Cloning and Characterization of AOEB166, a Novel Mammalian Antioxidant Enzyme of the Peroxiredoxin Family*

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Using two-dimensional electrophoresis, we have recently identified in human bronchoalveolar lavage fluid a novel protein, termed B166, with a molecular mass of 17 kDa. Here, we report the cloning of human and rat cDNAs encoding B166, which has been renamed AOEB166 for antioxidant enzyme B166. Indeed, the deduced amino acid sequence reveals that AOEB166 represents a new mammalian subfamily of AhpC/TSA peroxiredoxin antioxidant enzymes. Human AOEB166 shares 63% similarity with Escherichia coli AhpC22 alkyl hydroperoxide reductase and 66% similarity with a recently identified Saccharomyces cerevisiae alkyl hydroperoxide reductase/thioredoxin peroxidase. Moreover, recombinant AOEB166 expressed in E. coli exhibits a peroxidase activity, and an antioxidant activity comparable with that of catalase was demonstrated with the glutamine synthetase protection assay against dihydrothreitol/Fe3+/O2− oxidation. The analysis of AOEB166 mRNA distribution in 30 different human tissues and in 10 cell lines shows that the gene is widely expressed in the body. Of interest, the analysis of N- and C-terminal domains of both human and rat AOEB166 reveals amino acid sequences presenting features of mitochondrial and peroxisomal targeting sequences. Furthermore, human AOEB166 expressed as a fusion protein with GFP in HepG2 cell line is sorted to these organelles. Finally, acute inflammation induced in rat lung by lipopolysaccharide is associated with an increase of AOEB166 mRNA levels in lung, suggesting a protective role for AOEB166 in oxidative and inflammatory processes.

In cells and organisms that have evolved to live in an atmosphere rich in oxygen, the incomplete reduction of oxygen generates potent oxidizing agents (1). These include reactive oxygen species (ROS)1 and their toxic by-products, which may react with various cellular components such as lipids, proteins, and nucleic acids, leading to cell damage and possibly cell death (2, 3). In eukaryotes, two major intracellular sources of ROS are the mitochondrion, where electron transport coupled to oxidative phosphorylation takes place (4), and the peroxisome in which high amounts of hydrogen peroxide or superoxide anions are generated during β-oxidation of fatty acids and by the activity of various oxidases (5, 6). Moreover, other oxidative pathways in different subcellular compartments may account for ROS production, and ROS may also be generated extracellularly in the course of inflammatory processes (7).

Mammalian cells have developed complex mechanisms to protect themselves against oxidative attacks but also to maintain a redox balance in their different subcellular compartments (1). These antioxidant defense systems include enzymatic antioxidants (vitamin E, vitamin C, vitamin A, and uric acid), enzymes with antioxidant properties (catalase, superoxide dismutase, and glutathione peroxidase) as well as low molecular weight reducing agents (glutathione and thioredoxin). Recently, a new family of antioxidant enzymes, the AhpC/TSA peroxiredoxin family, has been discovered in prokaryotes and eukaryotes (8). These enzymes exhibit hydrogen peroxide and alkyl hydroperoxide reductase activities (9–12). Peroxiredoxins are considered to be involved in oxidative stress protection mechanisms but also in cell differentiation (13, 14), proliferation (14, 15), immune response (16), and apoptosis (17, 18).

Here, we report the cloning and initial characterization of AOEB166, a novel member of the mammalian peroxiredoxin family with mitochondrial and peroxisomal sorting signals. Although this new peroxiredoxin was first identified in human bronchoalveolar lavage fluid, AOEB166 presents the features of a highly conserved and widely expressed protein that might play an important antioxidant protective role in various tissues under nonpathological conditions but also during inflammatory processes.

EXPERIMENTAL PROCEDURES

cDNA Cloning and Sequence Analysis—A reverse cloning approach was used based on peptide microsequencing informations for cloning human AOEB166 cDNA. First strand cDNA was obtained with Moloney leukemia virus reverse transcriptase (Superscript II, Life Technologies, Inc.) from 2 µg of human lung RNA using oligo(dt)15 as primer accord-

1 The abbreviations used are: ROS, reactive oxygen species; AhpC, alkyl hydroperoxide reductase/LH s; DTT, dithiothreitol; GFP, green fluorescent protein; LPS, lipopolysaccharide; ORF, open reading frame; PBS, phosphate buffered saline; PCR, polymerase chain reac-

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBankTM/EBI Data Bank with accession number(s) AF110731 and AF110732.

