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Characterization of Human and Murine PMP20 Peroxisomal Proteins That Exhibit Antioxidant Activity *in Vitro**

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We have isolated the cDNAs encoding human and mouse homologues of a yeast protein, termed peroxisomal membrane protein 20 (PMP20). Comparison of the amino acid sequences of human (HsPMP20) and mouse (MmPMP20) PMP20 proteins revealed a high degree of identity (93%), whereas resemblance to the yeast *Candida boidinii* PMP20A and PMP20B (CbPMP20A and CbPMP20B) was less (30% identity). Both HsPMP20 and MmPMP20 lack transmembrane regions, as do CbPMP20A and CbPMP20B. HsPMP20 mRNA expression was low in human fetal tissues, especially in the brain. In adult tissues, HsPMP20 mRNA was expressed in the majority of tissues tested. HsPMP20 and MmPMP20 contained the C-terminal tripeptide sequence Ser-Gln-Leu (SQL), which is similar to the peroxisomal targeting signal 1 utilized for protein import into peroxisomes. HsPMP20 bound directly to the human peroxisomal targeting signal 1 receptor, HsPEX5. Mutagenesis analysis showed that the C-terminal tripeptide sequence, SQL, of HsPMP20 is necessary for its binding to HsPEX5. Subcellular fractionation of HeLa cells, expressing epitope-tagged PMP20, revealed that HsPMP20 is localized in the cytoplasm and in a particulate fraction containing peroxisomes. Double-staining immunofluorescence studies showed colocalization of HsPMP20 and thiolase, a *bona fide* peroxisomal protein. The amino acid sequence alignment of HsPMP20, MmPMP20, CbPMP20A, and CbPMP20B displayed high similarity to thiol-specific antioxidant proteins. HsPMP20 exerted an inhibitory effect on the inactivation of glutamine synthetase in the thiol metal-catalyzed oxidation system but not in the nonthiol metal-catalyzed oxidation system, suggesting that HsPMP20 possesses thiol-specific antioxidant activity. In addition, HsPMP20 removed hydrogen peroxide by its thiol-peroxidase activity. These results indicate that HsPMP20 is imported into the peroxisomal matrix via PEX5p and may work to protect peroxisomal proteins against oxidative stress. Because some portion of PMP20 might also be present in the cytosol, HsPMP20 may also have a protective effect in the cytoplasm.

Peroxisomes, also called microbodies, are single-membrane-bound organelles present in all mammalian cells with the exception of erythrocytes and are also found in plants, yeast, and most other eukaryotic cells. The peroxisome contains nearly 50 enzymes, many participating in various metabolic pathways (1, 2). Human peroxisomal enzymes are involved in numerous metabolic processes including β-oxidation of long and very long chain fatty acids, several steps in the synthesis of ether lipid, bile acids, and cholesterol, oxidation of D-amino acids, and α-oxidation (2, 3). Peroxisomes also contain catalase, which plays a central role in eliminating the hydrogen peroxide (H_2O_2) produced by peroxisomal oxidases.

"Newly synthesized" peroxisomal matrix proteins contain a peroxisomal targeting signal (PTS),[1] either PTS1 (4) or PTS2 (5), and are imported post-translationally (6) from the cytoplasm into the peroxisomes by the PTS1 and PTS2 receptors, respectively (7). The PTS1 sequence is a C-terminal tripeptide, Ser-Lys-Leu (SKL) or a variant (4), whereas the PTS2 sequence is an N-terminal peptide, (R/K)(L/V/I)X_5(H/Q)(L/A) (5). Most peroxisomal matrix proteins utilize PTS1, whereas a few utilize PTS2. However, either sequence is sufficient for peroxisomal targeting and is used by evolutionarily diverse organisms (8). Yeast peroxisome biogenetic mutants (*pex* mutants) have been used to identify over 20 genes (*PEX*) and their protein products (peroxins) that are required for peroxisomal protein import and biogenesis (8, 9). These genes include *PEX5* and *PEX7*, encoding the receptors for PTS1 and PTS2 sequences, respectively (10). Although human PEX5 isoforms are mainly present in the cytoplasm (11, 12), they shuttle between the cytosol and the peroxisomal membrane, bringing the PTS1-containing proteins into the peroxisomes (7, 13).

For some peroxisomal proteins, no apparent functions have been defined. Among these are the yeast PMP20 proteins. A data base search revealed that *Candida boidinii* PMP20 and the *Saccharomyces cerevisiae* counterpart contained the PTS1 sequences, Ala-Lys-Leu (AKL) and Ala-His-Leu (AHL), respectively. In addition, secondary structure analysis of all yeast PMP20 proteins reported suggests that there are no obvious membrane-spanning regions as previously reported for CbPMP20A and CbPMP20B (14). Initially, the *C. boidinii* PMP20 was defined as a membrane protein (15, 16). However, PMP20 is released from the membrane and was shown to be

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This paper is dedicated to William Troy and Charlene Engelhard for their continuing friendship and support for our research program.

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[1] The abbreviations used are: PTS, peroxisomal targeting signal; PMP, peroxisomal membrane protein; PCR, polymerase chain reaction; EST, expressed sequence tag; GST, glutathione *S*-transferase; TSA, thiol-specific antioxidant; DTT, dithiothreitol; MCO, metal-catalyzed oxidation; Hs, *Homo sapiens*; Mm, *Mus musculus*; Cb, *C. boidinii*; Sc, *S. cerevisiae*; bp, base pair(s); HA, hemagglutinin; GFP, green fluorescent protein; TK, thymidine kinase; EGF, epidermal growth factor; NRP/B, nuclear matrix protein B; PAGE, polyacrylamide gel electrophoresis.

present in the matrix by immunocytochemistry (17–23). In the present study, we have cloned two mammalian PMP20 cDNAs, determined the subcellular location of the PMP20 protein in mammals, and discovered a potential function for this class of proteins.

EXPERIMENTAL PROCEDURES

Cloning and Sequencing of Human PMP20 cDNA—When a nonredundant data base of human expressed sequence tag (EST) entries in GenBank™ was screened for human cDNAs similar to the CbPMP20A sequence, no significant matches were identified. However, a number of sequences (*e.g.* EST179427, EST185495, and EST186754) displayed some similarity to the coding region of the CbPMP20A sequence. Using the EST179427 sequence, we designed primers and amplified the putative sequence by the polymerase chain reaction (PCR) using as the template DNA from a human hippocampus cDNA library in the λZAPII vector (Stratagene, San Diego, CA). The PCR fragment was sequenced to confirm identity (99%) with the EST179427 sequence. The PCR fragment was radiolabeled using an [α-^{32}P]dCTP (NEN Life Science Products). Using this probe, the human hippocampus cDNA library was screened by hybridization. Positive clones were isolated, plaque-purified, excised, subcloned in pBluescript-SK (Stratagene, San Diego, CA), and sequenced on both strands. Using the same probe, a mouse brain cDNA library in the λgt11 vector (CLONTECH, Palo Alto, CA) was also screened, and positive clones were sequenced. Primers for PCR and sequencing were purchased from Genosys (The Woodlands, TX). Sequence alignment was performed by DNASTAR (Madison, WI).

