Targeting of the molecular chaperone oxygen-regulated protein 150 (ORP150) to mitochondria and its induction by cellular stress

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Arrington DD, Schnellmann RG. Targeting of the molecular chaperone oxygen-regulated protein 150 (ORP150) to mitochondria and its induction by cellular stress. Am J Physiol Cell Physiol 294: C641–C650, 2008. First published December 19, 2007; doi:10.1152/ajpcell.00400.2007.—Oxygen-regulated protein 150 (ORP150) is an inducible endoplasmic reticulum (ER) chaperone molecule that is upregulated after numerous cellular insults and has a cytoprotective role in renal, neural, and cardiac models of ischemia-reperfusion injury. ORP150 also has been shown to play a role in cellular Ca\(^{2+}\) homeostasis, and in turn, regulating calpain activity. In this study, we identified ORP150 in whole rat renal cortical mitochondria and matrix fractions, demonstrated the targeting of an ORP150-GFP construct to the mitochondria of NIH-3T3 cells, and showed that the NH\(_2\)-terminal 13 amino acids of ORP150 are sufficient for this translocation. ORP150 expression was found to be regulated by the anti-C/enhancer-binding protein homologous protein (CHOP)/GADD153 transcription factor and ORP150 levels increased in the mitochondria and ER of COS-7 cells after diverse stresses, including hypoxia, serum starvation, prolyl hydroxylase inhibition with dimethyloxaloylglycine, and exposure to tunicamycin, ethidium, bromide, and 2-deoxyglucose. Induction of the mitochondrial specific stress response in COS-7 cells through expression of an ornithine transcarbamylase mutant (ΔOTC) increased mitochondrial ORP150 levels and mitochondrial calpain activity. To determine whether mitochondrial ORP150 and mitochondrial calpain 10 interact, rat cortical mitochondria exposed to Ca\(^{2+}\) resulted in ORP150 cleavage in a calpain inhibitor-dependent manner, revealing that ORP150 is a substrate and may be regulated by calpain 10. These data reveal a novel cellular localization for ORP150 and that mitochondrial ORP150 is upregulated by CHOP/GADD153 in response to mitochondrial and ER stress. Our data also reveal that ORP150 is a substrate for mitochondrial calpain 10.

calpain; endoplasmic reticulum

OXYGEN-REGULATED PROTEIN 150 (ORP150), also known as glucose-regulated protein (GRP170), CBP-140, and Hyou1, was first described in 1996 by Kuwabara et al. (19) as a hypoxia-inducible protein expressed in cultured rat astrocytes. Since then, ORP150 has been reported to be a stress-inducible chaperone molecule localized to the ER in numerous cell types (1, 13, 19, 28). It is a 999 amino acid protein with both a signal peptide and an ER retention-like signal at the NH\(_2\)- and COOH-termini, respectively. ORP150 is conserved in numerous mammalian species and shares greater than 91% amino acid sequence identity to its Chinese hamster orthologue GRP170 and displays a high degree of similarity to the ATPase domain of the heat shock protein (HSP)70 family of proteins, suggesting the ability to bind ATP (8, 11). ORP150 is a highly glycosylated protein and migrates differentially on SDS-PAGE gels based on its level of secondary modification (150–170 kDa) (29). There are three predicted translation products arising from three separate transcription start sites defined by the presence or absence of stress signals such as hypoxia or ER stress (11). Upregulation of ORP150 has been observed after hypoxia, serum starvation, ischemia, in cancer, and after treatment with tunicamycin or 2-deoxyglucose (1, 5, 6, 12–14, 21–23, 27, 32). The functions of ORP150 have not been fully elucidated, but it appears to play a role in apoptosis (1, 6, 12–14, 21, 23, 24, 27, 30), insulin secretion (15, 16), protein transport (26), and wound healing (26, 31). Much of what is known about ORP150 arises from studies utilizing antisense, adenovirus-mediated, or whole animal knockdown of protein expression (1, 6, 14). These studies have shown the importance of ORP150 in protecting cells from ischemia-reperfusion injury in neural, cardiac, and renal in vivo models (1, 6, 12). Similarly, ORP150 has been shown to provide anti-apoptotic signals in neuronal cell models (14, 23) and in a variety of cancers such as prostate (22), breast (32), and bladder (3, 17) as well as decreasing the metastatic potential of these tumor lines (18).

