Mitochondrial Functions and Estrogen Receptor-dependent Nuclear Translocation of Pleiotropic Human Prohibitin 2*  

From the †Department of Biochemistry, Jichi Medical University, 3311-1 Yakushiji, Shimotsuke, Tochigi 329-0498, Japan and the **Graduate School, Women’s University of Nutrition, Saitama 350-0288, Japan

Proteins with multiple cellular functions provide biological diversity to eukaryotic cells. In the current studies, we identified the mitochondrial functions of human prohibitin 2 (PHB2), which was initially identified as a repressor of estrogen-dependent transcriptional activity. The mitochondrial complex of PHB2 consists of PHB1, voltage-dependent anion channel 2, adenine nucleotide translocator 2, and the anti-apoptotic Hax-1, which is a novel binding partner for PHB2. RNA interference-mediated knockdown of PHB2 in HeLa cells resulted in caspase-dependent apoptosis through down-regulation of Hax-1 and fragmentation of mitochondria. We also found that, in caspase-dependent apoptosis through down-regulation of PHB2, which is a novel binding partner for PHB2. RNA interference-mediated knockdown of PHB2 in HeLa cells resulted in caspase-dependent apoptosis through down-regulation of Hax-1 and fragmentation of mitochondria. We also found that, although PHB2 is predominantly expressed in the mitochondria of HeLa cells, it translocates to nucleus in the presence of estrogen receptor α and estradiol. Here, we first demonstrated the roles of mammalian PHB2 in mitochondria and the molecular mechanism of its nuclear targeting and showed that PHB2 is a possible molecule directly coupling nuclear-mitochondrial interaction.

In eukaryotes, biological diversity is acquired from a limited number of genes due to the existence of proteins with multiple cellular functions. A key factor is the dynamic regulation of these diverse cellular functions. One such multifunctional protein, prohibitin (PHB)1, was originally identified in mammals as a putative negative regulator of cell proliferation (1). PHB1 and PHB2 are closely related proteins and are highly conserved among yeast (2), plants (3), worms (4), flies (5), and mammals (6). Subcellular localization of PHBs has been confined to mitochondria in a variety of these species, although they also localize in nucleus in some mammalian cell lines (7, 8). To date, it is known that PHBs are localized in the mitochondrial inner membrane where they form a large protein complex (9).

addition, variety functions of PHBs have been suggested, including a role in cell cycle regulation (1, 10, 11), transmembrane signal transduction (12, 13), and control of life span (14).

In yeast, PHBs have been shown to exist in mitochondria and to function as chaperones that stabilize newly synthesized mitochondrial protein, possibly by negative regulation of the AAA protease (15, 16). Even in Caenorhabditis elegans, PHBs are essential for embryonic viability and germ line differentiation, and deficiency of these proteins results in altered mitochondrial biogenesis (17).

Alternatively, in mammals, knowledge of human PHBs focused on their transcriptional regulatory functions has been accumulated. Human PHB1 has also been shown to interact with the retinoblastoma protein, which results in the inhibition of the transcriptional activity of E2F (10). In a B-cell lymphoma line, stable overexpression of PHB1 protects the cells from camptothecin-induced apoptosis, possibly via down-regulation of E2F activity (18). Furthermore, human PHB2, also known as a repressor of estrogen receptor (ER) activity, has been shown to interact with and inhibit the transcriptional activity of the ER (13). These findings suggest the involvement of mammalian PHB proteins in the regulation of transcription in the nucleus.

On the contrary, in fibroblasts, mammalian PHB proteins mainly localize in mitochondria, and their expression is up-regulated by mitochondrial stress and down-regulated during cellular senescence (19). Therefore, it is thought that mammalian PHB proteins also have crucial roles in the mitochondria. Recently, human PHB1 was reported to associate with mitochondrial complex I, suggesting that PHB1 plays a role in complex assembly (20). Thus, mammalian PHBs have been suggested to be involved not only in the regulation of transcription but also cellular senescence, apoptosis, and mitochondrial respiratory activity (9, 18). The molecular functions of PHBs in mitochondria, especially PHB2, however, remain unclear.

In this study, to clarify the dynamic regulatory mechanism of the pleiotropic PHB2 in mammalian cells, we examined mitochondrial function of human PHB2 and then investigated its targeting mechanism to the nucleus. First, we identified the binding partners for PHB2 in the mitochondria by immunoprecipitation and mass spectrometric analysis. Hax-1, which was initially identified as an H51-binding protein (21) and as an inhibitor of apoptosis (22), was found to directly associate with PHB2 in mitochondria. RNA interference (RNAi)-mediated knockdown of PHB2 resulted in a reduction of the level of Hax-1 protein. In addition, caspase-mediated apoptotic cell death was enhanced in PHB2 knockdown cells. This induction of cell death was likely due to the down-regulation of Hax-1,
Pleiotropic Functions of Human PHB2

because its knockdown also caused the same manner of cell death without a reduction of PHB2 protein level. Furthermore, the knockdown of PHB2 caused the fragmentation of mitochondria by a mechanism independent of Hax-1.

Secondly, we found that, although PHB2 is predominantly expressed in the mitochondria of HeLa cells, it translocates to the nucleus in the presence of E2 and estradiol (E2). We further found that human PHB2 contains both an uncleavable mitochondrial targeting sequence (MTS) at its N terminus and an ERα-dependent nuclear localization sequence at its C terminus, suggesting that it is shuttled from the nucleus to mitochondria.

Taken together, our results show that human PHB2 has pleiotropic functions in the mitochondria, including inhibition of apoptosis via the PHB2-Hax-1 complex and regulation of the mitochondrial morphology. We also demonstrated that, in the presence of ERα and E2, PHB2 is translocated into the nucleus where it functions as a repressor of transcription. PHB2 is a versatile molecule that couples transcription in the nucleus and regulation of mitochondrial function in the mitochondria, suggesting that it plays a role in communication between these two organelles.