Northern Blot Analysis—Blots containing poly(A$^+$) RNA from various human fetal and adult tissues were purchased from CLONTECH (Palo Alto, CA). A gene-specific probe was generated by restriction digestion of the human PMP20 (HsPMP20) cDNA by *Pst*I (nucleotides 218–683). This 460-bp fragment was radiolabeled to a specific activity of 10^8–10^9 cpm/μg and was used as a probe for all Northern blots. The blots were hybridized with the probe according to the manufacturer's instructions. In addition, each blot was probed for β-actin or glyceraldehyde-3-phosphate dehydrogenase.

Vectors, Antibodies, Cell Culture, and Transfection—The DNA corresponding to the HA tag sequences (30 bp) and the coding region of human PMP20 cDNA (486 bp) were subcloned into the pcDNA3 vector (Invitrogen, Carlsbad, CA) to yield pcDNA3-HA-HsPMP20. We subcloned the PMP20 cDNA into the pTK-Hyg vector (CLONTECH, Palo Alto, CA) and fused it to the gene encoding the green fluorescent protein (GFP) under the control of the herpes simplex virus thymidine kinase (TK) promoter, to generate the plasmid pTK-GFP-HsPMP20-Hyg. The human *PEX5* (*HsPEX5*) cDNA was subcloned into the pcDNA3 vector (pcDNA3-HsPEX5). The vector sequence was confirmed by DNA sequencing. Anti-rat peroxisomal thiolase (24) and anti-human PEX5 antibodies were obtained, as described (25). Bovine anti-catalase and mouse monoclonal anti-catalase were obtained commercially (Rockland, Gilbertsville, PA). Polyclonal rabbit, monoclonal mouse anti-HA antibody, anti-epidermal growth factor (EGF) receptor antibody, and normal mouse IgG were obtained from a commercial vendor (Santa Cruz Biotechnology, Santa Cruz, CA). Goat anti-GST antibody was obtained from Sigma. Anti-nuclear matrix protein B (NRP/B) antibody was generated as described previously (26). HeLa and COS-7 cells were grown in Dulbecco's modified Eagle's medium containing 10% heat-inactivated fetal calf serum and antibiotics. Transfection of expression vectors into HeLa or COS-7 cells was performed using LipofectAMINE (Life Technologies, Inc.), and cells were assayed 48 h after each transfection.

Immunoprecipitation and Immunoblotting—Transfected COS-7 cells were washed with ice-cold phosphate-buffered saline and lysed in lysis buffer containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% (v/v) Nonidet P-40, 0.25% (w/v) sodium deoxycholate, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 10 mg/ml aprotinin, 10 mg/ml leupeptin. After protein normalization using the Bio-Rad protein assay, lysates (500 μg/sample) were immunoprecipitated with anti-HA antibody, anti-PEX5 antibodies, anti-GST antibody, or normal mouse IgG. Immunoprecipitates were separated by SDS-PAGE and transferred onto PVDF-Plus membranes (Micron Separations Inc., Westboro, MA). Bound proteins were immunoblotted with either anti-HA antibody or with anti-HsPEX5, as described (25). The blots were developed using enhanced chemiluminescence reagents (Amersham Pharmacia Biotech). Electrophoresis reagents were obtained from Bio-Rad.

Preparation of GST Fusion PMP20 Proteins—To construct wild-type human PMP20 protein (PMP20-WT) fused with GST at the N terminus, the coding region of HsPMP20 cDNA was amplified by PCR using *Pfu* DNA polymerase (Stratagene) and the following forward and reverse primers: 5′-ATG GCC CCA ATC AAG GTG GGA-3′, 5′-GCC TCA GAG CTG TGA GAT GAT-3′ with the attached restriction enzyme sites, *Bam*HI and *Xho*I, respectively. The DNA (~500 bp) fragment obtained from the PCR was gel-purified, digested, and ligated into the pGEX-4T-2 vector (Amersham Pharmacia Biotech). Constructs of mutant PMP20 proteins fused with GST where the PMP20 C-terminal tripeptide sequence SQL was either replaced with SKL (HsPMP20Q161K) or deleted (HsPMP20ΔSQL) were generated using the following reverse primers: 5′-GCC TCA GAG CTT TGA GAT-3′ or 5′-TCA GAT ATT GGG TGC CAG GCT-3′, respectively, in conjunction with the forward primer mentioned above. The sequences of all constructs were confirmed by DNA sequencing. GST fusion proteins were produced via isopropyl β-D-thiogalactopyranoside (U. S. Biochemical Corp.) induction, and purified using glutathione-Sepharose 4B beads (Amersham Pharmacia Biotech) according to the manufacturer's protocol. HsPMP20 was cleaved from the GST using thrombin (Amersham Pharmacia Biotech) according to the manufacturer's instructions.

Precipitation of GST Fusion Proteins and Far Western Analysis—For precipitation of GST fusion proteins, COS-7 cells transfected with pcDNA3-HsPEX5 were lysed, and 10 μg of various GST fusion proteins were bound to glutathione-Sepharose 4B beads. Bound proteins were separated by SDS-PAGE and immunoblotted with anti-PEX5 or anti-GST antibodies. For Far Western analysis, purified HsPMP20, GST fusion proteins containing HsPMP20-WT and HsPMP20ΔSQL, or GST alone were separated by 10% SDS-PAGE, transferred onto a nitrocellulose membrane, and subjected to Far Western blotting, using biotinylated HsPEX5 (25).

Cell Fractionation—The subcellular location of HsPMP20 was determined by cell fractionation (27). HeLa cells transiently transfected with pcDNA3-HA-HsPMP20 were washed with phosphate-buffered saline, resuspended in a hypotonic solution, passed through a 30-gauge needle, and centrifuged at 600 × *g* for 10 min to collect crude nuclei. These nuclei were further purified and used as the nuclear fraction. The supernatant was centrifuged at 10,000 × *g* for 10 min to collect the heavy membrane fraction (mitochondria, lysosomes, and peroxisomes). The supernatant was further centrifuged at 100,000 × *g* for 90 min, and the pellet and supernatant were used as the light membrane fraction (plasma membrane and microsomes) and cytoplasmic fraction, respectively. HeLa cells were standardized to represent an equal number of cells in each fraction and analyzed by SDS-PAGE and immunoblotting with anti-PEX5, anti-HA, anti-catalase, anti-peroxisomal thiolase, anti-EGF receptor, and anti-NRP/B antibodies.

