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Cloning and Expression of cDNA Encoding the Human 150 kDa Oxygen-Regulated Protein, ORP150

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We have cloned a cDNA encoding the human 150 kDa oxygen-regulated protein (ORP150) from hypoxia-treated astrocytoma U373 cDNA library. The deduced amino acid sequence of 999 residues contains a signal peptide and an ER retention-like signal at the N- and C-termini, respectively. It has a striking sequence similarity (91% identity) with Chinese hamster 170 kDa glucose-regulated protein (GRP170). The N-terminal half of ORP150 exhibits significant similarity to the ATPase domain of HSP70 family proteins with well-conserved ATP binding motifs. Northern blot analysis revealed that induction of ORP150 in U373 cells was not limited to hypoxia but also observed by 2-deoxyglucose or tunicamycin treatment. Furthermore, tissue specificity of expression of ORP150 was quite similar to that of GRP78. These findings suggest that ORP150 participates in quality control of proteins in the ER in response to diverse environmental stresses.

Astrocytes, the most abundant cell type in mammalian brain, play an important role in the maintenance and regeneration of neuronal functions. They retain cell viability even in extreme ischemia and proliferate in damaged brain, leading to tissue remodeling in the central nervous system (1, 2). Rat astrocytes exposed to hypoxia followed by reoxygenation were reported to release increased amounts of interleukin 6 (IL-6) that could promote neuronal survival in ischemic brain (3). On the other hand, induction of a set of stress proteins with molecular masses of about 28, 33, 78, 94, and 150 kDa was observed in cultured rat astrocytes exposed to hypoxia or hypoxia/reoxygenation (4). The 78 kDa protein identified as GRP78 (BiP) was found to be involved in the hypoxia-induced production of IL-6 in astrocytes (4). The 94 kDa protein whose expression was also induced by 2-deoxyglucose treatment may correspond to GRP94. Although other proteins remain to be characterized, these observations raise the possibility that the induction of these proteins by hypoxia is implicated in the astrocyte capacity to proliferate and generate neurotrophic mediators under environmental perturbation such as brain ischemia.

In the previous study (5), we observed induction of a 150 kDa protein in the endoplasmic reticulum (ER) of cultured astrocytes specifically by hypoxia and not by other stimuli including heat shock, hydrogen peroxide, cobalt chloride, 2-deoxyglucose, or tunicamycin. The selectivity in response to oxygen deprivation led us to designate this protein ORP150 (150 kDa oxygen-regulated protein). Furthermore, cerebral ischemia in mouse caused induction of ORP150 in the ischemic area (5), suggesting that induction of ORP150 is part of central nervous response to oxygen deprivation.

Here we report the cloning of human and rat ORP150 cDNAs from hypoxia-treated human astrocytoma U373 cells and rat astrocytes, respectively. Comparison of the predicted amino acid sequences encoded by the cDNAs with protein data bank revealed that ORP150 most likely represents human and rat homologues of Chinese hamster GRP170, recently reported to be a member of large and highly diverged class of HSP70-like proteins (6). Northern blot analysis revealed a marked similarity between ORP150 and GRP78 in stress inducibility in U373 cells and tissue specificity of expression.

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Abbreviations: ER, endoplasmic reticulum; G3PDH, glyceraldehyde-3-phosphate dehydrogenase; GRP, glucose-regulated protein; ORP, oxygen-regulated protein; UTR, untranslated region.

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MATERIALS AND METHODS

Cell culture and conditions for hypoxia. Astrocytes prepared from neonatal rats were cultured and exposed to hypoxia as described previously (5). Human astrocytoma U373 cells obtained from American Type Culture Collection were grown in Dulbecco's Modified Eagle Medium (Life Technologies, Inc., Gaithersburg, MD) supplemented with 10% fetal calf serum. Cultures which achieved 70% confluence were exposed to hypoxia using an incubator attached to an hypoxia chamber (Coy Laboratory Products, Ann Arbor, MI) (7).