Recently, ORP150 was found to be cytoprotective to the renal tubular epithelium after ischemia-reperfusion injury (6). In particular, the authors demonstrated enhanced renal protection in transgenic mice overexpressing ORP150 and increased renal injury in heterozygous ORP150\(^{-/-}\) mice (6). Transgenic mice and Madin-Darby canine kidney cells exposed to hypoxic conditions in this study had decreased caspase 3 activation. Similarly, the same investigators showed a protective role for ORP150 after myocardial infarction in the rat heart (1). Rat hearts infected with adenovirus expressing ORP150 exhibited a blunting in Ca\(^{2+}\) release, cellular calpain activity, cytochrome c release, and caspase 3 activation. The authors suggested that the anti-apoptotic effects of ORP150 were upstream of cytochrome c release and caspase activation and that it may be tied to the regulation of ER Ca\(^{2+}\) stores and the subsequent activation of the mitochondrial-mediated apoptotic pathway.

In the present study, we hypothesized that the localization and chaperoning functions of ORP150 may not be limited to the ER but may also reside in the mitochondria. The mitochondrial localization of ORP150 would provide insight into the cytoprotective and anti-apoptotic activities of this molecule observed in various in vitro and in vivo models. The preservation of mitochondrial energy production due to chaperone activity could also explain the potent protection observed during whole organ ischemia-reperfusion injury (1, 6, 12).
Fig. 1. Localization of oxygen-regulated protein 150 (ORP150) to the endoplasmic reticulum (ER) and mitochondria. The subcellular localization of ORP150 was assessed by immunoblot analysis. A: tissue from rat renal cortex was isolated and separated into mitochondrial and ER fractions by differential centrifugation. Equal amounts of protein from ER, whole mitochondria, and mitochondrial matrix were resolved by SDS-PAGE and probed with antibodies against glucose-regulated protein (GRP)170 (ORP150 orthologue), heat shock protein (HSP)60, and calnexin. B: COS-7 cells were cultured and treated with empty vector or pcDNA3.1-ORP150. Whole cell lysates were then resolved by SDS-PAGE and probed with antibodies against GRP170. Results are representative of three individual experiments.

METHODS AND MATERIALS

Isolation of ER, mitochondria, and mitochondrial subfractions. Kidney mitochondria were isolated from male Sprague-Dawley rats (250 g) (33). The Medical University of South Carolina IACUC reviewed and approved the animal protocol used in this study. Briefly, kidney cortex was minced and homogenized in buffer A (0.27 M sucrose, 5 mM Tris·HCl, 1 mM EGTA; pH 7.4). Nuclei and cellular debris were pelleted by centrifugation at 600 g for 5 min. The supernatant was centrifuged at 7,700 g for 5 min resulting in a crude mitochondrial pellet. The pellet was washed once in 0.27 M sucrose and resuspended in buffer B (in mM: 130 KCl, 9 Tris-PO4, 4 mM Tris·HCl, and 1 mM EGTA, pH 7.4). Crude mitochondria were then layered onto a sucrose-Percoll gradient and centrifuged at 20,000 g for 20 min. ER fractions were purified by ultracentrifugation of cytosolic fractions at 54,000 g for 1 h.

Purified mitochondria were subfractionated as described previously (34). Outer membrane rupture was achieved by hypotonic lysis in buffer C (10 mM KH2PO4, pH 7.4) for 20 min at 4°C. Mitoplasts were separated from the supernatant by centrifugation at 7,700 g for 5 min. The resulting pellet was resuspended in buffer D (in mM: 300 sucrose, 5 mM Tris·HCl, 20 MOPS, pH 7.4) and sonicated five times in 30-s bursts. Inner membrane and matrix fractions were then separated by centrifugation at 54,000 g for 30 min. Matrix fractions were frozen at −70°C for subsequent immunoblot analysis.

Immunoblotting. Isolated ER, mitochondria, and mitochondrial matrix fractions were subjected to SDS-PAGE and transferred to nitrocellulose membranes. Membranes were incubated with primary antibodies to hamster GRP170, polyclonal rabbit anti-hamster GRP170, a generous gift from Dr. John Subjeck (Roswell Park Cancer Institute, Buffalo, NY). This antibody recognizes both glycosylated (up to 170 kDa) and native ORP150 (150 kDa) and has been used for detecting mammalian ORP150 (29, 37). The primary antibodies used were polyclonal rabbit anti-hamster GRP170 (1:10,000), rabbit-anticalpain 10 (1:1,000, Biogenesis), anti-β-actin (1:1,000, Sigma), anti-calnexin (1:1,000, Calbiochem), anti-HSP60 (1:1,000, Calbiochem), anti-ND6 (1:1,000, Santa Cruz), anti-OTC (1:1,000) (gift from Dr. Gary Wright, Medical University of South Carolina, Charleston, SC), anti-green fluorescent protein (GFP) (1:1,000, Calbiochem), and mouse monoclonal anti-C/enhancer-binding protein homologous protein (CHOP, 1:1,000, Alexis Biochemicals). Primary antibody incubation was followed by a horseradish peroxidase-conjugated goat anti-rabbit or goat anti-mouse secondary antibody (1:1,000, Santa Cruz) incubation. Immunoreactive proteins were visualized by enhanced chemiluminesence (Amersham) and imaged using an Alpha Innotech imaging station.