EXPERIMENTAL PROCEDURES

Plasmid Construction—The coding regions of PHB1, PHB2, and Hax-1 were amplified by PCR from a human heart, HeLa cell cDNA library. Amino acids 1–50 and 45–272 of PHB1 and 1–50 and 51–299 of PHB2 were also amplified by PCR. The PCR products with 3× FLAG tag sequences or GFP-coding sequences at their 3′ termini were introduced into mammalian expression vector pCMV-SPORT (Invitrogen). Amino acids 1–100 and the full-length (1–299) of PHB2 were amplified by PCR and introduced into mammalian expression vector pEF4/Myb-His B (Invitrogen). The coding sequence of PHB2 was also inserted into the pGEX-4T-3 vector (Amersham Biosciences) to express it as a GST fusion protein. For RNAi, siRNA sequences were introduced into the pSilencer 3.1-H1 Puro vector (Ambion, Austin, TX).

Cell Culture and Transfection—HeLa and MCF7 cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin at 37 °C in an atmosphere containing 5% CO₂. Transfection was performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. Mitochondria were stained with rhodamine 123 (0.25 μg/ml) or MitoTracker Red CM-H₂XRos (250 nM, Molecular Probes, Eugene, OR) for 30 min at 37 °C. Cells expressing pSilencer 3.1-H1 Puro constructs were selected with 2 μg/ml puromycin (Sigma). Caspase activity was inhibited with 50 μM Z-VAD-FMK (Calbiochem, San Diego, CA). Cells expressing the ER expression plasmid were cultured in Opti-MEM I without phenol red (Invitrogen) supplemented with 10 nM ICI 182,780 (Tocris), and treated with 1 μM of E2 for 2 h. For inhibition of cytoplasmic translation, the cells were simultaneously cultured with 50 or 100 μg/ml cycloheximide (CHX).

Immunocytochemistry—HeLa cells were plated on 35-mm poly-L-lysine-coated glass-bottomed dishes (Matsunami Glass Ind.) and fixed for 20 min at room temperature with 4% paraformaldehyde and 0.4% Triton X-100 in PBS. The cells were incubated with antibodies against FLAG (Sigma, rabbit polyclonal), cytochrome c (BD Pharmingen, mouse monoclonal), ERα (Upstate Biotechnology, rabbit polyclonal), or Myc (BD Biosciences, mouse monoclonal) in PBST (PBS with 0.05% Tween 20) containing 2% horse serum. After washing three times with PBS, the cells were incubated with Alexa 488-conjugated anti-rabbit IgG ( Molecular Probes) and Cy3-conjugated anti-mouse IgG (Amersham Biosciences) in PBST containing 2% horse serum for 1 h at room temperature. Fluorescent images were captured and analyzed with a μRadiance™ Laser Scanning Confocal Microscope System (Bio-Rad).

Preparation and Fractionation of Mitochondria—Mitochondria were prepared from HeLa cells as previously described (24). To assess membrane association, mitochondria suspended in sucrose solution (0.25 M sucrose supplemented with 0.2 mM EDTA) were sonicated on ice. Intact mitochondria were removed by centrifugation at 4 °C for 10 min at 10,000 × g, and the supernatant containing sonicated mitochondria was further centrifuged at 4 °C for 30 min at 100,000 × g. The pellets were collected as the mitochondrial membrane fraction. The mitochondrial membrane fraction was then treated for 30 min on ice with 0.1 M Na₂CO₃ in sucrose solution. The solution was centrifuged at 4 °C for 30 min at 100,000 × g to separate the soluble proteins from the membranes.

For the protease protection assay, mitochondria were treated for 20 min at room temperature with 0.25 mg/ml trypsin (Sigma) in sucrose solution containing the indicated concentrations of digitonin (Sigma) or 1% Triton X-100. The reaction was stopped by adding trichloroacetic acid.

The submitochondrial fraction was prepared as follows: sonicated mitochondria in 0.45 M sucrose were layered onto a linear sucrose gradient (11 ml, 0.6–1.6 M sucrose in 10 mM HEPES-KOH, pH 7.4, and 0.2 mM EDTA) and centrifuged at 4 °C for 16 h at 100,000 × g. The gradient was collected in 25 0.5-ml fractions and then analyzed by Western blotting.

Cell Counting—The living cell number in a 6-well plate was determined by trypan blue staining. Cells were trypsinized and collected by centrifugation. The cell pellets were suspended in 0.4% trypan blue solution (Invitrogen), and living cells were counted.

Mass Spectrometry—Protein bands on acrylamide gels were stained with Cooamassie Brilliant Blue R-250 and cut out. In-gel digestion with trypsin was carried out as described by Shevchenko et al. (25). Proteins were identified using a Finnigan LTQ liquid chromatography-tandem mass spectrometry system (Thermo Electron Corp.).

Western Blotting—Samples were separated by electrophoresis on SDS-polyacrylamide gels (10% or 12% acrylamide) and then electrophoretically transferred to nitrocellulose membranes (Hybond ECL, Amersham Biosciences). The membranes were probed with primary and horseradish peroxidase-conjugated secondary antibodies, and immunoreactive bands were visualized with enhanced chemiluminescence reagents.
(Amersham Biosciences). The following primary antibodies were used: anti-PHB1 (1:200, NeoMarkers), anti-PHB2 (1:1000, Upstate Biotechnology), anti-Hax-1 (1:250, BD Transduction Laboratories), anti-cytochrome c (1:100, BD Pharmingen), anti-GAPDH (1:3000, Chemicon), anti-VDAC (1:2000, Calbiochem), anti-FLAG (1:2000, Sigma), anti-PDH2 (1:1000, Molecular Probes), anti-OPA1 (1:1000 (24)), and anti-Mfn1/hFzo1 (1:100, rabbit anti-sera against the peptide fragment, CVVLENELENTKQLPSSNEES, corresponding to the C-terminal portion of the protein).

