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Evidence for Mitochondrial Localization of P5, a Member of the Protein Disulphide Isomerase Family

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This report demonstrates for the first time that P5, a member of the protein disulphide isomerase (PDI) family, is present in the mitochondria. Various organelles were screened for proteins bearing the CGHC motif using an affinity column conjugated with the phage antibody 5E, which cross-reacts with PDI family proteins. P5 was found in bovine liver mitochondrial extract and identified by Western blot analysis using anti-P5 antibody and by mass spectrometric analysis. Results of cell fractionation, proteinase sensitivity experiments and immuno-electron microscopy supported the mitochondrial localization of P5 and also indicated the presence of ERp57, another PDI family protein, in mitochondria. Our findings will be useful for the elucidation of the translocation mechanism of PDI family proteins and their roles in mitochondria.

Key words: ERp57, protein disulphide isomerase, mitochondria, P5, single-chain antibody fragment.

Abbreviations: AIF, apoptosis inducing factor; bPDI, bovine PDI; bP5, bovine P5; ER, endoplasmic reticulum; hPDI, human PDI; hPDIR, human protein disulphide isomerase-related protein; hP5, human P5; hsc70, heat shock cognate70; hsp60, heat shock protein 60; IMS, intermembrane space; LC, liquid chromatography; MALDI-TOF/MS, matrix assisted laser desorption ionization-time of flight mass spectrometry; MS/MS, tandem mass spectrometry; PDI, protein disulphide isomerase; scFv, single-chain Fv fragment; Tim, Translocase of the inner mitochondrial membrane; Tom, Translocase of the outer mitochondrial membrane; TX, thioredoxin; yPDI, yeast PDI.

Protein disulphide isomerase (PDI) (EC5.3.4.1) catalyses the oxidation, reduction, and isomerization of disulphide bonds in proteins (*1*). PDI is a member of the thioredoxin (TX) superfamily and is believed to accelerate the folding of disulphide-bonded proteins by catalysing the disulphide interchange reaction, which is the rate-limiting step during protein folding in the luminal space of the ER (*2*). PDI and related proteins constitute a protein family (*1*) and have two or three TX sequences in their primary structures. Two TX sequences, Cys-Gly-His-Cys (CGHC), constitute the active sites of PDI (*3*). We previously cloned hP5 cDNA from a human placental cDNA library (*4*). Human P5 is a member of the PDI family and has isomerase and chaperone activities, although both activities are lower than those of hPDI (*5*).

PDI and related proteins have been shown to localize in the ER. Unexpectedly however, PDI and ERp57 have recently found in nucleoli isolated from higher vertebrates (*6*). Recently Rigobello *et al*. (*7*) reported that PDI is located in rat liver mitochondria; however, their purified protein was not clearly identified because it did not cross-react with monoclonal anti-PDI antibodies and its molecular weight differed from that of PDI. The detection of PDI family proteins in additional organelles would be helpful for understanding their physiological significance.

Polyclonal antibodies raised against individual PDI family proteins do not cross-react with other PDI family proteins. We previously isolated several clones that recognize sequences containing the CGHC motif or the CGHCK sequence (*8*) from a human synthetic phage display antibody library established by Griffiths *et al*. (*9*). The isolated phage antibody 5E reacts with hPDI, bPDI, hP5, hPDIR and yPDI (*8*).

These results suggest that the phage antibody is a powerful tool for screening novel PDI family proteins,

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for detecting their distribution, and for elucidating their functions, and motivated us to investigate the intra-cellular localization of PDI family members.

Initially we used affinity resin conjugated to the scFv 5E to screen organelles in bovine liver for proteins containing the CGHC motif. After extensive investigation, we found a protein in the mitochondria that cross-reacted with scFv 5E. The protein was also recognized by anti-hP5 antibodies and was identified as P5 by LC-MS/MS analysis. Results from several additional experiments all supported the mitochondrial localization of P5. The physiological significance of this unexpected localization is discussed below.

EXPERIMENTAL PROCEDURES

Materials—Bovine liver was purchased from Nippon Ham (Osaka, Japan). Adult male Sprague–Dawley rats were purchased from Clea Japan, Inc. (Osaka, Japan). The HiTrap^{TM} NHS-activated HP column and Ni^{2+}-chelating resin column were purchased from Amersham Bioscience (Uppsala, Sweden). Percoll was purchased from MP Biomedicals (Solon, OH, USA). Anti-P5 IgGs were prepared in our laboratories. Antibodies against AIF, calnexin, hsc70, PDI, Tom20 and F1-ATPase were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies against Grp75 and ERp57 were from StressGen (Victoria, BC, Canada) and those against ERp72, hsp60, cytochrome *c* and Tim23 were from BD Pharmingen (Tokyo, Japan). HRP-labelled anti-rabbit IgG, HRP-labelled anti-mouse IgG and HRP-labelled anti-goat IgG were from Biosource (Camarillo, CA, USA). All other chemicals were of reagent-grade quality.

Expression and Purification of scFv 5E and Recombinant hP5—Expression and purification of recombinant hP5, hPDI and scFv 5E were performed as previously described (*4*, *8*). Protein purity was confirmed by SDS-PAGE according to the method of Laemmli (*10*) and by Western blotting.