Amino acid sequence analysis of rat ORP150. Rat ORP150 was purified from cultured rat astrocytes exposed to hypoxia for 48 h and transferred to a polyvinyllidene difluoride (PVDF) membrane as described (5). The band corresponding to ORP150 was cut out and subjected to sequence analysis using a Model 476A sequencer (Perkin-Elmer Corp.), yielding a unique N-terminal 31 amino acid sequence, LAVMSVDLGSESMKVAIVKPGVPMEIVLNKE. The underlined sequence was used to design a degenerate PCR primer.

Construction and screening of cDNA libraries. Total RNA was isolated from hypoxia-treated rat astrocytes or U373 cells with acid guanidinium-phenol-chloroform method (8), and poly(A) RNA was purified by using oligo(dT)-magnetic beads BioMag$(dT)_{20}$ (PerSeptive Diagnostics, Cambridge, MA). Double stranded cDNA was synthesized with random hexamer and oligo(dT) primers by using a SuperScript Choice System kit (Life Technologies, Inc.) and was ligated to the EcoRI site of pSPORT1 (Life Technologies, Inc.) to construct a cDNA library. First, a 480 bp fragment encoding a part of rat ORP150 was obtained from the rat cDNA library by PCR using 5′-AARCCIGGIGTNCCNATGGA-3′ and 5′-AATACGACTCACTATAGGGA-3′ as primers. The former degenerate oligodeoxynucleotides encode KPGVPME found in the N-terminal sequence, and the latter corresponds to the antisense strand of the T7 promoter region in pSPORT1 vector. Second, the rat astrocytes cDNA library was probed with this 480 bp fragment, and a positive clone containing a 2,800 bp insert lacking the 3′ region of the coding sequence was obtained. Finally, to clone human ORP150 cDNA, the human U373 cDNA library was screened with 5′-GCACCCTTGAGGAAAATGCT-3′ (complementary to nucleotides 2699-2718 of rat 2,800 bp cDNA) as a probe by using a GeneTrapper cDNA Positive Selection kit (Life Technologies, Inc.) according to the manufacturer's instruction. The rat cDNA library was also screened in the same manner to obtain a cDNA encompassing the entire coding region. Nucleotide sequencing was carried out in both directions by a primer-walking strategy.

Northern blot analysis. A 4.5-kb EcoRI fragment of human ORP150 cDNA was labeled with [α-^{32}P] dCTP (3,000 Ci/mmol; Amersham Corp., Arlington Heights, IL) by using a DNA labeling kit (Pharmacia), and used as a hybridization probe. 20 μg of total RNA prepared from U373 cells exposed to various stresses were electrophoresed and transferred onto a Hybond N^+ membrane (Amersham Corp.). Multiple Tissue Northern Blots, in which each lane contained 2 μg of poly(A) RNA from the adult human tissues indicated, was purchased from Clontech. The filter was hybridized at 65 °C in the Rapid-hyb buffer (Amersham Corp.) with human ORP150, GRP78, glyceraldehyde-3-phosphate dehydrogenase (G3PDH), and β-actin cDNAs each labeled with [α-^{32}P] dCTP, washed with 0.1 × SSC containing 0.1% SDS at 65 °C, and followed by autoradiography.

RESULTS AND DISCUSSION

Cloning of ORP150 cDNA

Since the reported N-terminal 15 amino acid sequence of rat ORP150 (5) was not appropriate for designing mixed probes or primers because of large degeneracy, we further performed a sequence analysis of purified rat ORP150 to determine additional 16 residues. Based on this newly determined amino acid sequence, we first cloned rat ORP150 cDNA, which should facilitate cloning of the human counterpart.