Immunofluorescence labeling was performed as described previously (26). Briefly, HeLa cells transfected with pcDNA3-HA-HsPMP20 or with pTK-GFP-HsPMP20-Hyg were grown in chamber slides (Lab-Tec, Naperville, IL). Adherent cells were fixed with neutral buffered 4% (w/v) paraformaldehyde and then permeabilized with 0.5% Triton X-100. Double-label immunofluorescent staining was performed using mouse anti-HA antibody followed by goat anti-mouse IgG-fluorescein isothiocyanate (Vector Labs, Burlingame, CA) to decorate PMP20 in the HeLa cells. Peroxisomes were decorated with rabbit anti-thiolase antibody followed by goat anti-rabbit Texas Red IgG (Vector Labs). Immunostained preparations were examined using a Leica TCSNT confocal laser scanning microscope (Leica Inc., Exton, PA) fitted with air-cooled Argon and Krypton lasers. Fields of view were selected and brought into view under bright-field imaging conditions. Confocal micrographs of emission spectra (530 ± 15 nm and >590 nm) were recorded under dual-channel fluorescence imaging mode using excitation wavelengths of 488 and 568 nm. Images were collected from a 100× oil objective lens with 0.02 micron pixel size. Micrographs were examined using ImageSpace software (Molecular Dynamics, Sunnyvale, CA).

Assay of Antioxidant Activity of Human PMP20—The antioxidant activity of HsPMP20 was determined by monitoring the ability of the protein to inhibit the inactivation of glutamine synthetase (Sigma) by a thiol-catalyzed MCO system as described previously (28, 29). The assay was performed in a 50-μl reaction containing 50 mM imidazole-HCl (pH 7.0), 5 μg of glutamine synthetase, 3 μM $FeCl_3$, 10 mM dithiothreitol (DTT) and either 1 mM EDTA or 0–0.18 mg/ml HsPMP20 protein. For the nonthiol MCO system, DTT was replaced with 10 mM ascorbic acid. Following incubation at 30 °C for the indicated periods, the remaining activity of glutamine synthetase was measured by adding 5 μl of the reaction mixture to 2 ml of γ-glutamyltransferase assay mixture as described (28). The peroxidase activity of HsPMP20 was assayed as described previously (30). The reaction was initiated by adding 10 mM H_2O_2 to a 100-μl reaction containing 0.25 mM DTT, 0.15 mg/ml HsPMP20, 100 mM NaCl, and 50 mM HEPES (pH 7.0) at 37 °C. The concentration of the H_2O_2 remaining at the indicated time points was measured by the thiocyanate method as described previously (30).

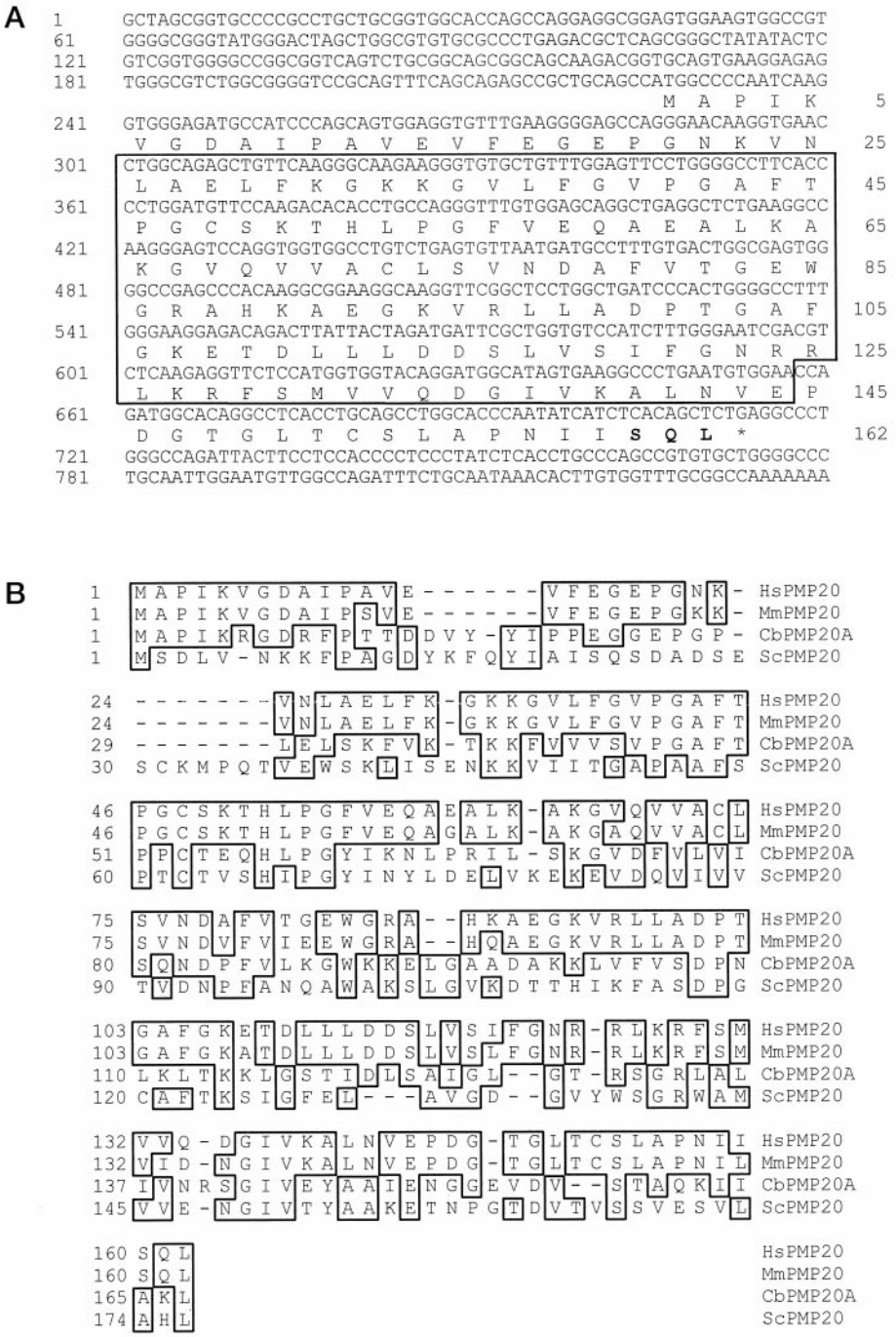

FIG. 1. *A*, nucleotide and deduced amino acid sequences of human PMP20. Nucleotide numbers are shown on the *left*. The amino acid numbers are shown on the *right*. The putative initiation codon is shown at nucleotides 226–228. The putative domain 210 (Prodom 36) is *boxed*. The PTS1-like sequence is shown in *boldface type*. The *asterisk* refers to the stop codon. *B*, amino acid alignment of the human PMP20 (HsPMP20), the mouse PMP20 (MmPMP20), and *C. boidinii* PMP20A (CbPMP20A) and *S. cerevisiae* PMP20 (ScPMP20) proteins. Sequence alignment was performed with DNASTAR using the Clustal method with the PAM250 residue weight table. Identical residues that are present in at least two of the four proteins are *boxed*.