Immunoprecipitation—The mitochondrial pellet from cells expressing PHB2-FLAG or Hax-1-FLAG was extracted with radioimmune precipitation assay buffer (20 mM Tris-HCl, pH 8, 150 mM NaCl, 1% sodium deoxycholate, and 1% Triton X-100). After sonication for 4 min on ice, the solution was centrifuged at 4 °C for 15 min at 10,000 × g. Immunoprecipitation was carried out by incubation of the supernatant with 5 μg of anti-FLAG antibody (Sigma, mouse monoclonal) and protein G-Sepharose (Amersham Biosciences) at 4 °C for overnight.

In Vitro Binding Assay—Using Escherichia coli XL2-blue, expression of GST-PHB2 was induced with 1 mM isopropyl β-D-galactopyranoside for 3 h. The fusion protein was then affinity purified with glutathione-Sepharose 4B (Amersham Biosciences). FLAG-tagged Hax-1 and PHB1 were synthesized in vitro using the TnT SP6 Coupled Reticulocyte Lysate System (Promega). The in vitro-translated products were mixed with GST or GST-PHB2 proteins in TNE buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 2 mM EDTA, and 1% Nonidet P-40) and then affinity purified with glutathione-Sepharose 4B.

RT-PCR—Total RNA was isolated from HeLa cells using TRIzol (Invitrogen) according to the manufacturer’s instructions. Two micrograms of the total RNA was subjected to RT-PCR (SuperScript II, Invitrogen) using random hexamer primers for the RT reaction.

RESULTS

Mammalian PHB Proteins Mainly Localize in the Mitochondria of HeLa Cells—In fibroblasts, mammalian PHB proteins are found mainly in the mitochondria (14), whereas they are also localized in the nucleus in MCF7 cells (7, 8). We expressed human PHB proteins with a FLAG tag at their C termini in HeLa cells and found that they are mainly present in the mitochondria where they colocalize with mitochondrial cytochrome c (Fig. 1A, left panel). When we expressed GFP fusion proteins of PHBs in MCF-7 or HeLa cells, like the FLAG-tagged proteins, PHB1-GFP and PHB2-GFP were expressed in the mitochondria of HeLa cells (Fig. 1A, right panel) along with cytochrome c (data not shown). We found that a portion of PHB2-GFP was also present in the nucleus of MCF7 cells, whereas PHB1-GFP was only present in the mitochondria in these cells (Fig. 1A, right panel). These results demonstrate that, in HeLa cells, PHB proteins mainly localize in the mitochondria.

Association of PHB2 with Hax-1 in Mitochondria—To clarify the function of human PHB2 in mitochondria, we next performed immunoprecipitation studies using a mitochondrial extract from HeLa cells expressing PHB2-FLAG. Immunoprecipitation with an anti-FLAG antibody (FLAG-IP) revealed that PHB2-FLAG specifically and reproducibly coprecipitates four proteins with molecular masses of 30–37 kDa (Fig. 1B), which were not detected in untransfected control extract (data not shown). Mass spectrometric analysis identified these proteins as PHB1, HS-associated protein X-1 (Hax-1) (21), voltage-dependent anion channel 2 (VDAC2), and adenine nucleotide translocator 2 (ANT2), respectively (Fig. 1B). We expected that PHB1 would coprecipitate with PHB2, because they form stable complex in mitochondria (9). ANT and VDAC are components of the permeability transition pore (PTP), and the association of PHB2 with ANT or VDAC has been reported as a possible mitochondrial nucleoid complex in Xenopus (26). Therefore, Hax-1 appears to be a novel binding partner of PHB2. The interaction between PHB2-FLAG and Hax-1 in the mitochondrial extract was confirmed by Western blotting with an anti-Hax-1 antibody following to FLAG-IP (Fig. 1C, upper panel). We further confirmed that Hax-1-FLAG coimmunoprecipitates with PHB2, PHB1, and VDAC (Fig. 1C, lower panel).

Next, to determine whether the interaction between PHB2 and Hax-1 is direct, we performed in vitro pull-down assays. GST-PHB2 directly bound to Hax-1 (Fig. 1D, lane 6), but PHB1, which was believed to directly interact with PHB2 (14), was not pulled down by GST-PHB2 (Fig. 1D, lane 4). In addition, the presence of PHB1 did not disturb the interaction between GST-PHB2 and Hax-1 (Fig. 1D, lane 7).

Hax-1 Is an Integral Protein of the Outer Mitochondrial Membrane—Hax-1, originally isolated as an HS-1 binding protein, is known to localize mainly in mitochondria (21). We confirmed that FLAG-tagged Hax-1 is mainly localized in the mitochondria of HeLa cells (Fig. 2A).

To clarify the submitochondrial localization of Hax-1, we initially examined whether Hax-1 is a membrane-integrated protein. Following sonication of HeLa cell mitochondria, most of the Hax-1 protein remained in the mitochondrial membrane pellets as did the integral inner membrane protein PHB1 and the inner membrane-associated protein cytochrome c (Fig. 2B, lane 3). As expected, alkali treatment of the mitochondrial membrane pellets released cytochrome c, an inner membrane-associated protein, from the membrane pellets (Fig. 2B, lanes 4 and 5). In contrast, Hax-1 as well as PHB1 and PHB2 remained in alkali-washed membrane pellets, indicating that they are integral mitochondrial membrane proteins (Fig. 2B, lane 5).

We further examined whether Hax-1 is integrated in the outer or inner mitochondrial membrane. Submitochondrial particles were fractionated by sucrose density gradient centrifugation, and the outer membrane and inner membrane fractions were collected. The effectiveness of the separation of these two membrane types was confirmed by Western blotting with antibodies to the outer membrane protein VDAC and the inner membrane protein PHB1. We found that Hax-1 was present in the outer membrane fraction as well as with VDAC (Fig. 2C), indicating that Hax-1 is integrated in the outer mitochondrial membrane.

