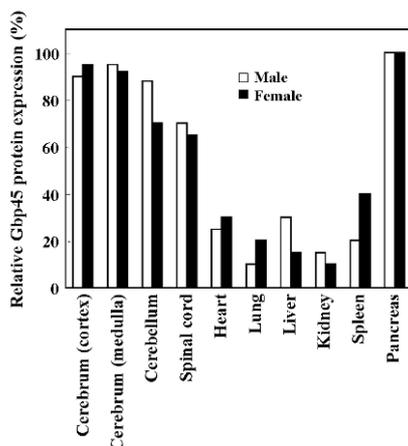


Fig. 4. The expression of Gbp45 protein in the mouse tissues. Western blotting analysis was performed on male and female mice as described in the text.

### Subcellular localization of Gbp45

The SOSUI membrane protein predictor (<http://bp.nuap.nagoya-u.ac.jp/sosui/>) predicted Gbp45 to be a soluble protein, while the PSORT protein-sorting signal and localization site predictor (<http://psort.nibb.ac.jp/>) suggested that it is a cytoplasmic protein. To determine the subcellular location of the Gbp45 protein, HEK-293 cells were transfected with the pEGFP-Gbp45 vector, and observed with confocal microscopy. GFP-tagged protein fluorescence was detected in the cytosolic compartment (Fig. 5A). To determine the location of the endogenous Gbp45, HEK-293 cells were analyzed by immunofluorescence staining of Gbp45. Again, immunofluorescence was mostly detected in the cytosolic compartment by a peptide competition-inhibitable mechanism (Fig. 5B).

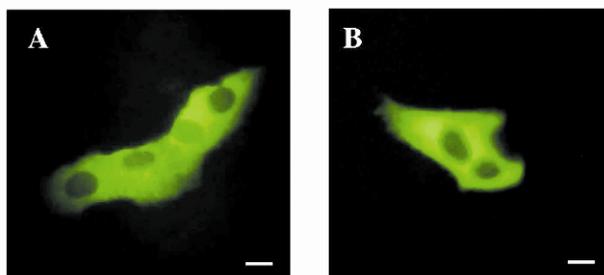


Fig. 5. The subcellular localization of the Gbp45 protein. A – HEK-293 cells were transfected with pEGFP-Gbp45. B – HEK-293 cells were stained with anti-Gbp45 antibody as described in the text. Bar = 10  $\mu$ m.

### The GTP-binding activity and GTPase activity of Gbp45

Thr<sup>43</sup> and Thr<sup>56</sup> were predicted as functional domains included in the G2 box of Gbp45. To determine the importance of Thr<sup>43</sup> (G2-1) and Thr<sup>56</sup> (G2-2), the GTP-binding activities of Gbp45 (Fig. 6A) and its mutants <sup>T43N</sup>Gbp45 (Fig. 6B) and <sup>T56N</sup>Gbp45 (Fig. 6C) were examined. The Scatchard plot analysis revealed that Gbp45, <sup>T43N</sup>Gbp45 and <sup>T56N</sup>Gbp45 respectively bound maximally 0.74, 0.15 and 0.32 mol of this nucleotide per mol proteins, with respective K<sub>d</sub> values of 0.88, 0.60 and 1.95 μM. The time and dose dependencies of the GTPase activity of these proteins was also examined (Fig. 7). The respective specific activities of Gbp45 and its mutants <sup>T43N</sup>Gbp45 and <sup>T56N</sup>Gbp45 were calculated to be 0.22, 0.13 and 0.17 mol Pi/mol of protein/min.