RESULTS

Human PMP20 Is Homologous to the Yeast PMP20—Because mammalian cDNA for PMP20 had not been described, we used the EST179427 sequence to probe a human hippocampus cDNA library and obtained four clones ranging in length from 0.45 to 1 kilobase pair. The longest cDNA (approximately 850 bp without the poly(A) stretch) encodes a protein consisting of 162 amino acids with an estimated molecular mass of 20 kDa (Fig. 1*A*). The sequence is likely to represent the full-length cDNA, because no cDNAs with a longer 5′-flanking region could be isolated. The codon at base pairs 226–228, along with the flanking nucleotides, correspond to the consensus sequence for an optimal translation initiation site (31, 32). The mouse PMP20 (MmPMP20) cDNA was cloned from a mouse brain library using the HsPMP20 cDNA as a probe. HsPMP20 and MmPMP20 share 88% nucleotide identity and 93% amino acid identity, and both have coding regions of 162 amino acids. The C-terminal tripeptide sequence of both HsPMP20 and MmPMP20 was Ser-Gln-Leu (SQL).

A search using the HsPMP20 polypeptide against GenBank™ via the BLAST 2.0 and FASTA programs detected a similarity with yeast PMP20 proteins, CbPMP20A, CbPMP20B, and ScPMP20. Alignment of the amino acid sequences of HsPMP20 with ScPMP20 and CbPMP20A revealed 67 and 65% similarity, respectively (Fig. 1*B*). The overall amino acid sequence identities between HsPMP20 and ScPMP20 or CbPMP20A were 27 and 35%, respectively (Fig. 1*B*). These proteins are of similar length and are more similar to each other than to any other known proteins. Based on this sequence similarity, it is most likely that HsPMP20, MmPMP20, ScPMP20, and CbPMP20A are homologous. Analysis of HsPMP20 and MmPMP20 using the Prosite data base revealed no glycosyla-

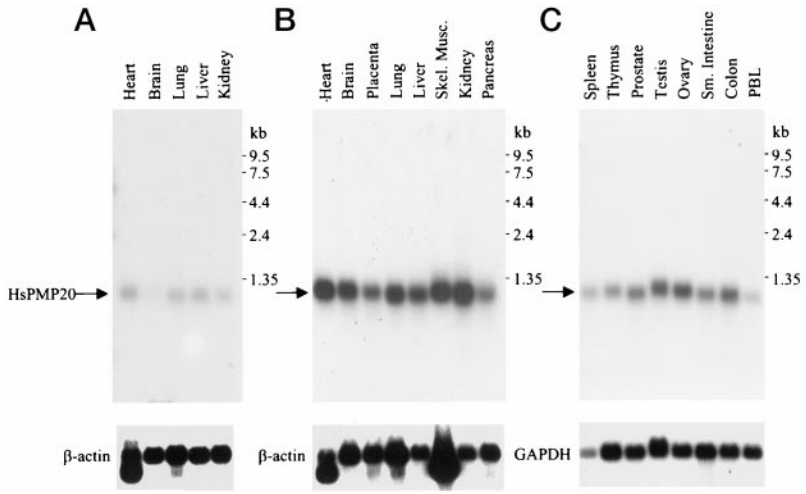

FIG. 2. **Expression of the HsPMP20 gene in various human tissues.** *A*, expression of human PMP20 in human fetal tissues by Northern blot analysis. *B* and *C*, expression of human PMP20 in human adult tissues by Northern blot analysis. The RNA blots were hybridized with an α-^{32}P-labeled human PMP20 gene-specific probe, followed by hybridization with β-actin or glyceraldehyde-3-phosphate dehydrogenase probes as controls for uniform RNA loading. *Skel. Musc.*, skeletal muscle; *Sm. Intestine*, small intestine; *PBL*, peripheral blood leukocytes.

tion sites or predicted transmembrane regions.

HsPMP20 Expression in Various Human Tissues—To analyze the mRNA expression levels of HsPMP20 in various human tissues, Northern blot analysis was performed. The expression level in adult tissues was higher than that in fetal tissues (Fig. 2, *B* and *C*). The sizes of the bands in fetal and adult tissues were similar to that of the cDNA from the library. HsPMP20 mRNA expression was abundant in adult heart, brain, lung, skeletal muscle, and kidney, whereas expression in spleen, thymus, and peripheral blood was relatively low. Rehybridization of the same blots with β-actin or glyceraldehyde-3-phosphate dehydrogenase probes showed that similar amounts of the RNA samples were present in each lane (Fig. 2, *lower panels*).

Subcellular Localization of HsPMP20—Cell fractionation of HeLa cells, expressing an HA-tagged PMP20, revealed that HsPMP20 protein localizes in a fraction enriched in mitochondria, lysosomes, and peroxisomes, and in the cytoplasmic fraction (Fig. 3). As expected, HsPEX5 was located mainly in the cytoplasmic fractions and partly in the heavy membrane fraction (Fig. 3). Similar results were obtained with HeLa cells expressing GFP-HsPMP20 (data not shown). Antibodies to peroxisomal catalase and 3-ketoacyl-CoA thiolase were used as controls. Both markers were localized in the heavy membrane fraction (Fig. 3) and were also faintly detected in the cytosolic fractions, consistent with their known ability to leak out from peroxisomes during tissue homogenization (11). As expected, the EGF receptor and NRP/B nuclear matrix protein were mainly localized in the light membrane and nuclear fractions, respectively (Fig. 3).

Double-staining immunofluorescence studies using HeLa cells that were transfected with either pcDNA3-HA-HsPMP20 or pTK-GFP-HsPMP20-Hyg revealed HA-HsPMP20 in punctate structures (Fig. 4, *B* and *C*), and there was very strong colocalization of HsPMP20 with thiolase (Fig. 4, *G* and *I*), as well as with catalase (data not shown). Thus, these results support the conclusion of the biochemical analysis (Fig. 3) and show that the epitope-tagged HsPMP20 colocalizes with genuine peroxisomal matrix proteins.