To obtain a specific probe for cloning rat ORP150 cDNA, a cDNA library prepared from cultured rat astrocytes that had been exposed to hypoxia was subjected to PCR. The amplified 480 bp fragment thus obtained was sequenced and found to contain a 39 bp nucleotide sequence encoding part of the N-terminal sequence KPGVPMEIVLNKE. Northern blot analysis using this fragment as a probe revealed that mRNA with a length of about 4 kb was induced in cultured rat astrocytes upon hypoxia (data not shown). The rat cDNA library was then screened with this PCR fragment as a probe, and a 2,800 bp cDNA containing most of the coding region but lacking the C-terminal portion was isolated. To obtain human ORP150 cDNA, a cDNA library prepared from hypoxia-treated U373 cells was screened by the Gene Trapper system. Briefly, the cDNA library was converted to single stranded DNA, and the desired cDNA clone was then enriched by hybridization with a 20-mer oligonucleotide derived from the 3′ region of the 2,800 bp rat cDNA clone. A full-length 4,503 bp human cDNA was isolated and found to contain a 2,997 bp open reading frame predicted to encode a polypeptide of 999 amino acids with a calculated molecular mass of 111,330 Da (Fig. 1). The first ATG was thought to be the translation initiation site, since a TGA nonsense codon was located 99 bp upstream in the same frame and the flanking nucleotides matched Kozak's criteria (purine at position −3 and G at +4) (9). A polyadenylation signal (AATAAA) was found 20 bp upstream of the poly(A) tail. A 3,252 bp rat cDNA containing an open reading frame with the same length but lacking most of the 3′ untranslated region was isolated in the same manner (the sequence is available from GenBank under accession number U41853).

We confirmed that the cloned cDNA encodes ORP150 by transient expression in COS-7 cells transfected with human ORP150 cDNA ligated into pSV-SPORT1 (Life Technologies, Inc.). Western blotting of the cell lysate using antibody raised against purified rat ORP150 (5) gave an immunoreactive band at a position of 150 kDa (data not shown). The deduced amino acid sequences of human and rat ORP150 exhibit high similarity (over 91% identity) to each other and to those of Chinese hamster GRP170 (GenBank U34206) for the entire molecule. Furthermore, amino acids 347-999 of human ORP150 showed 90% identity with the amino acid sequence of mouse ER-resident Ca^{2+}-binding protein CBP-140 deduced from partially cloned cDNA (10). Although ORP150, GRP170, and CBP-140 were identified from different species on different criteria, such high