Protease protection assay was performed to further define the submitochondrial localization of Hax-1. Hax-1 as well as the mitochondrial matrix protein pyruvate dehydrogenase E2

Pleiotropic Functions of Human PHB2
subunit and the intermembrane space protein cytochrome c or optic atrophy 1 (OPA1) remained in intact mitochondria after trypsin treatment (Fig. 2D, lane 2). Selective disruption of the outer membrane with digitonin decreased Hax-1, cytochrome c, and OPA1 levels following trypsin treatment but not effect the level of pyruvate dehydrogenase E2 (Fig. 2D, lane 4), demonstrating that, like cytochrome c and OPA1, Hax-1 localizes in the intermembrane space. PHB proteins have been reported to be mitochondrial inner membrane proteins, and it has been suggested that their C termini are exposed to the intermembrane space in yeast (15). However, PHB proteins are known to be resistant to protease treatment after disruption of the outer membrane (19, 27), which may be due to their tight folding. Our experiments are consistent with these previous studies that PHB proteins are resistant to proteolytic degradation following disruption of the outer membrane.

Knockdown of PHB2 Induces Reduction of Hax-1 Protein and Apoptosis—To clarify the function of human PHB2 in mitochondria and the significance of its interaction with Hax-1, we performed RNAi-mediated knockdown studies. Small interfering RNA (siRNA) for PHB1 or PHB2 based on a short hairpin RNA expression vector containing a puromycin-resistant gene was expressed in HeLa cells. Semiquantitative RT-PCR showed that the expression levels of PHB1 and PHB2 mRNAs were specifically reduced by siRNAs for PHB1 (siPHB1) and PHB2 (siPHB2), respectively (Fig. 3A). Western blotting confirmed that siPHB1 and siPHB2 decreased the levels of PHB1 and PHB2 but did not affect the expression of other mitochondrial proteins (cytochrome c, VDAC, and pyruvate dehydrogenase E2 subunit) or cytosolic glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Fig. 3B). Although the siRNAs were specific for each PHB protein at the mRNA level (Fig. 3A), the protein levels of PHB1 and PHB2 were reduced by siPHB2 and siPHB1, respectively. This is not surprising because the two PHB proteins are interdependent in yeast and C. elegans so that deletion of one decreases the protein level of the other (17, 27).

We next examined the effect of PHB2 knockdown on the expression of Hax-1. We found that knockdown of PHB2 reduced the level of Hax-1 protein (Fig. 3B), although the siRNA did not change the level of Hax-1 mRNA (Fig. 3A). On the contrary, knockdown of Hax-1 specifically reduced Hax-1 protein.
expression with no effect on the expression of PHB proteins (Fig. 3C). Thus, it was indicated that PHB2 regulates Hax-1 expression at the protein level.

Hax-1 is known to inhibit apoptosis, and its expression is decreased during apoptosis possibly due to cleavage by the Omi/HtrA2 protease (22, 28, 29). We suspected that knockdown of PHB proteins would induce apoptosis in HeLa cells, because it down-regulated the level of the anti-apoptotic protein Hax-1. Indeed, 4 days after transfection, a subpopulation of PHB or Hax-1 knockdown cells was not stained with rhodamine 123 (Fig. 4A), which indicates decrease of mitochondrial membrane potential, and, 5 days after transfection, also showed release of cytochrome c from mitochondria (Fig. 4B).

In addition, 5 days after transfection, the cell number of PHB or Hax-1 knockdown cells was reduced by the siRNAs compared with the empty siRNA vector (Fig. 4C). The reduction of cell number was likely to be caused by cell death, because it was inhibited by the caspase inhibitor Z-VAD-FMK (Fig. 4C), indicating that caspase-dependent apoptosis occurs in these cells. Therefore, it appears that knockdown of PHB2 induces apoptotic cell death through down-regulation of Hax-1 expression. Treatment with Z-VAD-FMK, however, did not prevent the down-regulation of Hax-1 (Fig. 4D), suggesting that Hax-1 functions in the apoptotic cascade upstream from caspase activation.

**Knockdown of PHB Protein Causes the Fragmentation of Mitochondria**—We next investigated the RNAi effect on the morphology of mitochondria by staining living PHB knockdown cells with MitoTracker Red. This analysis revealed that ~30% of the PHB knockdown cells contained fragmented mito-

![FIGURE 2. Mitochondrial Hax-1 integrated in the outer membrane localized in the intermembrane space. A, C-terminally FLAG-tagged Hax-1 was expressed in HeLa cells. The HeLa cells were fixed and immunostained with anti-FLAG (α-FLAG) and anti-cytochrome c antibodies (α-cyt. c). As shown in the merged image (Merge), Hax-1 colocalizes with mitochondrial cytochrome c. Scale bars, 10 μm. B, mitochondria from HeLa cells were sonicated (sonic.) and separated into supernatants (sup.) and membrane-pellets (ppt.) by centrifugation at 100,000 × g. The membrane pellets were further treated with Na2CO3 (alkali) and separated into “sup.” and “ppt.” by centrifugation at 100,000 × g. Samples were analyzed by Western blotting using antibodies (α) against PHB1, PHB2, Hax-1, and cytochrome c (cyt. c). C, the outer membrane (OM) and the inner membrane (IM) fractions were prepared by sucrose density gradient centrifugation of submitochondrial particles. Proteins were analyzed by Western blotting with indicated antibodies. The Western blot analysis revealed that Hax-1 and the outer membrane protein VDAC are present in the outer membrane fraction. D, mitochondria were treated with various concentrations of trypsin, digitonin, and Triton X-100 and then analyzed by Western blotting with indicated antibodies. The protease protection assay showed that Hax-1 and cytochrome c are localized in the intermembrane space.

![FIGURE 3. RNAi-mediated knockdown of PHB2 down-regulates Hax-1. Five days after transfection, protein or mRNA expression in HeLa cells transfected with siRNA for PHB1 (siPHB1), PHB2 (siPHB2), or Hax-1 (siHax-1) was compared with untransfected cells (−) and/or cells expressing only the puromycin resistance vector (puro-vec.). A, the expression of mRNAs for PHB1, PHB2, mitochondrial transcription factor A (TFAM), single-stranded DNA-binding protein (SSBP), and Hax-1 was examined by semiquantitative RT-PCR. B, the effect of siPHB1 or siPHB2 on protein expression was determined by Western blotting with indicated antibodies. C, RNAi-mediated knockdown of Hax-1.