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RESULTS

Cloning of Human and Rat AOEB166 cDNAs—Human AOEB166 was initially identified during the two-dimensional electrophoresis mapping of proteins from pooled human bronchoalveolar lavage fluid. This unknown protein was shown to have a molecular mass of 17 kDa and a pI of 6.9 (23). Thus, a partial amino acid sequence of 37 residues at the N terminus (Fig. 1A) was used to design degenerate oligonucleotide primers which allowed the PCR amplification of a human lung cDNA fragment. Based on the sequence of this amplicon, 5′- and 3′-RACE was performed, and the longest PCR products were cloned and sequenced. The composite cDNA sequence contained a poly(A) tract at the 3′-end, 36 bases of a 5′-leader sequence, an open reading frame of 645 bases, and a 116-base-long 3′-tailer sequence containing a AATAAA polyadenylation signal (Fig. 1A). Two Kayak consensus sequences for translation initiation (24) were found in the 5′-untranslated sequence. The molecular reading frame (ORF) of the longest sequence encoded a polypeptide of 214 residues (GenBankTM accession number AF110731). The screening of 5′- and 3′-amplons obtained by RACE on rat liver cDNA led to the identification of a composite cDNA sequence containing a poly(A) tract at the 3′-end, 39 bases of a 5′-leader sequence, an ORF of 642 bases, and a
Chromosomal Assignment—According to the Genebridge 4 radiation hybrid panel, the human AOEB166 gene was mapped to chromosome 11q13, about 7 cR from marker D11S913 and between markers D11S1963 and D11S4407.

Deduced Amino Acid Sequences—Analysis of the amino acid sequences of human and rat AOEB166 reveals several interesting features (Fig. 1B). First, the amino acid sequences are well conserved between the two species downstream from the second methionine (Met53 for human AOEB166 and Met52 for rat AOEB166) because they are 90% identical. However, amino acid sequences diverge upstream from Met52–53. A more precise analysis of human and rat amino acid sequences between Met1 and Met52–53 showed that these sequences are different in amino acid composition, they both display mitochondrial presequence features (25, 26). Indeed, this sequence in human and rat is composed of abundant amino acid residues with positive charges, very few negative charges, and frequent hydroxylated residues. The existence of a SQL peroxisomal targeting sequence of the peroxisomal targeting signal 1 family (27–29) was noted at the C-terminal of human and rat AOEB166. Also, three cysteines in AOEB166 (Fig. 1B) were identified, and the functional significance of these residues in AOEB166 for its antioxidant activity will be discussed below.

AOEB166 Is a Novel Mammalian Peroxiredoxin—Protein data bases were screened using BLAST 2.0 (gapped BLAST at the NCBI), and a search for identical or homologous polypeptides revealed that AOEB166 is a novel mammalian protein not yet characterized. Interestingly, sequence homology was noted with several proteins of different phyla, but none were from vertebrates. Among proteins with significant homology and known function or subcellular localization, we found that human AOEB166 (without its predicted mitochondrial presequence) had 68–65% similarity (36–35% identity) with, respectively, PMP20A and PMP20B peroxisomal membrane proteins of yeast \textit{Candida boidinii} (GenBank accession numbers J04984 and J04985), 66% similarity (26% identity) with \textit{YLR109w} ORF of \textit{Saccharomyces cerevisiae} (Fig. 1C; GenBank accession number Z73281) recently identified as a thioredoxin peroxidase/alkyl hydroperoxide reductase (12, 30), and 63% similarity (25% identity) with \textit{E. coli} alkyl hydroperoxide reductase AhpC22 protein (GenBank accession number D13187). To identify homologies between human AOEB166 and the known members of the human peroxiredoxins, we selected one member of the five known subfamilies of human peroxiredoxins, and we performed an amino acid alignment (Fig. 2A). Notably, AOEB166 conserve amino acids especially around Cys100 of human AOEB166, which has been directly implicated in catalysis of peroxides in peroxiredoxins (9, 10, 31–35). However, as for the so-called one-cysteine peroxire-
doxin ORF06 (36), many residues in human AOEB166 differ from the consensus found for the other peroxiredoxins. Interestingly, human AOEB166 possesses two other cysteines at positions 125 and 204 (Fig. 1) that could be involved in the catalysis of peroxides and in dimerization because most of peroxiredoxins exist as homodimers or heterodimers (36). However, AOEB166 seems to diverge phylogenetically from known mammalian peroxiredoxins as illustrated by the alignment but also by the phylogenetic tree presented in Fig. 2. For these reasons, we propose that AOEB166 proteins represent a new peroxiredoxin subfamily named peroxiredoxin V. As discussed by Jin et al. (37) and as illustrated in the phylogenetic tree of Fig. 2 B, the other subfamilies are subfamilies I, II, III, and IV and 1-Cys.