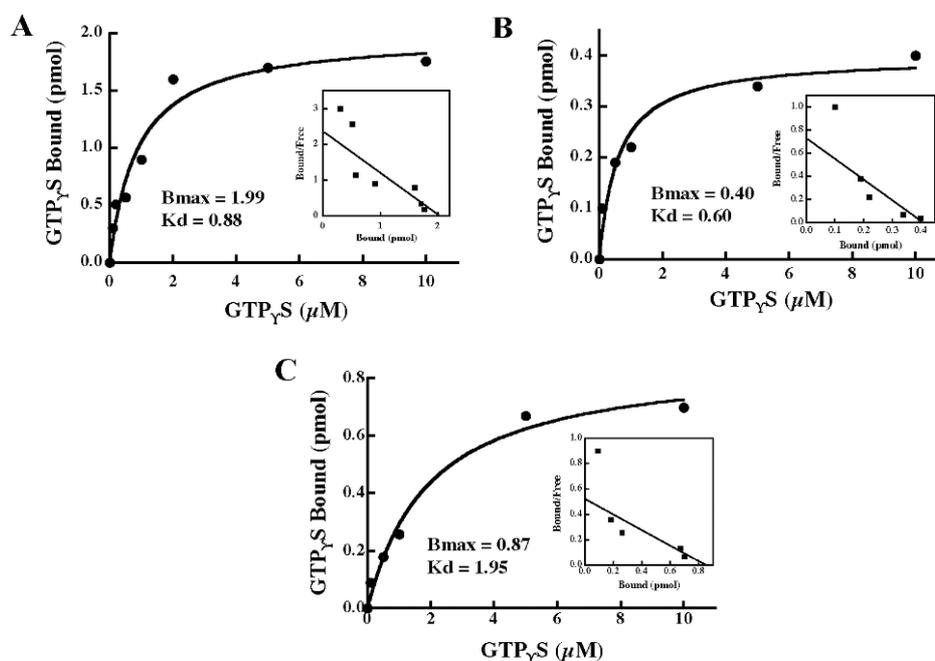


Fig. 6. The GTP-binding activity of Gbp45 and its mutants. The GTP-binding activities of wild-type Gbp45 and its mutants <sup>T43N</sup>Gbp45 and <sup>T56N</sup>Gbp45 were examined as described in the text. A – Dose-dependent effect. 120 ng of Gbp45, <sup>T43N</sup>Gbp45 and <sup>T56N</sup>Gbp45 was incubated with various doses of [<sup>35</sup>S]GTP<sub>γ</sub>S for 30 min at 30°C under the standard conditions. B – Scatchard plots. The results shown are representative of three independent experiments.

### The biological activities of Gbp45

To explore the biological activities of Gbp45, pEGFP-Gbp45, pEGFP-<sup>T43N</sup>Gbp45 and pEGFP-<sup>T56N</sup>Gbp45 vectors were transiently transfected into HEK-293 cells. The expression of the transfected GFP-tagged proteins is shown in phase-contrast images in Fig. 8A. The transfection efficiencies yielded by analyzing GFP fluorescence intensity and GFP protein expression were almost

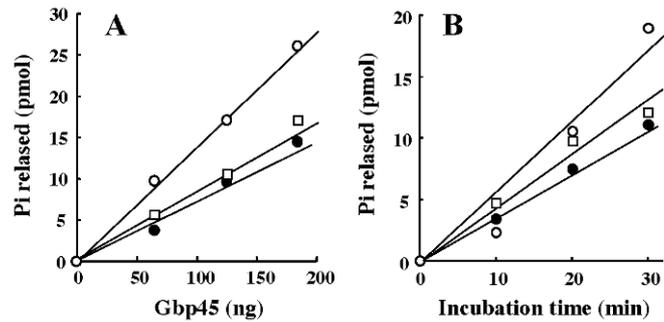


Fig. 7. The GTPase activity of Gbp45 and its mutants. This is a dose-dependent effect: various doses of wild-type Gbp45 (open circles) and its mutants <sup>T43N</sup>Gbp45 (closed circles) and <sup>T56N</sup>Gbp45 (open squares) were incubated with 1  $\mu$ M [ $g$ -<sup>32</sup>P]GTP for 30 min at 30°C under the standard conditions (A). Time course: the GTPase activity of 120 ng of wild-type Gbp45 (open circles) and its mutants <sup>T43N</sup>Gbp45 (closed circles) and <sup>T56N</sup>Gbp45 (open squares) was assayed with 1  $\mu$ M [ $g$ -<sup>32</sup>P]GTP for various periods of time at 30°C (B). The results shown are representative of three independent experiments.