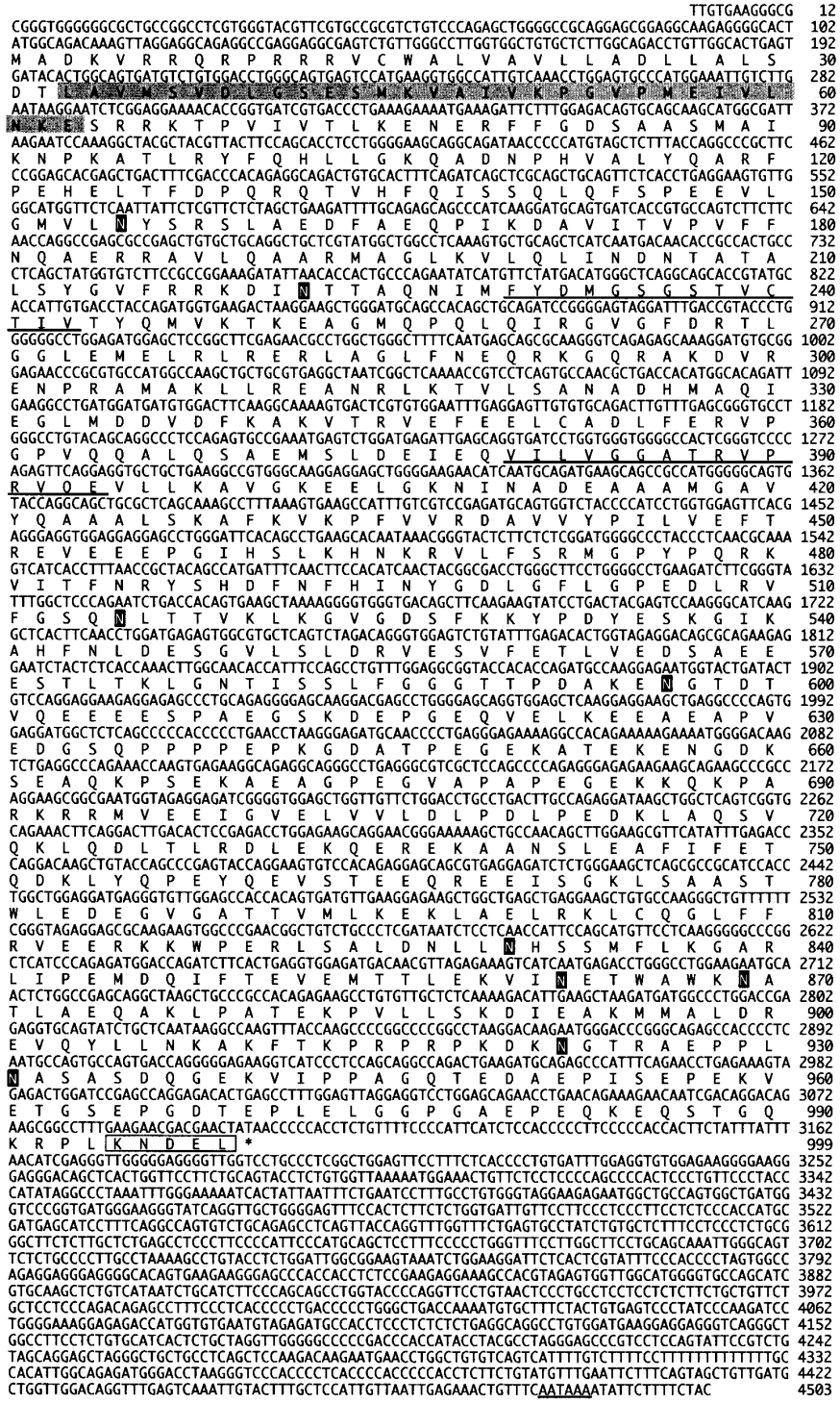

FIG. 1. Nucleotide sequence of human ORP150 cDNA and the predicted amino acid sequence. The top line shows the nucleotide sequence of the cDNA for human ORP150, and the second line shows the predicted amino acid sequence. The shaded amino acids are identical with the N-terminal 31 amino acid sequence of purified rat ORP150. The underlined amino acid sequences correspond to the HSP70 family signatures 2 and 3. The potential N-linked glycosylation sites are highlighted; the ER-retention signal-like sequence at the C-terminal is boxed. The nucleotide sequence underlined represents the potential polyadenylation signal. GenBank Accession Number is U65785.

similarities suggest that they represent functionally homologous (orthologous) proteins.

The nucleotide sequence similarity of the coding regions between human ORP150 and hamster GRP170 cDNAs was 88%, consistent with the striking conservation at the amino acid level. In contrast, both the 5′ and 3′ untranslated regions (UTRs) of these cDNAs exhibited marked diversities. The 3′ UTR of the human ORP150 cDNA (1,400 bp) was much longer than that of the hamster GRP170 cDNA (845 bp), and their overall sequence similarity was only 39%. The 5′ UTR of hamster GRP170 cDNA (54 bp) also exhibited only 44% similarity to the corresponding region of human ORP150 cDNA. Although UTR sequences are usually less conserved among species than coding sequences, such remarkable divergency suggests regulatory consequences.