Association of HsPMP20 with HsPEX5—The PTS1 sequence is known to be SKL or a variant (4). Because the C-terminal tripeptide sequence of HsPMP20 was similar to SKL, the ability of HsPMP20 to bind to the human PTS1 receptor, HsPEX5, was examined. HsPMP20 fused at its N terminus with an HA epitope (HA-HsPMP20) was expressed together with HsPEX5 in COS-7 cells upon transient transfection. Cells co-transfected with the control vector (pcDNA3-HA) or pcDNA3-HA-HsPMP20 together with pcDNA3-HsPEX5 were lysed and immunoprecipitated with mouse anti-HA monoclonal antibodies, rabbit anti-PEX5 antibodies, or control antibodies. Bound proteins were analyzed by Western blotting. When both HsPMP20 and HsPEX5 were expressed, anti-HA antibodies co-immunoprecipitated HsPEX5 (Fig. 5*A*). In addition, anti-PEX5 polyclonal antibodies co-immunoprecipitated HA-HsPMP20 (Fig. 5*B*). These results indicate that HsPMP20 and HsPEX5 proteins interact in cells.

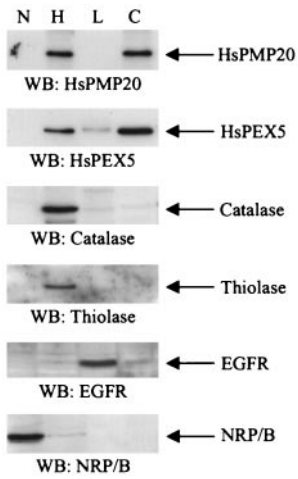

FIG. 3. **Subcellular location of HsPMP20; cell fractionation of HeLa cells transfected with pcDNA3-HA-HsPMP20.** Subcellular fractions, standardized to represent equal numbers of cells in each fraction, were separated by 10% SDS-PAGE and analyzed by Western blotting (*WB*) with anti-HA, anti-PEX5, anti-catalase, anti-peroxisomal 3-ketoacyl-CoA thiolase, anti-EGF receptor, and anti-NRP/B antibodies. *N*, purified nuclear fraction; *H*, heavy membrane fraction; *L*, light membrane fraction; *C*, cytoplasmic fraction.

Direct Binding of HsPEX5 to the C-terminal Tripeptide Sequence of HsPMP20—To examine whether HsPMP20 and HsPEX5 can directly bind to one another, Far Western blotting was employed. Purified HsPMP20, bovine serum albumin, and GST fusion proteins were blotted onto a nitrocellulose membrane and subjected to Far Western analysis (Fig. 6*A*, *upper panel*). The biotinylated HsPEX5 protein could bind to both HsPMP20 (Fig. 6*A*, *lower panel*, *lane 1*) and HsPMP20 fused at the N terminus with GST (Fig. 6*A*, *lower panel*, *lane 3*). The biotinylated HsPEX5 protein was not able to bind to the GST fusion protein lacking the C-terminal tripeptide (HsPMP20ΔSQL) (Fig. 6*A*, *lower panel*, *lane 4*) or to GST alone (Fig. 6*A*, *lower panel*, *lane 5*). To determine the importance of the SQL motif, COS-7 cells transfected with *HsPEX5* cDNA were lysed and precipitated with various GST fusion proteins. Co-precipitates were separated by