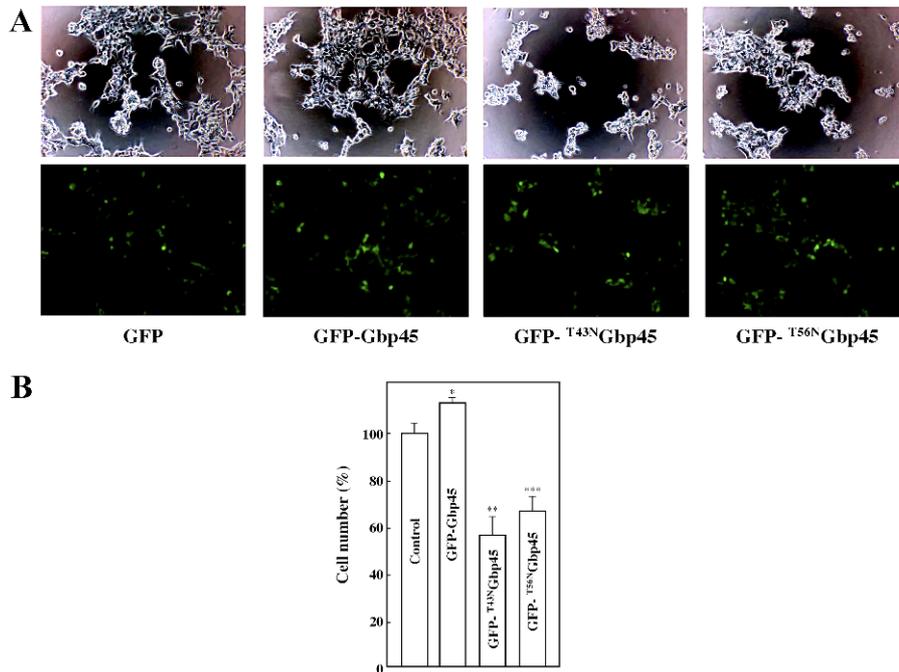


Fig. 8. The effects of overexpression of wild-type Gbp45 and its mutants <sup>T43N</sup>Gbp45 and <sup>T56N</sup>Gbp45. pEGFP-Gbp45, pEGFP-<sup>T43N</sup>Gbp45, and pEGFP-<sup>T56N</sup>Gbp45 vectors were transiently transfected into HEK-293 cells. The expression of the transfected GFP-tagged proteins is shown in phase-contrast images (A). The proliferation of cells transfected with these vectors is shown in B. The values are the means  $\pm$  SD (n = 7). \* $P$  < 0.05 vs. wild-type, \*\* $P$  < 0.01 vs. wild-type, \*\*\* $P$  < 0.01 vs. wild-type.

the same (data not shown). The rate of proliferation of GFP-Gbp45-expressing cells was about 1.2-fold that of empty vector-transfected cells (Fig. 8B). When transfected with pEGFP-<sup>T43N</sup>Gbp45 and pEGFP-<sup>T56N</sup>Gbp45 vectors, about 60% and 70% of cells, respectively, were viable.

By transfecting pRNA-U6.1/Neo-Gbp45, a hairpin siRNA expression vector targeting Gbp45 mRNA, into HEK-293 cells, we could obtain cells (HEK-293-siRNA-Gbp45) in which Gbp45 was stably silenced. However, a control vector, pRNA-U6.1/Neo, exhibited no silencing response in HEK-293-siRNA (Fig. 9A). The growth rate of HEK-293-siRNA-Gbp45 was lower than that of the cells transfected with the empty vector (Fig. 9B). When treated with the mitochondrial electron transport inhibitors Rotenone or Antimycin A, HEK-293-siRNA-Gbp45 exhibited a decreased resistance to the cytotoxic activity of these agents (Fig. 9C).

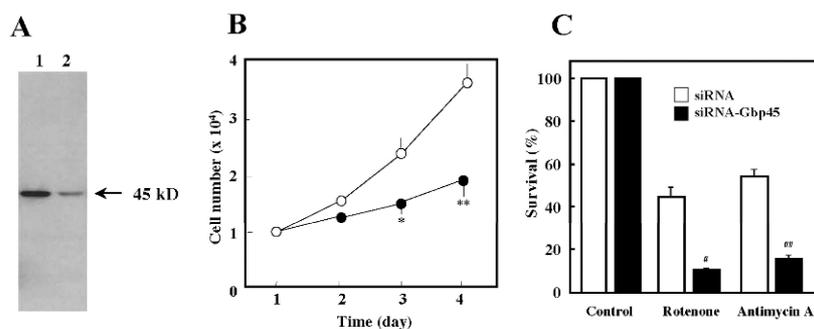


Fig. 9. The effect of silencing Gbp45 on cell growth and mitochondrially mediated cell death. A – HEK-293 cells transfected with empty vector pRNA-U6.1/Neo (HEK-293-siRNA) or pRNA-U6.1/Neo-Gbp45 (HEK-293-siRNA-Gbp45) were analyzed by Western blotting using anti-Gbp45 antibody. Lane 1: HEK-293-siRNA; lane 2: HEK-293-siRNA-Gbp45. B – At the indicated times, the number of cells was counted and plotted. Open squares: HEK-293-siRNA; closed squares: HEK-293-siRNA-Gbp45. The values are the means  $\pm$  SD (n = 7). \* $P$  < 0.01 vs. HEK-293-siRNA group on day 3, \*\* $P$  < 0.01 vs. HEK-293-siRNA group on day 4. C – The cells were placed for 24 h in a medium containing Rotenone (1  $\mu$ M) or Antimycin A (0.1  $\mu$ M). Cell mortality was determined via the trypan blue exclusion assay. Open bars, HEK-293-siRNA; closed bars, HEK-293-siRNA-Gbp45. The values are the means  $\pm$  SD. # $P$  < 0.01 vs. HEK-293-siRNA group treated with Rotenone, ## $P$  < 0.01 vs. HEK-293-siRNA group treated with Antimycin A.