NOVEMBER 24, 2006 • VOLUME 281 • NUMBER 47 • JOURNAL OF BIOLOGICAL CHEMISTRY 36405
Pleiotropic Functions of Human PHB2

A. Rh123 staining

vec. | siPHB1 | siPHB2 | siHax-1
---|---|---|---
Fl |  |  |  
Tl |  |  |  

B. α-cyt. c

vec. | siPHB1 | siPHB2 | siHax-1
---|---|---|---
Fl |  |  |  
Tl |  |  |  

C. X 10^3

Living cell number in 6 well plate

vec. | siPHB1 | siPHB2 | siHax-1
---|---|---|---
- | + | + | + 

D. puro-vec. | siPHB2
---|---
- | + 
α-Hax-1 | α-GAPDH
---|---
- | + 
α-VAD-FMK
---|---
- | + 

CHONDRIA (Fig. 5, A and B), whereas cells expressing the empty siRNA vector or Hax-1 knockdown cells showed tubular mitochondria with almost no morphological changes. Therefore, it appears that PHB proteins are involved in the regulation of mitochondrial morphology independently of Hax-1 function. On the other hand, overexpression of each PHB protein did not activate mitochondrial fusion and show long tubular mitochondria (Fig. 1 A and data not shown). These results indicate that PHB proteins are not directly involved in the mitochondrial fusion machinery as OPA1 and Mfn1/hFzo1. To address the mechanism of involvement of PHB proteins in the mitochondrial morphology, we examined whether PHB proteins regulate the protein levels of such mitochondrial fusiogenic proteins. Protein blot analysis shows knockdown of PHB2 induces significant reduction of OPA1 protein, but not of Mfn1/hFzo1, whereas that of Hax-1 induces no effect (Fig. 5 C). Because the fragmented mitochondria were induced by knockdown of OPA1, it is likely that PHB proteins regulate the mitochondrial morphology by stabilizing OPA1 protein.

ERα-dependent Nuclear Translocation of PHB2—In this study, we showed that PHB2 mainly localizes and functions in the mitochondria of HeLa cells. Human PHB2 is also known as a repressor of ER activity, however, it remains unclear how PHB2 is delivered to the nucleus. Because MCF7 expresses the PHB2 ligand, ERα, but HeLa cells do not, we hypothesized that the nuclear distribution of PHB2 is driven by ERα. To determine the validity of this hypothesis, we expressed PHB2-GFP along with ERα in HeLa cells and observed its subcellular distribution. In the absence of E2, PHB2-GFP mainly localized in the mitochondria (Fig. 6 A, upper panel, –E2). Surprisingly, PHB2-GFP translocated into the nucleus in the presence of ERα and E2 (Fig. 6 A, upper panel, +E2). This ERα-dependent nuclear translocation was specific for PHB2, because the distri-

FIGURE 4. Knockdown of PHB2 induces apoptotic cell death. A, 4 days after transfection with indicated siRNA, the HeLa cells were stained with rhodamine 123 (Rh123), which labels mitochondria depending on the mitochondrial membrane potential. Arrowheads indicate cells not stained with rhodamine 123. B, 5 days after transfection, the HeLa cells were immunostained with anti-cytochrome c antibodies (α-cyt. c). Arrowheads indicate cells that release cytochrome c from mitochondria. Fl, fluorescence image; Tl, transmission image. Scale bars, 10 μm. C, 3 days after transfection with indicated siRNAs, 2 × 10^5 cells were seeded on 6-well plates, and selected by puromycin. From 4 days after transfection, the cells were treated with (+) or without (−) the caspase inhibitor Z-VAD-FMK, and the cell number was determined on the 5th day after transfection. D, cells were treated as described in C and examined by Western blotting with indicated antibodies.

FIGURE 5. Effect of PHBs knockdown on the morphology of mitochondria. A, 6 days after transfection with PHBs or Hax-1 siRNA, the HeLa cells were stained with MitoTracker Red. Squared regions in each panel are enlarged. Scale bars, 10 μm. B, cell populations with fragmented mitochondria in A, C, 5 days after transfection with indicated siRNA, the expression of OPA1, Mfn1/hFzo1, and GAPDH was examined by Western blotting.
Pleiotropic Functions of Human PHB2

The molecular function of mammalian PHB proteins, especially PHB2, in mitochondria has not been well understood. In addition, although mammalian PHB proteins have been reported to interact with transcription factors and function as transcriptional repressors, how they are targeted to the nucleus has not been determined. In this study, we analyzed the mitochondrial PHB2 protein complex and identified the anti-apoptotic protein Hax-1 as a novel binding partner for PHB2. We also clarified the pleiotropic functions of PHB2 on mitochondria, including regulation of apoptosis by forming a complex with Hax-1 and maintenance of mitochondrial morphology. Furthermore, we identified the molecular mechanism by which PHB2 is targeted to the nucleus.

PHB2 Protein Complex in Mitochondria

Our immunoprecipitation analyses showed that PHB2 forms a complex with known mitochondrial proteins, PHB1, Hax-1, ANT2, and VDAC2 in mitochondria. An in vitro binding assay showed us that PHB2 directly interacts with Hax-1. Unexpectedly, we did not detect a direct interaction between PHB2 and PHB1, suggesting that PHB2 has a weaker physical interaction with PHB1 than with Hax-1. The effective association between PHB1 and PHB2 might require an additional cellular factor, because it has only been demonstrated by immunoprecipitation in whole cell lysates (14). It is unlikely that PHB1 interacted

nucleus in the presence of ERα and E2 (Fig. 7C). Therefore, the C-terminal domain is necessary for the ERα-dependent nuclear targeting of PHB2.