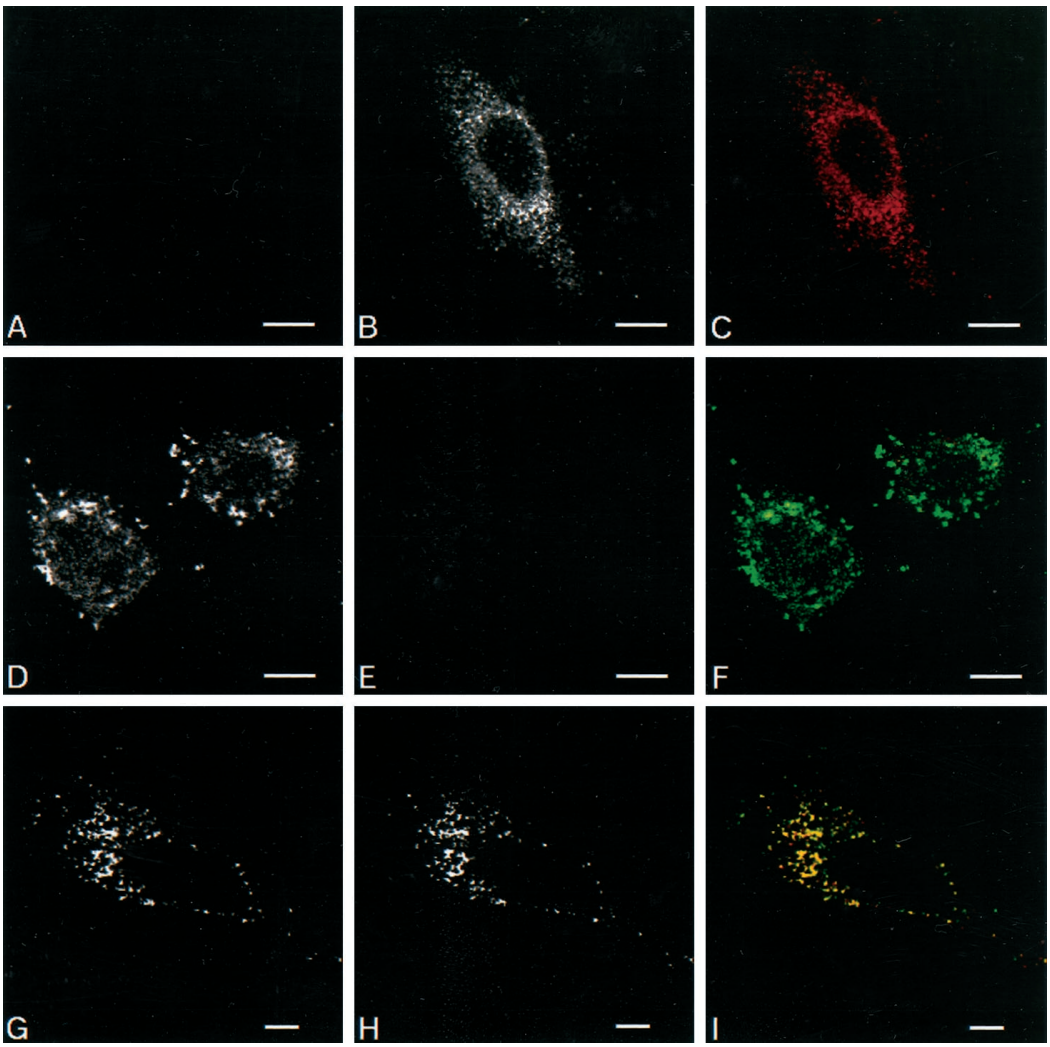

FIG. 4. **Immunofluorescence confocal microscopy analysis of HsPMP20.** Confocal micrographs of HeLa cells transfected with pcDNA3-HA-HsPMP20 and immunodecorated for HsPMP20 alone (*A–C*), thiolase alone (*D–F*), or following double immunodecoration for HsPMP20 and thiolase (*G–I*). Micrographs were recorded under similar conditions with imaging parameters adjusted to reduce cross-talk of fluorochrome emission spectra by Texas Red into the fluorescein isothiocyanate channel (*A*) and for fluorescein isothiocyanate into the channel used to detect Texas Red (*E*). Color composite micrographs reveal vesicular decoration of peroxisomes in cells stained for HsPMP20 alone (*red*, *C*) or thiolase alone (*green*, *F*) and show strong colocalization in cells double-labeled for both HsPMP20 and thiolase (*yellow*, *I*). *A–F*, bar indicates 10 microns. *G–I*, *bar* indicates 5 microns.

SDS-PAGE and immunoblotted with anti-HsPEX5 antibodies. GST fusion proteins containing the wild-type and the mutant HsPMP20, whose C-terminal tripeptide SQL was replaced with SKL (PMP20Q161K), also precipitated HsPEX5 (Fig. 6*B*, *upper panel*). PMP20ΔSQL or GST alone failed to precipitate HsPEX5. The amount of GST fusion proteins used for the assay was similar (Fig. 6*B*, *lower panel*). These results indicate that HsPEX5 binds directly to the SQL sequence at the free C terminus of HsPMP20 in the same manner as HsPEX5 binds to the typical PTS1 sequence.

HsPMP20 Exhibits a Thiol-specific Antioxidant Activity—In the Prodom data base (Prodom release 36), three yeast PMP20 proteins, CbPMP20A, CbPMP20B, and ScPMP20, are reported to contain the structural domain termed Prodom domain 210. The yeast PMP20 proteins share this domain with 72 other proteins, most of which are antioxidant proteins. Of the antioxidant proteins containing the domain 210, yeast PMP20 proteins showed a higher homology to thiol-specific antioxidant (TSA) proteins. Alignment analysis performed by DNASTAR showed that the amino acid sequence of domain 210 in HsPMP20 and in other TSA proteins was 56% identical and 76% similar. MmPMP20 also showed a high homology to these TSA proteins.

Therefore, the thiol-specific antioxidant activity of HsPMP20 was investigated. Antioxidant activity of HsPMP20 was analyzed by monitoring the ability of the protein to inhibit the inactivation of glutamine synthetase using an MCO system. HsPMP20 exerted, in a dose-dependent manner, an inhibitory effect on the inactivation of glutamine synthetase using a thiol-MCO system (DTT/Fe^{3+}/O_2) (Fig. 7*A*) but not using a nonthiol MCO system (ascorbate/Fe^{3+}/O_2) (Fig. 7*B*), suggesting that HsPMP20 does carry TSA activity. Furthermore, the protective activities of catalase and HsPMP20 on the inactivation of glutamine synthetase in the DTT/Fe^{3+}/O_2 system were compared. Catalase and HsPMP20 exerted protective effects in a dose-dependent manner (Fig. 7*C*). Both proteins could completely inhibit the inactivation of glutamine synthetase, and the concentration of proteins required to preserve 50% of the glutamine synthetase activity was 8 μg/ml for catalase and 40 μg/ml for HsPMP20. HsPMP20 removed H_2O_2 in the presence of DTT, suggesting that HsPMP20 acts as a TSA protein (Fig. 7*D*).

DISCUSSION

We have isolated two mammalian cDNAs encoding PMP20 and characterized the activity of the human protein. Compar-

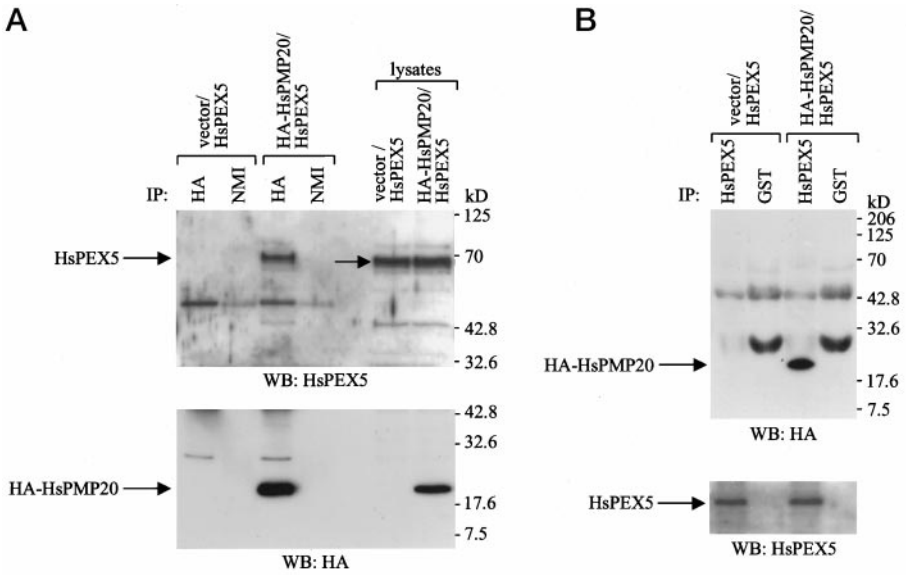

FIG. 5. **Association of HsPMP20 with the human PTS1 receptor, HsPEX5.** *A*, plasmids pcDNA3-HA and pcDNA3-HsPEX5 (HA/HsPEX5) or pcDNA3-HA-HsPMP20 and pcDNA3-HsPEX5 (HA-HsPMP20/HsPEX5) were co-transfected into COS-7 cells. Cell lysates (500 μg/sample) were immunoprecipitated (*IP*) with anti-HA antibodies (*HA*) or normal mouse IgG (*NMI*) antibodies as controls. Each sample was separated by SDS-PAGE, and immunoprecipitated proteins were analyzed by immunoblotting with anti-HA or anti-HsPEX5 antibodies. In addition, 100 μg of each lysate were analyzed by Western blot (*WB*) analysis. *B*, COS-7 cells co-transfected with vectors expressing HA-PEX5 or HA-HsPMP20/PEX5 were lysed and immunoprecipitated with rabbit anti-PEX5 antibody or goat anti-GST antibody as a control antibody. Each sample was separated by SDS-PAGE, and immunoprecipitated proteins were analyzed by immunoblotting with anti-HA or anti-PEX5 antibodies. The two faint bands at ~50 kDa and ~30 kDa are the heavy and light chains of IgG that were used for each immunoprecipitation and cross-reacted with secondary anti-mouse IgG antibody.