Plasmid construction. cDNA for human ORP150 (BC072436) was obtained from ATCC in the pCMV-SPORT6 shuttle vector. Full-length ORP150 was amplified by PCR (sense: 5′-TGGGAGGCCCGCGAGGCAGCACGCTC-3′; antisense: 5′-TCATCAGGCCATGAGCGCGGC-3′) and subcloned into pcDNA3.1-TOPO-TA-CT-GFP (pcDNA3.1-ORP150-GFP) (Invitrogen) producing an ORP150-GFP fusion product (COOH-terminal GFP). ORP150 was also subcloned into the pcDNA3.1 vector lacking the CT-GFP. GFP control plasmids coding for cytosolic GFP and mitochondrially targeted GFP (cytochrome oxidase IV signal sequence) were generous gifts from Dr. Douglas Sweet (Medical University of South Carolina, Charleston, SC).

Fig. 2. ORP150 translocates to the mitochondria (Mito) via an NH2-terminal targeting signal. Localization of ORP150 was confirmed by colocalization of ORP150-GFP and the mitochondrial-specific dye MitoTracker Red. NIH-3T3 fibroblasts were used for localization experiments because of their distinct elongated mitochondrial morphology. A: fibroblasts were transfected with a control mitochondrially targeted green fluorescent protein (Mito-GFP) construct and subsequently stained with MitoTracker Red. B: fibroblasts were transfected with pcDNA3.1-ORP150-GFP and subsequently stained with MitoTracker Red. C: fibroblasts were transfected with the pcDNA3.1-5S-GFP or pcDNA3.1-5SINV-GFP constructs. pcDNA3.1-5S-GFP-transfected cells were then subsequently stained with MitoTracker Red. GFP and MitoTracker Red-stained cells were then visualized by confocal microscopy. D: fibroblasts were transfected with pcDNA3.1-ORP150-GFP and incubated for 48 h. Mitochondrial and ER fractions were subsequently isolated and subjected to immunoblot analysis for the presence of GFP-tagged ORP150 or native ORP150 (anti-GFP and anti-GRP170, respectively). Fraction purity was assessed by immunoblot analysis using antibodies specific for HSP60 (Mito) and calnexin (ER). Results are representative of three independent experiments.
Complementary DNA sequences coding for the NH2-terminal 13 amino acids of ORP150 were generated, annealed, and ligated into pcDNA3.1-TOPO-TA-CT-GFP (pcDNA3.1-TS-GFP) to assess NH2-terminal sufficiency for mitochondrial targeting. The negative control for this experiment was obtained via ligation of the above sequence into pcDNA3.1-TOPO-TA-CT-GFP (pcDNA3.1-TSINV-GFP) in the reverse orientation.

**Cell culture and transfection.** NIH-3T3 fibroblasts were maintained in Dulbecco’s Modified Essential Medium (DMEM) containing 10% fetal bovine serum until confluent. Cells were split and
plated onto 35-mm confocal dishes (MatTek) at a density of 250,000 cells/plate. At 70% confluence, cells were transiently transfected with 4 µg pcDNA3.1-ORP150-GFP, pcDNA3.1-TS-GFP, or pcDNA3.1-TSINV-GFP plasmid constructs using Lipofectamine 2000 (Invitrogen). Cells were incubated for 24 h and, when indicated, exposed to 50 nM MitoTracker Red (Molecular Probes) for 20 min before confocal microscopy imaging. Cells were imaged using a Zeiss LSM 5 confocal microscope.

**Induction of mitochondrial stress.** COS-7 cells were maintained in DMEM containing 10% fetal bovine serum. At 70% confluence, cells were treated with various concentrations of ethidium bromide for 7 days to disrupt the mitochondrial genome as previously described (7, 9). ORP150 expression was subsequently determined by immunoblot analysis.