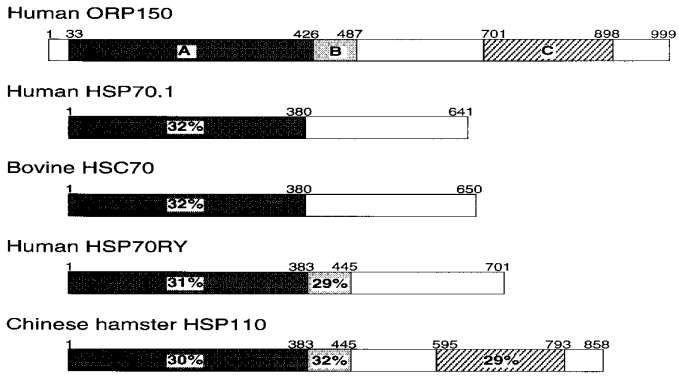

FIG. 2. Amino acid sequence similarities among human ORP150 and HSP70 family proteins. Region A containing the ATPase domain exhibits a modest similarity among HSP70 family proteins, and the similarity with HSP70RY and HSP110 further extends to region B. Similarity is also found for region C between human ORP150 and Chinese hamster HSP110. The first 32 amino acids of ORP150 represent the putative signal peptide.

Structural Features of ORP150

The N-terminal amino acid sequence obtained with purified rat ORP150 corresponded to amino acids 33-63 deduced from both the human and rat cDNAs, indicating that the first 32 residues represent the signal peptides for secretion. The C-terminal KNDEL sequence, which resembles KDEL sequence, a signal to retain the ER-resident proteins (11), may function as an ER-retention signal. The existence of a signal peptide at the N-terminus and the ER-retention signal-like sequence at the C-terminus suggests that ORP150 resides in the ER, consistent with the previous results of immunocytochemical analysis of rat hypoxic astrocytes (5). The deduced amino acid sequence contains nine potential N-linked glycosylation sites and more than 20 sites of phosphorylation by serine/threonine kinase, suggesting that at least part of the discrepancy between observed and calculated molecular masses of ORP150 is due to post-translational modifications such as glycosylation and phosphorylation.

Analysis of protein data bases with the BLAST program (12) showed that the N-terminal half of ORP150 has a modest similarity to the ATPase domain of numerous HSP70 family sequences. An extensive analysis with pairwise alignments (13) revealed that amino acids 33-426 of human ORP150 was 32% identical to amino acids 1-380 of both inducible human HSP70.1 (14) and constitutive bovine HSC70 (15), typical members of HSP70 family (Fig. 2). An additional region similar to HSP70RY and hamster HSP110, which both belong to a new subfamily of large HSP70-like proteins (16), extended further to residue 487. A protein sequence motif search with PROSITE (17) showed that ORP150 contains two of the three HSP70 protein family signatures: FYDMGSGSTVCTIV and VILVGGATRVPRVQE which completely matched with the HSP70 signatures 2 and 3, respectively (Fig. 1, underlined), and VDLG (amino acids 38-41) which matched with the first four amino acids of the signature 1. Furthermore, the N-terminal region of ORP150 contained a putative ATP-binding site consisting of the regions (amino acids 36-53, 197-214, 229-243, 378-400, and 411-425) corresponding to the five motifs specified by Bork et al. (18). Although the C-terminal putative peptide-binding domains of HSP70 family are generally less conserved (19), the C-terminal region flanked by nucleotides 701 and 898 shared appreciable similarity with HSP110 (amino acids 595-793; 29% identity).

Stress Inducibility of ORP150 mRNA

In view of the striking sequence similarity between ORP150 and GRP170 characterized as a glucose-regulated protein (20), we examined whether ORP150 mRNA was inducible by stresses other than hypoxia. Total RNAs prepared from astrocytoma U373 cells exposed to various stresses were analyzed by Northern blotting using 4.5 kb of human ORP150 cDNA as a probe. As shown in Fig. 3, the ORP150 mRNA level was highly enhanced upon 24-48 h of exposure to hypoxia. In parallel experiments, treatment with 2-deoxyglucose (25 mM, 24 h) or tunicamycin (5 μg/ml, 24 h) enhanced ORP150 mRNA to the levels comparable to that induced by hypoxia. The induction levels were also comparable with those observed for mRNA of a typical glucose-regulated protein GRP78. Heat shock treatment failed to enhance ORP150 mRNA appreciably.