We further examined whether the C-terminal domain of PHB2 is sufficient for the ERα-dependent nuclear translocation. N-terminal deletion mutants of PHB proteins (PHB1-(45–272)-GFP and PHB2-(51–299)-GFP, respectively) were not expressed in the mitochondria (Fig. 7D), demonstrating that the N-terminal portions of PHB proteins are the sole determinant of mitochondrial targeting. Using the deletion mutants, we found that the C-terminal domain of PHB2 (amino acids 51–299), which uniformly distributes in the nucleus and cytoplasm, accumulates in the nucleus in the presence of ERα and E2 (Fig. 7D). On the other hand, the C-terminal domain of PHB1 (amino acids 45–272) did not specifically accumulate in the nucleus under these conditions. Therefore, the C-terminal domain of PHB2 appears to be sufficient for ERα-dependent nuclear translocation in the presence of E2.

Thus, human PHB2 localizes in the mitochondria through MTS at its N termini and in the nucleus through its C-terminal domain, suggesting the shuttling of PHB2 between the two organelles. Supporting this idea, the MTS of human PHB2 seemed to be non-cleavable as like as that in yeast and C. elegans, because PHB2-FLAG expressed in HeLa cells and in vitro-translated PHB2-FLAG had the same molecular masses (Fig. 7E). The shuttling of PHB2 was further supported by a chase assay, in which PHB2-GFP accumulated in the nucleus was reduced after removal of E2 (Fig. 7F). In the cell, mitochondrial signal of PHB2-GFP around the nucleus seemed to be increased (Fig. 7F), convincing the translocation of PHB2 from the nucleus to mitochondria.

DISCUSSION

The molecular function of mammalian PHB proteins, especially PHB2, in mitochondria has not been well understood. In addition, although mammalian PHB proteins have been reported to interact with transcription factors and function as transcriptional repressors, how they are targeted to the nucleus has not been determined. In this study, we analyzed the mitochondrial PHB2 protein complex and identified the anti-apoptotic protein Hax-1 as a novel binding partner for PHB2. We also clarified the pleiotropic functions of PHB2 on mitochondria, including regulation of apoptosis by forming a complex with Hax-1 and maintenance of mitochondrial morphology. Furthermore, we identified the molecular mechanism by which PHB2 is targeted to the nucleus.
Pleiotropic Functions of Human PHB2

A  C-terminal domain of PHB2 is sufficient for its ERα-dependent nuclear targeting. A, the deletion constructs of PHB proteins used in this study. Predicted MTS (green), transmembrane domain (TM; strong dark blue); weak (light blue), and the coiled-coil domain (red) are shown. B, when expressed as C-terminally FLAG-tagged proteins, amino acids 1–50 of PHB1 and PHB2 were sufficient for mitochondrial localization. The two proteins were expressed in HeLa cells, and the cells were immunostained with antibodies (α) to FLAG and cytochrome c (cyt. c). C, localization of C-terminally Myc-tagged PHB2 or its deletion mutant (amino acids 1–100) in HeLa cells when expressed alone (−) or when cotransfected with ERα and treated with E2. The cells were immunostained with an anti-Myc antibody. The C-terminal domain of PHB2 was required for its ERα-dependent nuclear targeting. Arrowhead shows a cell in which PHB2-Myc translocated to the nucleus. D, localization of PHB-GFP fusion proteins lacking their N-terminal mitochondrial target sequences when expressed alone (−) or when cotransfected with ERα and treated with E2. The colorization of the fusion proteins was determined by fluorescence microscopy. The C-terminal domain of PHB2 was sufficient for the ERα-dependent nuclear targeting of PHB2. E, an extract of HeLa cells expressing PHB2-FLAG and the in vitro-translated PHB2-FLAG were analyzed by Western blotting with an anti-FLAG antibody (α-FLAG), F, a chase assay of PHB2-GFP in the same cell. PHB2-GFP and ERα were coexpressed in HeLa cells. After incubation with E2 for 60 min, E2 was removed (time, 0 min), and the cells were further cultured for 90 min in the presence of ICI 182,780, an antagonist for E2 (1 μM) and CHX (100 μg/ml). Arrowheads show a cell in which PHB2-GFP translocated from the nucleus to mitochondria. Scale bars, 10 μm.

with Hax-1, because the interaction between PHB2 and Hax-1 was not disturbed by PHB1 and because PHB1 was not pulled down with the PHB2-Hax-1 complex. Based on these results, it appears that Hax-1 binds directly and specifically to PHB2.

Hax-1 seems to be integrated in the outer mitochondrial membrane and exposed to the intermembrane space. This conclusion agrees well with the fact that, during apoptosis, Hax-1 is cleaved in mitochondria by the Omi/HtrA2 protease, which is thought to be present in the mitochondrial intermembrane space (33). Because the C terminus of PHB proteins may be tightly folded and exposed to the intermembrane space (15, 16), it is likely that the outer membrane protein Hax-1 directly binds to the inner membrane protein PHB2 within the intermembrane space.

Mitochondrial Functions of PHB2

Anti-apoptotic Function via Regulation of Hax-1 Protein Stability—Using RNAi-mediated knockdown of PHB proteins, we demonstrated that, as in yeast and C. elegans, human PHB proteins are interdependent. From yeast to human, it is possible that PHB proteins stabilize each other in mitochondria. Furthermore, knockdown of PHB2 protein reduced the level of Hax-1 protein. Because PHB proteins bind to newly synthesized mitochondrial proteins and stabilize them in yeast (15, 16), we suspect that Hax-1 is stabilized in mitochondria by the mammalian PHB complex, especially by PHB2. The substantial reduction of Hax-1 in PHB2 knockdown than PHB1 knockdown supports the direct involvement of PHB2 in the stabilization of Hax-1. By analogy with yeast regulation, wherein PHB proteins protect newly synthesized protein from the AAA protease (15), Hax-1 may be protected from cleavage by the Omi/HtrA2 protease by inclusion in the PHB complex.