Mitochondrial stress also was initiated via induction of the mitochondrial stress response (MSR) (39). COS-7 cells were transiently transfected with 4 µg pcAGGS-vector-only control, pcAGGS-OTC, or pcAGGS-OTC plasmid constructs using Lipofectamine 2000 (plasmid constructs were a generous gift from Dr. Nicholas Hoogenraad, La Trobe University, Melbourne, AU). The mitochondrial expression of the ornithine transcarbamylase (OTC) and ΔOTC protein products was monitored by immunoblot analysis 36 h after transfection. Induction of the MSR was validated by immunoblot analysis of the mitochondrial chaperone HSP60 and the stress-induced transcription factor CHOP/GADD153.

COS-7 cells also were challenged with various chemical stressors to elucidate the inducible nature of ORP150. Cells were treated with tunicamycin (10 µg/ml), 2-deoxyglucose (10 mM), or the prolyl hydroxylase inhibitors dimethylxoyalyl-glycine (DMOG) (250 µM) or ethyl-3,4-dihydroxybenzoate (EDHB) (500 µM) for 24 h. In some experiments, COS-7 cells were grown in serum-free media or under hypoxia for a period of 24 h. Expression of ORP150 was subsequently evaluated by immunoblot analysis.

**Electrophoretic mobility shift assays.** Complimentary oligonucleotides encompassing the CHOP binding sequence were synthesized (wild type, 5′-CTCGGCTCAGCAACCTCCCTCAGCTATT-3′, and complimentary strand; mutant, 5′-CTCGGCTCAGTGTCGCTCCCTGCGTATT-3′, and complimentary strand). All duplex oligonucleotides were annealed by combining them in a 1:1 molar ratio in 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, and 50 mM KCl, and heated to 100°C for 5 min and then cooled to room temperature. The 5′-ends of the annealed oligonucleotides were phosphorylated with [γ-32P]ATP using polynucleotide kinase and purified by passage through NuTrap Probe purification columns (Stratagene). A 20,000 counts/min aliquot of the probe was incubated for 30 min at 25°C with an aliquot of COS-7 nuclear extract containing 10 µg of protein and 2 µg of dl-dC (Pharmacia) in 4% vol/vol glycerol, 2 mM MgCl2, 0.5 mM EDTA, 0.5 mM dithiothreitol, 50 mM NaCl, 10 mM Tris·HCl (pH 9.0), and 300 µg/ml BSA in a total volume of 20 µl. The reaction mixtures were resolved by electrophoresis on a 5% polyacrylamide gel. Dried gels were analyzed by phosphorimaging. In antibody binding assays, monoclonal antibodies were used against CHOP (Alexis Biochemicals) or control monoclonal anti-β-actin (2 µg, Sigma) and were incubated for 30 min on ice with the nuclear extracts before the addition of the labeled oligonucleotides.

**ORP150 degradation.** The sensitivity of ORP150 to calpain 10-mediated cleavage was determined using isolated rat renal cortical mitochondria. Briefly, isolated rat renal mitochondria were incubated with 1 µM Ca2+ for 5 min at room temperature in the presence and absence of 10 µM calpeptin. ORP150 degradation was subsequently evaluated by immunoblot analysis.

**Calpain activity.** Calpain activity was assayed spectrophotometrically using the calpain-selective substrate SLVVY-AMC (Bachem), as previously described (4). Mitochondrial extracts (200 µg) were diluted in buffer B and incubated with 10 mM CaCl2 (to ensure maximal calpain activation) in the presence of 50 µM SLVVY-AMC. Calpain activity was measured under linear conditions as a function of AMC hydrolysis using excitation and emission wavelengths of 355 and 444 nm, respectively. The fluorescence of mitochondria incubated in the absence of substrate was equivalent to buffer B alone.

**RESULTS**

**ORP150 is dualy targeted to the ER and mitochondria.** Evaluation of the NH2-terminus of ORP150 revealed an enrichment in positively charged residues indicative of classical mitochondrial targeting signal. The protein localization algorithm MITOP2 (http://ihg.gsf.de/mitop2/start.jsp) was used to further evaluate the possibility that ORP150 may be a mitochondrial protein and returned a probability score of 54%. ER and mitochondrial fractions from rat kidney cortex were probed for ORP150 using a primary antibody against the hamster GRP170, the hamster orthologue of mammalian ORP150. Immunoblot analysis revealed the presence of one immunoreactive protein eluting at ~170 kDa in ER, mitochondria, and the mitochondrial matrix fraction (Fig. 1A), suggest-
ing the presence of fully glycosylated ORP150. Purity of mitochondrial and ER fractions was assessed by immunoblot using antibodies directed against HSP60 and calnexin, respectively (Fig. 1A). Antibody specificity was verified by overexpression of ORP150 in COS-7 cells using the pcDNA3.1-ORP150 vector, followed by immunoblot analysis using anti-GRP170. This blot indicated an increase in band intensity in cells overexpressing ORP150 (~170 kDa) (Fig. 1B). These results provide strong evidence that the GRP170 antibody only recognizes ORP150 and that ORP150 is localized to the mitochondria. The immunodetection of ORP150 in all future experiments demonstrates immunoreactivity at ~170 kDa unless otherwise specified.