## DISCUSSION

In this study, we identified Gbp45, which was previously uncharacterized as a GTP-binding protein, although with the increase in the number of sequences listed in public databases, a variety of new GTP-binding proteins have recently been described in the literature. Gbp45 cDNA was found to overlap with a hypothetical human protein, PTD004, the sequence of which had previously been deposited in the databases. The gene for PTD004 was identified as

a homologue of yeast-44.2 kD protein (Zhang *et al.*, 1998, direct submission). Recently, the gene for PTD004 was found to be one of the ATPases, hOLA1 (human Obg-like ATPase 1) [9]. The protein was characterized and found to bind and hydrolyze ATP. Although this protein contains GTP-binding motifs, it does not share sequence similarity with other GTP-binding proteins, and has thus been classified as a member of a novel group, the GTP1/Obg family, the functions of which remain uncertain. Only suggestions exist concerning the functions of the members of this family. For example, Sikora-Borgula *et al.* [10] recently reported that homologues of CgtA, the common GTP-binding protein of *V. harveyi*, play roles in the coupling of DNA replication to cell growth and cell division. As homologues of the *V. harveyi* CgtA protein found in different organisms exhibit high levels of similarity to this protein, it might be speculated that this type of role is also played by other members of the GTP1/Obg subfamily, including GTP-binding protein PTD004.

Using the protein sequence of Gbp45 to search the NCBI/EMBL protein database revealed that proteins homologous to it exist in many eukaryotic species from *A. thaliana* to humans. Although these were hypothetical proteins (without experimental evidence for their actual existence), this high degree of evolutionary conservation suggests that Gbp45 may have important biological functions. This was confirmed by our findings that cell proliferation was accelerated by overexpression of Gbp45, and that cell viability was decreased by the expression of mutants of the protein.

The amounts of Gbp45 in the cerebrum, cerebellum, spinal cord and pancreas were significantly larger than those in the heart, lung, liver and spleen. This protein is thus expressed relatively strongly in the tissue of the nervous system. The subcellular localizations of Gbp45 predicted by PSORT were the cytosol (probability 56.5%), mitochondria (17.4%), and nucleus (13%). Indeed, the results of immunocytochemical analysis and examination with GFP-tagged protein suggested that Gbp45 is probably located primarily in the cytosolic compartment.

Thr<sup>43</sup> (G2-1) and Thr<sup>56</sup> (G2-2) were predicted as functional domains included in the G2 box of the GTP-binding protein. The GTP-binding activity and GTPase activity of the mutants <sup>T43N</sup>Gbp45 and <sup>T56N</sup>Gbp45 was significantly decreased compared with that of the wild-type. Thr<sup>43</sup> and Thr<sup>56</sup> thus play critical roles in the biological activities of Gbp45. It should be noted that the rate of proliferation of Gbp45-overexpressing cells was slightly increased. On the other hand, expression of <sup>T43N</sup>Gbp45 and <sup>T56N</sup>Gbp45 markedly decreased the number of viable cells. In the experiment using hairpin siRNA expression vectors, silencing Gbp45 reduced the cell proliferation rate. These findings revealed that Gbp45 plays an essential role in cell growth. Furthermore, T43N and T56N mutants possibly affect the cellular status not only with their dominant negative activity but also their cytotoxic effect. Further study on the details is needed.

The mitochondrial electron transport chain is both the cellular source of ATP generated via oxidative phosphorylation and the main source of ROS including

O<sub>2</sub><sup>-</sup> by mitochondrial electron transport inhibition [11, 12]. Hydrogen peroxide, which is itself a dangerous oxidant due to its production of hydroxyl radicals via events such as the Fenton reaction, is generated both in the spontaneous dismutation of O<sub>2</sub><sup>-</sup> or in the catalytic dismutation of it by superoxide dismutase. In this study, the cells with stably reduced expression of Gbp45 exhibited a decreased resistance to the cytotoxic activity of the mitochondrial electron transport inhibitors Rotenone or Antimycin A. This indicated that Gbp45 plays important roles related to mitochondrial electron transport inhibition, which causes ATP depletion and uncontrolled ROS production. It should be noted that Gbp45 expression had no effect on oligomycin-induced cell death (oligomycin is an inhibitor of ATP production; data not shown). The molecular mechanisms based on these phenomena are the subject of our current investigations. In summary, this study identified a novel and highly conserved GTP-binding protein, Gbp45, which may be representative of a new group of proteins. Investigation of Gbp45 will likely reveal new pathways of signaling in the cell cycle and cell death.

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