Knockdown of PHB2 induced apoptotic cell death as shown by the inhibition of cell death by the caspase inhibitor Z-VAD-FMK and release of cytochrome c from mitochondria. The down-regulation of Hax-1 caused by knockdown of PHB2 was unaffected by Z-VAD-FMK, suggesting that the down-regulation of Hax-1 is not induced as a consequence of apoptosis. This further supports the direct involvement of PHB2 in the regulation of Hax-1. Because down-regulation of Hax-1 is sufficient for the induction of cell death, it appears that PHB2 functions as an anti-apoptotic factor by maintaining Hax-1 expression.

Maintenance of Mitochondrial Morphology—Knockdown of PHB proteins caused the fragmentation of mitochondria. Similarly, in yeast, PHB proteins have been suggested to participate in the regulation of mitochondrial morphology, because they genetically interact with Mdm12p, a protein required for normal mitochondrial morphology (27), and because PHB mutants...
Mitochondrial PHB2 Localizes to the Nucleus in an ERα-dependent Manner

We found that, in HeLa cells, mitochondrial PHB2 translocates to the nucleus in the presence of ERα and E2. Therefore, E2-dependent binding of ERα to PHB2 is thought to be essential for its nuclear targeting. In fact, we found that the C-terminal domain of PHB2 that contains the previously reported ERα-binding site (32) was necessary and sufficient for the ERα-dependent nuclear translocation. The ERα-dependent nuclear translocation occurred with PHB2 but not PHB1, demonstrating a highly specific interaction between PHB2 and ERα.

PHB proteins contained an atypical MTS at their N termini that was sufficient for their mitochondrial targeting. Because the N-terminal region of PHB2 did not respond to ERα and E2, it appears that the nuclear targeting and mitochondrial targeting of PHB2 occurs independently. Considering the existence of separate mitochondrial targeting and ERα-dependent nuclear localization sequences intramolecularily, we suspect that PHB2 shuttles between the mitochondria and nucleus. By the chase assay of PHB2-GFP in the same cell, it was further supported that PHB2 shuttles between the two organelles.

Several proteins have been reported to localize both in the mitochondria and nucleus. Among these, however, there are few examples of conditional translocation between the two organelles. Two such proteins, apoptosis-inducing factor and endonuclease G, are localized in mitochondria and translocate to the nucleus following stimulation with inducers of apoptosis (36). In these cases, unlike PHB2, the proteins are thought to possess MTS that are processed after their import into the mitochondria (37, 38), suggesting that, once released from the mitochondria, they cannot re-enter. Therefore, PHB2 likely has a distinct mode of translocation from these molecules in that it potentially shuttles between the organelles via non-cleavable targeting sequence.

Fig. 8 shows a summary of the location and functions of PHB2. Through an unconventional N-terminal MTS, PHB2 is imported into mitochondria where it plays multiple roles, such as inhibition of apoptosis through PHB2:Hax-1 complex and maintenance of mitochondrial morphology via OPA1. On the other hand, binding of the ERα-E2 complex causes mitochondrial PHB2 to translocate to the nucleus where it inhibits ERα-dependent transcription. Then, how PHB2 in the mitochondria detects ERα and E2 in the first place? Recently, ERα has also been shown to localize in the mitochondria (39). Therefore, it is not surprising that the first interaction between PHB2 and ERα occurs in the mitochondria. If so, it is possible that PHB2 is delivered from the mitochondria to nucleus by ERα. Alternatively, if PHB2 might shuttle from the mitochondria to cytoplasm, because it possesses an unprocessed MTS, the possibility should not be excluded that PHB2 firstly binds to ERα in the cytoplasm.

Recently, a PHB2 knock-out mouse was produced, although the homozygous animals did not develop and died in the embryonic stage (40). An essential role of PHB2 in early development agrees well with findings in C. elegans (17). Thus, both the transcriptional function and mitochondrial functions of PHB2 may be required for development. Alternatively, it was recently reported that ERs are present and function in the mitochondria (39). E2-dependent interaction between ERα and PHB2 might occur in the mitochondria and be important for the mitochondrial targeting and functions of ERα.

PHB2 is a versatile molecule that regulates transcription in the nucleus as well as regulation of mitochondrial functions in the mitochondria. PHB2 also possesses a non-cleavable N-terminal MTS, indicating a possibility of shuttling between the mitochondria and nucleus.
two organelles. Considering above, PHB2 would be a candidate that couples nuclear-mitochondria interaction. Further experiments to prove the physiological significance of the nuclear-mitochondrial interaction by PHB2 should be required.

Acknowledgments—We are grateful to Dr. Pierre Chambon for the cDNA. We are also grateful to our laboratory members for helpful discussions.