**ORP150 is targeted to mitochondria via an NH2-terminal targeting peptide.** Human ORP150 was subcloned into a TOPO-TA-CT-GFP vector (Invitrogen) to produce an ORP150-GFP fusion protein containing an intact NH2-terminus and a COOH-terminal GFP moiety. This construct was chosen to avoid modification of the NH2-terminus, which may result in the loss of putative mitochondrial localization motifs. NIH-3T3 cells were transfected with the pcDNA3.1-ORP150-GFP construct for 24 h, exposed to MitoTracker Red (20 min), and imaged via confocal microscopy. Cells also were transfected using GFP constructs specific for cytosol (data not shown) or mitochondria (Fig. 2A) to serve as positive GFP controls for these compartments. The transfection experiments revealed that the ORP150-GFP construct localized to the mitochondria of NIH-3T3 cells (Fig. 2B).

To test the hypothesis that the NH2-terminus of ORP150 is responsible for its mitochondrial localization, oligonucleotides coding for the first 13 NH2-terminal amino acid residues were annealed and ligated into the TOPO-TA-CT-GFP vector. Negative controls included the same oligonucleotides inserted in the reverse orientation. NIH-3T3 cells transfected with these constructs displayed cytosolic targeting and mitochondrial targeting for the reverse and forward orientations, respectively (Fig. 2C). These results reveal that the NH2-terminal 13 amino acids of ORP150 are sufficient for mitochondrial targeting.

NIH-3T3 fibroblasts were transfected with pcDNA3.1-ORP150-GFP and incubated for 48 h, and ORP150 was measured with immunoblot analysis to confirm mitochondrial targeting. Briefly, ER and mitochondrial subfractions were probed for the presence of GFP-tagged and native ORP150. ORP150-GFP was primarily observed in the mitochondrial fraction, whereas native ORP150 was present in both mitochondrial and ER fractions (Fig. 2D). Very little ER-localized ORP150-GFP was observed with confocal imaging or immunoblot analysis (Fig. 2, B and D). This observation may be a result of the masking of the ER retention signal (KNDEL) by the COOH-terminal GFP moiety.

**Fig. 4.** The mitochondrial stress response (MSR) induces expression of ORP150. The MSR can be induced via mitochondrial expression of a mutated form of ornithine transcarbamylase (ΔOTC). ΔOTC forms insoluble protein aggregates once inside the mitochondria and induces the expression of numerous mitochondria-specific chaperone molecules. A: diagrammatic representation of the wild-type OTC and mutant ΔOTC from which the carbamyl phosphate-binding domain (amino acids 30–114) has been deleted. B: transient expression of wild-type OTC and ΔOTC in mitochondrial extracts of COS-7 cells. The p- and m-proteins represent precursor and signal peptide-cleaved mature forms of both OTC and ΔOTC. C: induction of MSR was validated by Western blot analysis. HSP60 and anti-C/enhancer-binding protein homologous protein (CHOP) protein levels were increased significantly in ΔOTC-transfected COS-7 cells. D: effects of the MSR on ORP150 expression was evaluated by Western blot analysis. ORP150 protein levels were significantly elevated in ΔOTC-transfected COS-7 cells. Equal protein loading in mitochondrial and ER fractions was verified by immunoblot analysis using antibodies against complex I protein ND6 and calnexin, respectively. Results are representative of three independent experiments.
ORP150 expression increased after mitochondrial stress. ORP150 is a chaperone molecule best known for its induction in the ER under hypoxic conditions (19). Given its presence in the mitochondria, we sought to determine whether this stress-responsive molecule also would be induced in mitochondria under various cellular insults/stresses. First, we induced cellular stress using etidium bromide treatment because this treatment has been shown to disturb mitochondrial function by decreasing mitochondrial gene transcription (35). COS-7 cells were treated with various concentrations of etidium bromide for 7 days. ORP150 expression increased in the presence of etidium bromide in a concentration-dependent manner (Fig. 3). Equal protein loading and fraction purity was verified by immunoblot analysis using the nuclear-encoded mitochondrial protein HSP60 and resident ER protein calnexin, respectively (Fig. 3).