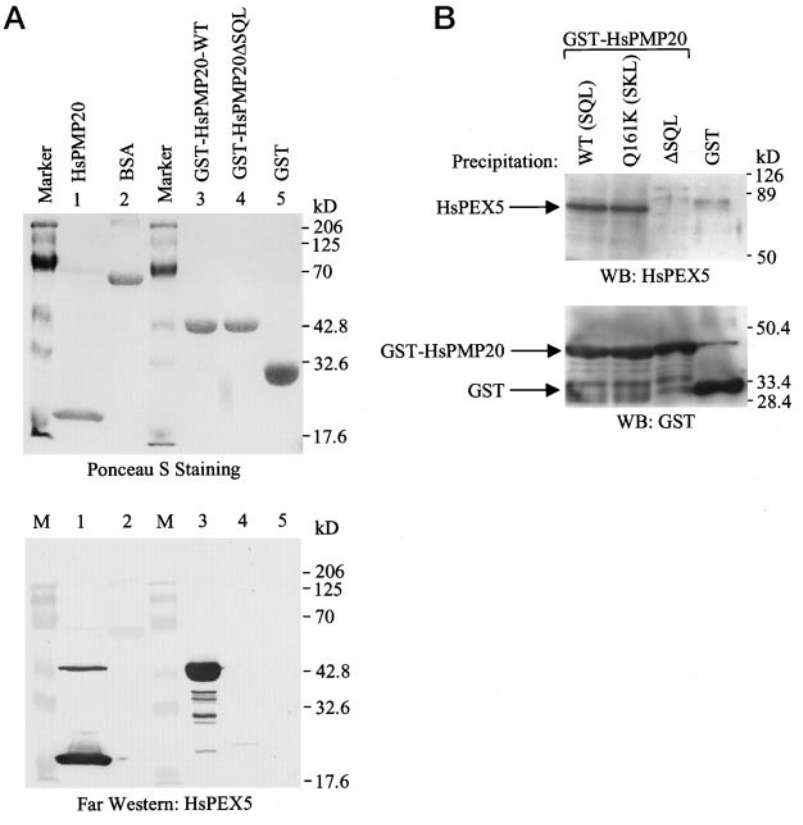

FIG. 6. **The SQL sequence at the C terminus is required for the association of HsPMP20 with HsPEX5.** *A*, Far Western analysis. Purified HsPMP20 and GST fusion proteins containing the wild-type HsPMP20 (GST-HsPMP20) or a mutant HsPMP20 that lacks the C-terminal tripeptide SQL (GST-HsPMP20ΔSQL) were separated by SDS-PAGE transferred onto a nitrocellulose membrane and then subjected to Far Western blotting with HsPEX5 protein. Bovine serum albumin (*BSA*) and GST alone were used as controls. Cleaved GST-HsPMP20 fusion is shown. The *upper band* in *lane 1* that binds to HsPEX5p is due to traces of GST-HsPMP20-WT, not detectable by Ponceau S staining and present in the HsPMP20 sample. *B*, COS-7 cells co-transfected with pcDNA3-HsPEX5 and plasmids expressing either GST alone or various fusions of GST with wild-type and mutant forms of HsPMP20 were bound to glutathione-Sepharose beads. The bound proteins were analyzed by immunoblotting with antibodies to either HsPEX5 (*top panel*) or GST (*lower panel*); *WB*, Western blot.

ison of the deduced amino acid sequences of human and mouse PMP20s revealed 93% homology, indicating that PMP20s are highly conserved between these species, whereas their similarity to yeast PMP20 proteins was relatively limited. Human PMP20 mRNA was found in all human adult tissues examined. Interestingly, the expression of human PMP20 mRNA was very low in fetal brain, and increased post-natally in the adult brain.

Cell fractionation experiments reveal that HsPMP20 protein is present in the heavy membrane fraction corresponding to mitochondria, lysosomes, and peroxisomes, as well as in the cytoplasmic fraction. In these experiments, several control proteins such as HsPEX5, catalase, thiolase, EGF receptor, and NRP/B showed the expected associations with various subcellular fractions. Immunofluorescence studies confirmed the co-