**Northern and Dot Blot Analysis of Human AOEB166**—Northern blot analysis of AOEB166 mRNA expression in human tissues and cell lines revealed a hybridizing region at approximately 1 kilobase (Fig. 3, A and B). AOEB166 mRNA is ubiquitously expressed in all tissues examined as well as in the cell lines. Master dot blots (CLONTECH) normalized for eight housekeeping genes were used to estimate the levels of AOEB166 mRNA in 30 different human tissues (Fig. 3 C). Interestingly, expression was significantly different among the tissues. The highest levels of expression were detected in thyroid gland, trachea, kidney, lung, adrenal gland, heart, and colon. Lower but still detectable levels were observed in pancreas, peripheral leukocytes, lymph node, and whole brain.

**Antioxidant and Peroxidase Activity of Human AOEB166**—Antioxidant activity of human AOEB166 was measured on *E. coli* recombinant protein without its predicted mitochondrial presequence (Fig. 5). The protection of glutamine synthetase from inactivation by thiol-dependent metal-catalyzed oxidation has been extensively used previously to determine antioxidant...
properties of different peroxiredoxins (8–10). As shown in Fig. 5B, inactivation of glutamine synthetase in absence of DTT as thiol reductor is completely prevented in presence of 0.2 mg/ml of AOEB166. However, when DTT is replaced by ascorbate, a reductor lacking thiol, recombinant AOEB166 is unable to protect glutamine synthetase from inactivation as expected for a peroxiredoxin (Fig. 5C). To compare the potency of catalase to that of AOEB166, we measured protection activity at various concentrations of the proteins (Fig. 5D). Bovine catalase and AOEB166 exhibited 50% of protection at the same concentration of about 0.04 mg/ml. Peroxidase activity of recombinant human AOEB166 measured by the consumption of H$_2$O$_2$ was very similar to the previously reported activity of hORF06, the human 1-Cys member of the mammalian peroxiredoxins (36).

**AOEB166 Gene Expression during Lung Inflammation**—AOEB166 was first identified at high levels in bronchoalveolar lavage fluids of patients suffering from various lung diseases (23). We therefore assessed the possibility that AOEB166 is regulated at the transcriptional level in rat lung during inflammation induced by LPS instillation. As shown in Fig. 7, AOEB166 mRNA levels increased in rat lungs with inflammation. Higher expression was reached after 24 h and was still high 72 h after LPS instillation.

**DISCUSSION**

We have recently identified the AOEB 166 protein in human bronchoalveolar lavage fluid as a novel protein (23). Here, we show that, structurally and functionally, AOEB166 is a new member of the AhpC/TSA peroxiredoxin family, a recently identified group of antioxidant enzymes evolutionarily conserved in all phyla (8). Structurally, the antioxidant function of peroxiredoxins is dependent upon conserved cysteine residues responsible for peroxide reduction and dimerization (9, 38). In the peroxiredoxin prototype TSA from *S. cerevisiae*, two active cysteines are present in positions 47 and 170. These cysteines and their neighboring residues are highly conserved in four peroxiredoxin subfamilies (Prx I, II, III, and IV) (37) (see also Fig. 2). By contrast, the 1-Cys subfamily is defined by few peroxiredoxins that have conserved only the corresponding Cys$^{47}$ and its surrounding residues. AOEB166 does not fit perfectly these subfamilies. Indeed, like Prx I, II, III, IV and 1-Cys, AOEB166 possesses a cysteine corresponding to Cys$^{47}$ of *S. cerevisiae* TSA (Cys$^{100}$ for human AOEB166) but has no cysteine residue corresponding to Cys$^{170}$ of Prx I, II, III, and IV. AOEB166 does not enter either into the 1-Cys subfamily because it contains two other cysteines, Cys$^{25}$ and Cys$^{204}$, lacking in 1-Cys subfamily and that may be involved in antioxidant activities and/or dimerization. Also, in AOEB166 the amino acids surrounding Cys$^{100}$ are much less conserved than in the other peroxiredoxins (Fig. 2A). For these reasons, AOEB166 represents the prototype for a new mammalian peroxiredoxin subfamily (Prx V in Fig. 2B).