Subsequently, we chose to induce what is known as the mitochondrial stress response (MSR), as described by Zhao et al. (39). The MSR is induced via transfection of cells with a mutant form of OTC, a protein normally expressed in liver mitochondria. The mutant form of OTC is incapable of folding correctly after mitochondrial import and induces the expression of mitochondria-specific chaperone molecules such as HSP60, chaperonin 10, chaperonin 60, and mtDnaJ (39). Diagrammatic representation of the wild-type OTC and mutant ΔOTC from which the carbamyl phosphate-binding domain (amino acids 30–114) has been deleted is depicted in Fig. 4A. Briefly, COS-7 cells were transiently transfected with wild-type OTC, mutant ΔOTC, and vector-only expression vectors for 36 h. Protein expression was verified by immunoblot analysis, which revealed mature and precursor wild-type and mutant OTC in the mitochondria (Fig. 4B). Induction of known MSR proteins (HSP60 and CHOP/GADD153) in the ΔOTC-treated cells was confirmed by immunoblot analysis (Fig. 4C). Finally, the expression of ORP150 was evaluated under the same conditions and was found to be increased in both the mitochondrion and ER fractions of ΔOTC-treated cells (Fig. 4D). Equal protein loading and fraction purity of mitochondrial and ER samples was verified by immunoblot using ND6 and calnexin, respectively. These results reveal that two independent mitochondrial stressors result in the induction of ORP150.

ORP150 expression is increased after cellular insult. The next series of experiments were designed to determine whether diverse chemical and environmental stressors could induce ER and mitochondrial ORP150 protein expression. COS-7 cells were treated for 24 h in the presence of tunicamycin (10 μg/ml), 2-deoxyglucose (10 mM), and the prolyl hydroxylase inhibitors DMOG (250 μM) and EDHB (500 μM). Cells also were grown in the absence of serum or under hypoxic conditions (10 μg/ml tunicamycin, 10 mM 2-deoxyglucose, 250 μM dimethylglyoxal-glycine (DMOG), and 500 μM ethyl-3,4-dihydroxybenzoate (EDHB)). Cells cultured in the absence of fetal bovine serum or under hypoxic conditions were also harvested at 24 h. Samples were subsequently prepared for immunoblot analysis and probed with antibodies against GRP78. Fully glycosylated ORP150 was elevated after various treatments (−170 kDa), whereas native unmodified ORP150 (150 kDa) was detected in the samples pretreated with tunicamycin. Equal protein loading and purity of mitochondrial and ER fractions was verified by immunoblot analysis using antibodies against HSP60 and calnexin, respectively. Results are representative of three independent experiments.

ORP150 expression is regulated by the transcription factor CHOP/GADD153. ORP150 expression is regulated by hypoxia and the unfolded protein response (UPR) via presence of an ER stress element (ERSE) spanning intron 1 and exon 2 of the ORP150 gene (11). The transcription factor CHOP is known to induce numerous stress response genes including mitochondrial chaperones Cpn60/10, ClpP, and mtDnaJ (39) and has been shown to be upregulated through the MSR and UPR (25, 39). We identified a CHOP binding element (TGCAACC) in the 5’ promoter region (−2645 bp upstream of exon 1A) of the ORP150 gene and therefore hypothesized that MSR-mediated ORP150 expression in the ER and mitochondria can be mediated by CHOP induction. Electrophoretic mobility shift assays (EMSA) were conducted in which a 30-bp ORP150 promoter fragment containing the CHOP-binding element (wild-type) and a mutant probe (mutant) containing an altered CHOP site were incubated with nuclear extracts isolated from cells transfected with various constructs or incubated with tunicamycin and subjected to PAGE and PhosphorImager analysis. An increase in the level of a high-molecular-weight complex was observed in nuclear extracts from the OTC and ΔOTC COS-7 cell transfection groups, with the largest amount of binding observed in cells expressing ΔOTC (Fig. 6, lanes 6–8). As a positive control, nuclear extracts from tunicamycin-treated cells, an agent known to induce CHOP expression (36), led to an increase in the high-molecular-weight complex (Fig. 6, lane A). Briefly, ORP150 expression was evaluated under the same conditions and was found to be increased in both the mitochondrion and ER fractions of ΔOTC-treated cells (Fig. 4D). Equal protein loading and fraction purity of mitochondrial and ER samples was verified by immunoblot using ND6 and calnexin, respectively. These results reveal that two independent mitochondrial stressors result in the induction of ORP150.
Nuclear extracts from cells treated with tunicamycin or cells expressing the various constructs did not produce high-molecular-weight complexes when incubated with mutant probe DNA (Fig. 6, lanes 1–4).