Apparently, some of the environmental stresses that cause accumulation of unfolded proteins in the ER induced ORP150 mRNA in U373 cells. Similar induction was also observed in HeLa cells (data not shown). Thus, the induction of ORP150 is not restricted to hypoxia at

least in some established cell lines, in contrast to highly selective induction observed with cultured rat astrocytes (5) and human mononuclear phagocytes (21). This finding may be interpreted to mean that actively growing cells need a higher level of ORP150 expression to cope with perturbation of the ER functions caused by accumulation of unfolded or misfolded proteins.

Tissue Specificity of ORP150 Expression

Expression of ORP150 was studied by Northern blot analysis of poly(A) RNA prepared from eight different human tissues using ^{32}P-labeled ORP150 cDNA as a probe. ORP150 mRNA was found to be highly expressed in the liver and pancreas, whereas little expression was observed in kidney and brain (Fig. 4). Furthermore, the tissue specificity of ORP150 expression was quite similar to that of GRP78. The higher expression observed in the tissues that contain well-developed ER and synthesize large amounts of secretory proteins is consistent with the finding that ORP150 is localized in the ER (5).

In conclusion, both the characteristic primary protein structure and the similarity found with GRP78 in stress inducibility and tissue specificity suggest that ORP150 plays an important role in protein folding and secretion in the ER, perhaps as a molecular chaperone, in concert with other GRPs to cope with environmental stress.

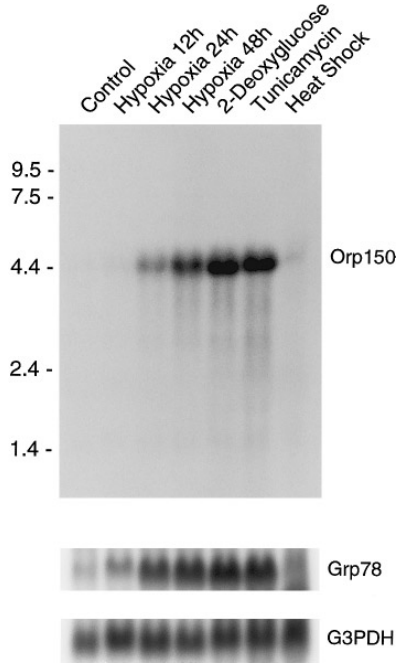

FIG. 3. Stress inducibility of ORP150 mRNA in human astrocytoma U373 cells. Total RNA was extracted and Northern blot analysis was performed using ^{32}P-labeled human ORP150 cDNA as a probe. Human GRP78 cDNA and G3PDH cDNA each labeled with [α-^{32}P] dCTP were rehybridized to the same filter. Hypoxia, treated for the indicated time period; 2-deoxyglucose, 25 mM for 24 h; tunicamycin, 5 μg/ml for 24 h; heat shock, at 43 °C for 1 h and recovery at 37 °C for 2 h. Sizes (in kilobases) are indicated on the left.

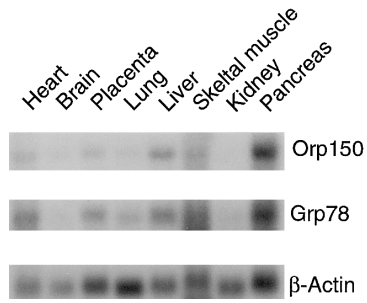

FIG. 4. Tissue specificity of ORP150 expression. Multiple Tissue Northern Blots (Clontech, Inc.), in which each lane contained 2 μg of poly(A) RNA from the adult human tissues indicated, were hybridized with ^{32}P-labeled human ORP150 cDNA. Human GRP78 cDNA and β-actin cDNA each labeled with [α-^{32}P] dCTP were rehybridized to the same filter.

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