Functionally, the antioxidant activity of AOEB166 has been confirmed *in vitro* by testing the ability of the recombinant protein to protect glutamine synthetase from the dithiothreitol/Fe$^3+/O_2$ oxidation. Like other peroxiredoxins, AOEB166 requires a thiol-containing reductor (DTT) to exert its antioxidant activity and is inactive or less active in presence of other electron donors such as ascorbate. The cellular thiol-containing reductor is still to be identified, but two good candidates as direct electron donors for AOEB166 are thioredoxin and glutathione, which are physiological reductors of several members of the peroxiredoxin family (9, 12, 30, 37, 39). The antioxidant activity of recombinant AOEB166 was quantitatively comparable with that of catalase, which suggested that hydrogen peroxide was indeed a substrate for AOEB166. This was corroborated by the time-dependent removal of hydrogen peroxide by recombinant AOEB166 in the *in vitro* peroxidase assay. Of interest, tert-butyl hydroperoxide is also consumed by the recombinant protein, which demonstrates that AOEB166 is able to reduce organic peroxides like its *S. cerevisiae* orthologue (12, 30). Thus, these data suggest that AOEB166 might afford a protection not only against hydrogen peroxide but also against alkyl hydroperoxides in mammalian cells.

Analysis of deduced amino acid sequences of both human and rat AOEB166 reveals the presence of a predicted mitochondrial presequence at the N terminus as well as a SQL peroxisomal targeting signal type 1 at the C terminus in the same protein.
Furthermore, we demonstrate that in fusion with the green fluorescent protein, these targeting sequences are functional and sort the protein to mitochondria and peroxisomes in HepG2 cells. Members of the Prx III subfamily have been also identified in mitochondria (40). AOEB166 represents therefore the second peroxiredoxin subfamily to be localized in mitochondria. Interestingly, AOEB166 is the only peroxiredoxin reported so far to be addressed to the peroxisomes. The functional significance of AOEB166 localization in organelles to which other antioxidant proteins with similar enzymatic activities (glutathione peroxidase, catalase) are sorted is still to be investigated.

The fact that AOEB166 is well conserved among species and is expressed in all tissues and cell lines examined in this study...
is consistent with an important physiological function for that protein. In that respect, it is interesting to note that AOEB166 expression is highest precisely in those tissues, such as thyroid gland, lung, or kidney, that are particularly exposed to oxidative stress (41, 42). Although at this stage the implication of AOEB166 in physiopathological processes remains speculative, the significant increase of AOEB166 gene expression in rat lungs with lipopolysaccharide-induced inflammation suggests that the protein may play in vivo a protective role against oxidative damage. Furthermore, several observations indicate that AOEB166 might be implicated in various pathophysiological situations by mechanisms that do not necessarily imply directly its antioxidant activity but rather its high conservation during evolution. In particular, the AOEB166 gene is located to human chromosome 11q13, which is a region of genetic linkage for atopic hypersensitivity (asthma, hay fever, and eczema) (43) and AOEB166 presents a high homology to a major allergen of Aspergillus fumigatus (GenBank™ accession number U58050) (44). An attractive hypothesis would be that IgE antibodies directed to the allergen would cross-react with AOEB166 and therefore initiate autoimmunity. This mechanism has been postulated for allergen manganese superoxide dismutase of A. fumigatus, which also exhibits a high homology to human manganese superoxide dismutase (45).

In conclusion, our data show that AOEB166 represents a new subfamily of the peroxiredoxin mammalian antioxidant enzymes with functional mitochondrial and peroxisomal targeting signals. The protein, highly conserved throughout species and widely distributed in the body, presents several features, suggesting that it may play an important protective role against oxidative damages caused by peroxides in organelles that are major sources of ROS.

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Addendum—Since this article was submitted, a third paper describing S. cerevisiae YLR109w thioredoxin peroxidase function has been published (47).

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