Next, we assessed whether the complex observed was due to binding of CHOP to this promoter region. Nuclear extracts of cells expressing /H9004 OTC were isolated and incubated with monoclonal antibodies against CHOP or a control protein (anti-/H9252-actin) before the EMSA was conducted. A band shift was identified in extracts incubated with a monoclonal CHOP antibody and was absent in those incubated with anti-/H9252-actin (Fig. 6, lanes 11–12), suggesting that the band shift was dependent on the binding of CHOP to the promoter region outlined in the MATERIALS AND METHODS. These results suggest that induction of the MSR leads to increased cellular CHOP and subsequent upregulation of ORP150.

ORP150 is a mitochondrial calpain substrate. Recent studies using cardiac ischemia as a model system revealed that the overexpression of ORP150 negatively regulates cytosolic calpain activity by blocking the ischemia-induced release of ER Ca²⁺ stores (1). Given that ORP150 (this work) and calpain 10 (2) are both localized to the mitochondrial matrix, we sought to determine whether these proteins interacted within isolated mitochondria. Mitochondrial calpain 10 activity was assessed in COS-7 cells after transfection with vector or /H9004 OTC and was significantly elevated in /H9004 OTC-transfected cells (Fig. 7A).

To further evaluate ORP150/calpain 10 interactions, we investigated the possibility that ORP150 is a calpain 10 substrate. Using the calpain substrate prediction algorithm PEST-FIND (http://srs.nchc.org.tw/emboss-bin/emboss.pl?_action=input&_ap=p = pestfind), we determined that ORP150 is a candidate substrate for calpain-mediated proteolysis. Subsequently, degradation experiments were performed in which isolated rat renal mitochondria were treated with Ca²⁺ (1 μM) in the presence and absence of 10 μM calpeptin. ORP150 was found to be cleaved in the presence of Ca²⁺, and this degradation was significantly inhibited by pretreatment with calpeptin (Fig. 7B). These results reveal that mitochondrial calpain 10 activity increases in response to the MSR and ORP150 induction, and that mitochondrial calpain 10 cleaves ORP150 in a Ca²⁺-dependent manner.

DISCUSSION

There are two major classes of stress proteins: 1) GRPs, which are induced by inhibition of glycosylation, defects in Ca²⁺ homeostasis, chronic hypoxia, glucose deprivation, and reductive stress; and 2) HSPs, which are induced by heat, oxidative stress, and ethanol, among varied other environmental stressors (29). The GRPs are primarily ER proteins, whereas the HSPs are more ubiquitously localized to the cytosol, nucleus, and mitochondria, reflecting the sensitivities of these compartments to various environmental stresses. Despite their varied localizations, both classes act to preserve the normal integrity and functional viability of damaged proteins. Their activities range from, but are not limited to, protein stabilization, protein folding and unfolding, the chaperoning of molecules between cellular compartments, and protein complex assembly (38).

Since the discovery of ORP150 in 1996 as a hypoxia-inducible element in cultured astrocytes, much has been elucidated about its protective role in cell death (19). Numerous
studies have implicated a role for ORP150 in blocking cell death in various models (1, 6, 12–14, 24, 27, 30). Many of these studies demonstrated increases in cytosolic Ca\(^{2+}\) (1, 14), cytochrome c (1), and active caspase 3 (1, 6, 14) in stressed cells, all of which were found to be decreased when ORP150 expression was induced. More striking, were reports of decreased calpain activity in ORP150 transgenic animals after induction of cardiac ischemia-reperfusion injury or glutamate-mediated neuronal toxicity (14). Whereas the exact mechanism of ORP150 action/protection has not been determined, investigators have placed the site of action of ORP150 at the ER due to its COOH-terminal KNDEL sequence (10, 19). We hypothesized that ORP150 might also be localized to mitochondria and that it may be regulated by Ca\(^{2+}\)-induced calpain 10 activity. Mitochondrial localization of ORP150 could provide a novel means of regulating cell death through modulation of the intrinsic apoptotic pathway or through modulation of cellular calpain activity.