REFERENCES
1. Nuell, M. J., Stewart, D. A., Walker, L., Friedman, V., Wood, C. M., Owens, G. A., Smith, J. R., Schneider, E. L., Dell‘Orco, R., Lumpkin, C. K., Danner, D. B., and McClung, J. K. (1991) *Mol. Cell. Biol.* 11, 1372–1381
2. McClung, J. K., Jupe, E. R., Liu, X. T., and Dell‘Orco, R. T. (1995) *Exp. Gerontol.* 30, 99–124
3. Snedden, W. A., and Fromm, H. (1997) *Plant Mol. Biol.* 33, 753–756
4. Loukas, A., and Maizels, R. M. (1998) *DNA Seq.* 9, 323–328
5. Eveleth, D. D., Jr., and Marsh, J. L. (1986) *Nucleic Acids Res.* 14, 6169–6183
6. Sato, T., Saito, H., Swensen, J., Olifant, A., Wood, C., Danner, D., Sakamoto, T., Takita, K., Kasumi, F., and Miki, Y. (1992) *Cancer Res.* 52, 1643–1646
7. Fusaro, G., Dasgupta, P., Rastogi, S., Joshi, B., and Chellappan, S. (2003) *J. Biol. Chem.* 278, 47853–47861
8. Kurtev, V., Margueron, R., Kroboth, K., Ogris, E., Cavailles, V., and Seiser, C. (2004) *J. Biol. Chem.* 279, 24834–24843
9. Nijtmans, L. G., Artal, S. M., Grivell, L. A., and Coates, P. J. (2002) *Cancer Res.* 62, 143–155
10. Wang, S., Nath, N., Fusaro, G., and Chellappan, S. (1999a) *Oncogene* 18, 3501–3510
11. Wang, S., Nath, N., Fusaro, G., and Chellappan, S. (1999b) * Mol. Cell. Biol.* 19, 7447–7460
12. Terashima, M., Kim, K. M., Adachi, T., Nielsen, P. J., Reth, M., Kohler, G., and Lamers, M. C. (1994) *EMBO J.* 13, 3782–3792
13. Montano, M. M., Ekena, K., Delage-Mourroux, R., Chang, W., Martini, P., and Katzenellenbogen, B. S. (1999) *Proc. Natl. Acad. Sci. U.S.A.* 96, 6947–6952
14. Coates, P. J., Jamieson, D. J., Smart, K., Prescott, A. R., and Hall, P. A. (1997) *Curr. Biol.* 7, 607–610
15. Steglich, G., Neupert, W., and Langer, T. (1999) *Mol. Cell. Biol.* 19, 3435–3442
16. Nijtmans, L. G., de Jong, L., Artal-Sanz, M., Coates, P. J., Berden, J. A., Back, J. W., Muijsers, A. O., van der Spek, H., and Grivell, L. A. (2000) *EMBO J.* 19, 2444–2451
17. Artal-Sanz, M., Tsang, W. Y., Willems, E. M., Grivell, L. A., Lemire, B. D., van der Spek, H., and Nijtmans, L. G. (2003) *J. Biol. Chem.* 278, 32091–32099
18. Fusaro, G., Wang, S., and Chellappan, S. (2002) *Oncogene* 21, 4539–4548
19. Coates, P. J., Neutruil, R., Mcgregor, A., Picket, S. M., Crouch, D. H., Hall, P. A., and Wright, E. G. (2001) *Exp. Cell Res.* 265, 262–273
20. Bourges, I., Ramus, C., Mouson de Camaret, B., Beugnet, R., Remacle, C., Cardol, P., Hofhaus, G., and Issartel, J. P. (2004) *Biochem. J.* 383, 491–499
21. Suzuki, Y., Demoliere, C., Kitamura, D., Takeshita, H., Deuschle, U., and Watanabe, T. (1997) *J. Immunol.* 158, 2736–2744
22. Cilenti, L., Soundararajan, M. M., Kryazis, G. A., Stratigos, V., Singh, S., Gupta, S., Bonventre, J. V., Almenri, E. S., and Zervos, A. S. (2004) *J. Biol. Chem.* 279, 50295–50301
23. Gamble, S. C., Odontiadis, M., Waxman, J., Westbrook, J. A., Dunn, M. J., Wait, R., Lam, E. W., and Bevan, C. L. (2004) *Oncogene* 23, 2996–3004
24. Satoh, M., Hamamoto, T., Seo, N., Kagawa, Y., and Endo, H. (2003) *Biochem. Biophys. Res. Commun.* 300, 482–493
25. Shevchenko, A., Wilm, M., Vorm, O., and Mann, M. (1996) *Anat. Chem.* 68, 850–858
26. Bogenhagen, D. F., Wang, Y., Shen, E. L., and Kobayashi, R. (2003) *Mol. Cell. Proteomics* 2, 1205–1216
27. Berger, K. H., and Yaffe, M. P. (1998) *Mol. Cell. Biol.* 18, 4043–4052
28. Sharp, T. V., Wang, H. W., Kouni, A., Hollyman, D., Endo, Y., Ye, H., Du, M. Q., and Bosshoff, C. (2002) *J. Virol.* 76, 802–816
29. Mirnoohmandasdegh, A., Tartler, U., Michel, G., Baer, A., Walz, M., Wolf, R., Ruzicka, T., and Hengge, U. R. (2003) *J. Invest. Dermatol.* 120, 1045–1051
30. Chen, H., and Chan, D. C. (2005) *Hum. Mol. Genet.* 14, Spec. No. 2, R283–R289
31. Tatsuta, T., Model, K., and Langer, T. (2005) *Mol. Biol. Cell* 16, 248–259
32. Delage-Mourroux, R., Martini, P. G., Choi, I., Kraichely, D. M., Hoeksema, J., and Katzenellenbogen, B. S. (2000) *J. Biol. Chem.* 275, 35848–35856
33. Suzuki, Y., Imai, Y., Nakayama, H., Takahashi, K., Takio, K., and Takahashi, R. (2001) *Mol. Cell* 6, 613–621
34. Piper, P. W., Jones, G. W., Bringloe, D., Harris, N., MacLean, M., and Mollapour, M. (2002) *Aging Cell* 1, 149–157
35. Olichon, A., Baricault, L., Gerli, A., Guillaud, E., Valette, A., Belenguer, P., and Lenaers, G. (2003) *J. Biol. Chem.* 278, 7743–7746
36. van Gurp, M., Festjens, N., van Loo, G., Saelens, X., and Vandenabeele, P. (2003) *Biochem. Biophys. Res. Commun.* 304, 487–497
37. Cote, J., and Ruiz-Carrillo, A. (1993) *Science* 261, 765–769
38. Otera, H., Ohkawa, S., Nakamura, Z., Ishihara, N., and Mihara, K. (2005) *EMBO J.* 24, 1375–1386
39. Chen, J. Q., Yager, J. D., and Russo, J. (2005) *Biochim. Biophys. Acta* 1746, 1–17
40. Park, S. E., Xu, J., Frolova, A., Liao, L., O‘Malley, B. W., and Katzenellenbogen, B. S. (2005) *Mol. Cell. Biol.* 25, 1989–1999