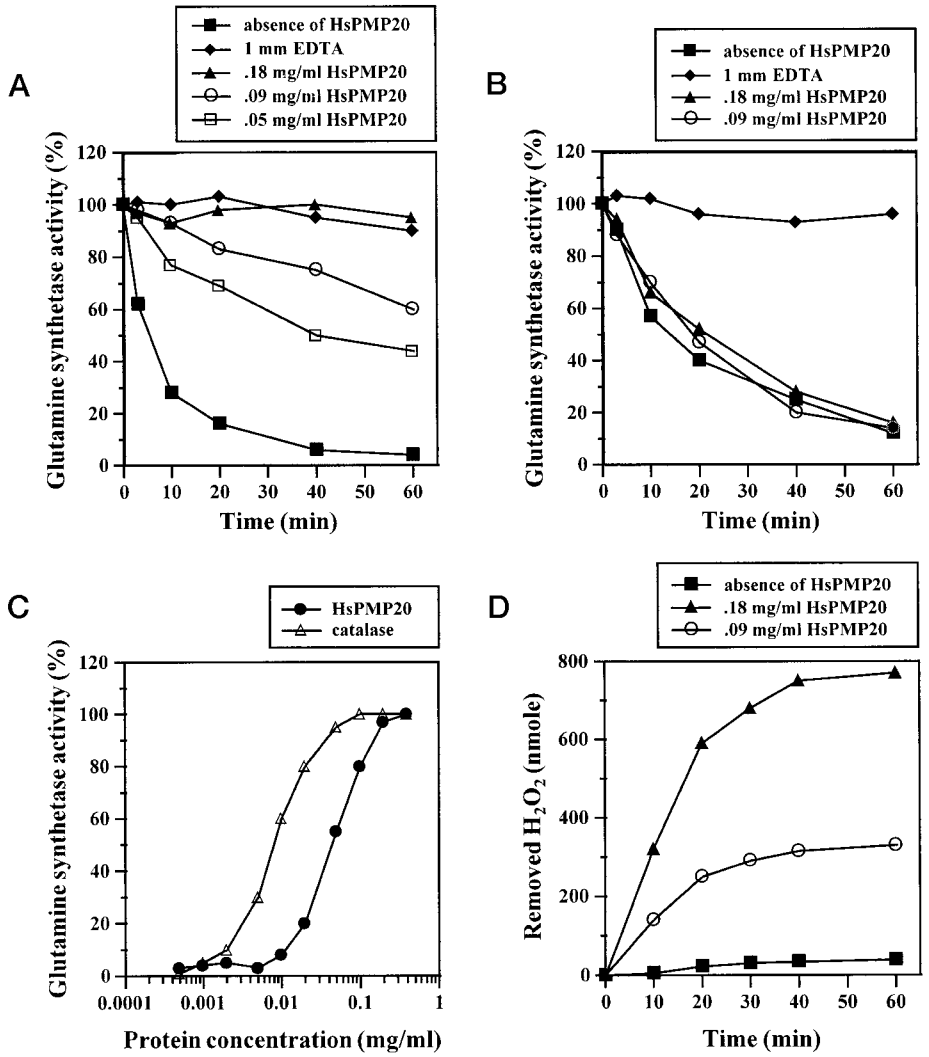

FIG. 7. **Thiol-specific antioxidant activity of HsPMP20.** *A*, protection of glutamine synthetase by HsPMP20 in the thiol-MCO system (DTT/Fe^{3+}/O_2). The inactivation mixture (50 μl) contained 5 μg of glutamine synthetase, 10 mM DTT, 50 mM imidazole-HCl (pH 7.0), and either 1 mM EDTA or 0.18 mg/ml, 0.09 mg/ml, 0.05 mg/ml, 0 mg/ml HsPMP20, as shown. At the indicated times, aliquots (10 μl) were removed and assayed for remaining glutamine synthetase activity. *B*, protection of glutamine synthetase by HsPMP20 in the nonthiol MCO system (Ascorbate/Fe^{3+}/O_2). The inactivation mixture (50 μl) contained 5 μg of glutamine synthetase, 10 mM ascorbic acid, 50 mM imidazole-HCl (pH 7.0), and either 1 mM EDTA or 0.18 mg/ml, 0.09 mg/ml, 0 mg/ml HsPMP20. At the indicated times, aliquots (10 μl) were removed and assayed for remaining glutamine synthetase activity. *C*, protection of glutamine synthetase by catalase and HsPMP20 against the DTT/Fe^{3+}/O_2 system. Variable amounts of catalase and HsPMP20 were added into the inactivation mixture as described for *A*. Remaining glutamine synthetase activity was measured 30 min after the reaction. *D*, removal of H_2O_2 by HsPMP20. Peroxidase reactions were performed in a 50-μl reaction mixture containing 0.5 mM H_2O_2, 0.25 mM DTT, 50 mM HEPES (pH 7.0), and 0 mg/ml, 0.18 mg/ml, or 0.09 mg/ml HsPMP20.

localization of HsPMP20 with thiolase and catalase. We could not analyze the endogenous HsPMP20 localization, because we could not generate specific antibodies for HsPMP20. HsPMP20 was colocalized with thiolase and catalase, both markers for peroxisomal staining. It could be that the cytosolic localization observed is partly due to overexpression of the epitope-tagged HsPMP20.

Consistent with the localization of HsPMP20 to the peroxisomal matrix, this protein has a functional C-terminal PTS1 sequence, SQL. When HA-HsPMP20 and HsPEX5 were coexpressed, anti-HA monoclonal antibodies co-immunoprecipitated HsPEX5. In addition, anti-PEX5 antibodies co-immunoprecipitated HA-HsPMP20. Furthermore, Far Western analysis revealed that HsPEX5 protein could bind to both the purified HsPMP20 and GST-HsPMP20 but not to GST-HsPMP20ΔSQL. In addition, both GST-HsPMP20 and GST-HsPMP20Q161K could precipitate HsPEX5, and the amount of HsPEX5 bound to their C-terminal tripeptides, SQL and SKL, was similar. The mutant HsPMP20, lacking the C-terminal tripeptide, could not bind to HsPEX5. These results indicate that HsPMP20 and HsPEX5 can directly bind to each other in mammalian cells and that the tripeptide sequence SQL of HsPMP20 is solely required for binding to HsPEX5, the PTS1 receptor. This interaction between the PTS1 sequence of HsPMP20 and HsPEX5 would explain how HsPMP20 is targeted to peroxisomes. In view of the variants of the PTS1 sequence that function as PTSs, the ability of SQL to interact with HsPEX5 is not really surprising (33).

It has been shown that the yeast PMP20 proteins and numerous AhpC/TSA family proteins share the Prodom domain 210 with various antioxidant proteins, especially with the TSA from various eukaryotes and prokaryotes. The amino acid alignment of the putative domain 210 of HsPMP20 exhibited homology to proteins of the AhpC/TSA family (56% identical, 76% similar), as do CbPMP20A, CbPMP20B, and ScPMP20. MmPMP20 also showed a homology to these thiol-specific antioxidant proteins. HsPMP20 exhibited antioxidant activity in the thiol-MCO system but not in the nonthiol MCO system, suggesting that HsPMP20 carries TSA activity. Requirement of a thiol-reducing equivalent in the antioxidant activity suggests that HsPMP20 might function as an antioxidant enzyme containing functional cysteines. Peroxisomes contain several oxidases that use oxygen as an electron acceptor to oxidize organic substrates in the process of forming H_2O_2. Because peroxisomes lack an electron transport chain, electrons released during the oxidation of fatty acids are used to form H_2O_2, which is highly toxic to the cell. H_2O_2 is efficiently converted to H_2O within the peroxisomes by catalase. Abnormality in catalase import into peroxisomes is reported to lead to a severe neurological disorder. Like catalase, TSA owes its protective action to the removal of H_2O_2 (29). Both catalase and HsPMP20 exerted a protective effect, in a dose-dependent manner, in the thiol-MCO system where the H_2O_2 generated inactivates glutamine synthetase. In addition, we have shown that HsPMP20 re-

moves H_2O_2 by its thiol-peroxidase activity in the presence of DTT. Therefore, we propose that HsPMP20 is a novel member of the AhpC/TSA family and is present in the peroxisomal matrix and possibly in the cytoplasm. HsPMP20 might play a role as a protector against oxidative stress in peroxisomes, as well as assist in the function of peroxisomal enzymes within the peroxisome. The source of thiols in peroxisomes, which are required for the TSA activity of HsPMP20, is still unknown. Further experiments should reveal the function of HsPMP20 as an antioxidant protein in peroxisomes.

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