In the present study, we identified the mitochondrial localization of ORP150. Endogenous ORP150 expression in the rat kidney cortex was found to be localized to the ER and mitochondria, including the mitochondrial matrix, suggesting that ORP150 is not simply associated with the outer mitochondrial membrane. Expression of ORP150 tagged with GFP at its COOH-terminus, to avoid blocking any putative NH\(_2\)-terminal mitochondrial targeting signals, resulted in mitochondrial, but not ER, localization. This finding was further strengthened by observation that GFP-tagged ORP150 was primarily localized to the mitochondrial fraction of transfected NIH-3T3 cells. The lack of ER staining under these conditions is likely due to blockade of the COOH-terminal ER retention signal (KNDEL) by the GFP moiety.

We tested the hypothesis that the NH\(_2\)-terminal 13 amino acids of ORP150 are responsible for mitochondrial localization and determined that this signal sequence is sufficient for the targeting of ORP150 to the mitochondrion. It is likely that this NH\(_2\)-terminal mitochondrial targeting signal competes with the COOH-terminal ER retention signal (KNDEL) for the normal trafficking of ORP150 within the cell.

The expression of mitochondria-specific chaperone molecules can be induced by a variety of stressors, including heat shock, oxidation, mitochondrial inhibition, and via accumulation of mitochondrial protein aggregates (20). We utilized two independent methods of mitochondrial stress induction to evaluate their effects on the expression of mitochondrial ORP150. Chronic dosing of COS-7 cells with ethidium bromide induced a concentration-dependent increase in mitochondrial ORP150 expression. Similarly, the induction of the MSR using a truncated deletion mutant of ornithine transcarbamylase increased ORP150 in both the ER and mitochondrial fractions. These data reveal that mitochondria-specific stressors induce ORP150 and may serve as a protective mechanism to preserve mitochondrial viability. In our hands, the induction of ORP150 by the MSR is not specific to the mitochondria: significant protein upregulation was also found in the ER. However, we postulate that this observation is due to the competing NH\(_2\)- and COOH-terminal localization motifs found in ORP150.

ER-localized ORP150 expression can be modulated by a variety of chemical agents and cell treatments, including hypoxia, hyperosmotic culture conditions, and culture in the presence of tunicamycin and 2-deoxyglucose (1, 5, 6, 12–14, 21–23, 27). We showed that a number of these treatments as well as serum starvation and treatment with prolyl hydroxylase inhibitors (to mimic hypoxia) also induced ORP150 expression in the mitochondria and the ER, suggesting that numerous diverse cellular insults can regulate ORP150 in both organelar fractions.

We also sought to determine the mechanism of mitochondrial-stress driven ORP150 induction. In the work by Kaneda et al. (11), the production of three mRNAs was shown to be driven by alternative promoters that had preferential induction of one species under stress conditions, including hypoxia and tunicamycin treatment. In this study, promoter analysis revealed the presence of three transcription start sites, which begin at exons 1A, 1B, and 2. The differential transcription of the ORP150 gene during stress is thought to be controlled by the presence of an ER stress element (ERSE) located in the promoter region. Indeed, upon analysis of the ORP150 promoter region, a 19-bp sequence was identified as being almost identical to that of the ERSE (CCATN\(_6\)CCACG) with only 1-bp mismatch. Kaneda et al. (11) also showed that this sequence interacts with the transcription factor ATF6 and preferentially produces cDNAs containing exon 1B due to overlap of the ERSE sequence with the S\(^{\prime}\) end of exon 1A.
Induction of the unfolded protein response (UPR) is initiated under periods of ER stress to upregulate cellular chaperones required to properly fold and maintain damaged cellular proteins (38). Recent reports have also demonstrated the upregulation of transcription factors such as CHOP/GADD153 and XBP-1 in response to the UPR (36). We showed the induction of nuclear CHOP/GADD153 protein levels after the induction of ΔOTC-mediated MSR in COS-7 cells. Furthermore, CHOP bound to a putative CHOP binding element located 2,645 bp upstream of exon 1A as shown by an electrophoretic mobility shift assay. Taken together, the enhanced expression of ORP150 and CHOP, and the ability of CHOP to bind elements upstream of the ORP150 promoter under these conditions, suggest that enhanced CHOP expression may modify ORP150 levels during periods of mitochondrial stress. These finding are also significant because induction of the UPR also upregulates cellular CHOP.

Our studies also revealed a smaller molecular weight protein that immunoreacted with the GRP170 antibody after tunica-elastica disruption, calpain 10 becomes activated and cleaves ORP150. As has been observed for numerous other mitochondrial chaperone proteins (38). Recent reports have also demonstrated the upregulation of CHOP/GADD153 and the ability of CHOP to bind elements upstream of the ORP150 promoter under these conditions.

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