Preparation and Detection of Proteins Bearing CGHC-Motif from Bovine Liver—The mitochondrial fraction was prepared from bovine liver by the method of Tangen *et al*. (*11*) and proteins were extracted by Triton X-100 treatment. The resulting extract was applied to a scFv 5E-Sepharose column prepared as described by Horibe *et al*. (*12*). Unbound proteins were thoroughly washed out with 20 mM sodium phosphate buffer (pH 7.4) containing 100 mM NaCl, followed by 100 mM HCl, and the bound proteins were eluted with 10 mM NaOH. Following SDS–PAGE, proteins were detected using a silver-staining kit (Wako Pure Chemicals, Osaka, Japan) and by Western blot analysis using the rabbit anti-hP5 and anti-bPDI antibodies. Thereafter, proteins were detected with the NBT-BCIP combo (Invitrogen CA, USA). Purification of P5 from bovine liver mitochondria was carried out using an anti-hP5 antibody-Sepharose column. The preparation of the column and the mitochondrial fraction, and the washing and eluting conditions were as described above.

Mass Spectrometric Analysis—SDS–PAGE gel fragments containing proteins were excised and subjected to in-gel trypsin digestion, as described previously (*13*). Generated peptides were analysed using a MALDI-TOF/MS system (Voyager DE-STR, Applied Biosystems, CA, USA) (*13*). For nano-flow LC-MS/MS analysis, the peptide mixture was separated on a fritless Mightysil-C18 (3-μm particles; Kanto Chemical, Osaka, Japan) column (0.150 mm i.d., 450 mm length), using a gradient of acetonitrile (0–40%) in 0.1% formic acid at a flow rate of 100 μl/min with a Direct nano-LC system (Nanosolution, Tokyo, Japan). Eluted peptides were sprayed directly into a quadropole time-of-flight hybrid mass spectrometer (Q-Tof2, Micromass, Manchester, UK). MS/MS spectra were acquired by data-dependent collision-induced dissociation, and the obtained MS/MS data were analysed using MASCOT software (Matrix Science, Wyndham Place, UK) for peptide assignment (*14*, *15*).

Subcellular Fractionation and Purification of Mitochondria—Subcellular fractionation was performed as described previously (*16*), except that one Complete EDTA-Free tablet (Roche, Mannheim, Germany) was added instead of Trasylol. The HEMS buffer consisted of 10 mM HEPES-KOH (pH 7.4), 1 mM EDTA, 220 mM mannitol, 70 mM sucrose and one Complete EDTA-Free tablet. The pellets from the 8,000*g* centrifugation were washed three times with excess amounts of HEMS buffer to obtain the crude mitochondrial fraction. The supernatants were subjected to ultracentrifugation at 100,000*g* for 60 min at 4°C to obtain the microsomal fraction.

To obtain the pure mitochondrial fraction, the crude mitochondrial fraction was subjected to percoll step-density gradient centrifugation, as previously described (*17*), with minor modifications. Briefly, 2.5 ml of the crude mitochondrial fraction (2.0 mg protein) were layered onto the step-gradient solutions consisting of 70% (v/v) percoll (5 ml, bottom) and 30% (v/v) percoll (5 ml, top) in 10 mM HEPES-KOH (pH 7.4), 1 mM EDTA, 1 mM PMSF and 300 mM sucrose, and ultracentrifuged with a swing rotor (R40ST, Hitachi, Tokyo, Japan) at 40,000 r.p.m. for 30 min at 4°C. After centrifugation, two bands were apparent: fraction 1 in the middle of the 30% layer and fraction 2 at the interface between the 30% and 70% layers. Each band was collected carefully using a Pasteur pipette and washed three times after the addition of excess HEMS buffer. These pure fractions were resuspended in a small amount (50–100 μl) of HEMS buffer.

Assays—The proteinase-sensitivity assay, hypotonic treatment and salt- and alkali-sensitivity assays were performed as described previously (*18*), except that percoll-purified mitochondria (100 μg) were treated with 25 μg/ml of proteinase K. Each sample (typically 50–100 μg) obtained above was subjected to SDS–PAGE following by Western blot analysis using the antibodies indicated in the figures. Detection was performed by ECL (GE Healthcare, Buckinghamshire, UK) and analysed by LAS-1000 plus (Fujifilm, Tokyo, Japan). Protein concentrations were determined with a BCA assay kit (Pierce, Rockford, IL, USA).

Immuno-Electron Microscopy—Mouse liver was perfusion-fixed through the heart with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 10 min and

processed for ultra-thin cryosectioning. Frozen sections were incubated with anti-p5 rabbit polyclonal antibody or non-immunized rabbit IgG at the same concentration, followed by incubation with anti-rabbit IgG coupled with 5-nm gold particles. Sections were negatively stained, embedded in polyvinyl alcohol (*19*) in place of methyl cellulose, and examined under a Hitachi H7600 electron microscope.

RESULTS

PDI Family Protein P5 is Detectable in Bovine Liver Mitochondria—PDI and its relatives have been shown to localize in the ER. PDI and ERp57 were also recently found in nucleoli (*6*). These observations prompted us to use the isolated phage antibody 5E to detect proteins bearing the CGHC motif (*8*) in other organelles. The bovine liver mitochondrial extract was applied to the scFv 5E column and bound proteins were eluted. As shown in Fig. 1A, several bands that specifically bound to the scFv 5E were detected by silver staining. Since Rigobello *et al*. (*7*) had reported that PDI was localized in mitochondria, we analysed these proteins by Western blotting using anti-bPDI antibody; however, PDI was not detected (Fig. 1B, lanes 2–4). The concentration of PDI in the ER has been estimated to be 0.1–0.2 mM (*20*). Thus, PDI is an extremely abundant protein in the ER. The fact that no protein was detected in any of these fractions by the anti-PDI antibody indicated that the mitochondrial preparations were not contaminated with ER (see also Fig. 4B). However, in the fraction eluted from the scFv 5E affinity column a few bands were detected that cross-reacted with anti-hP5 antibody, with the major band at a position very close to that of recombinant hP5 lacking a signal sequence (Fig. 1C). Other bands were probably due to non-specific reactions. Collectively, these results suggested that the mitochondrial protein of interest was probably P5.

To confirm this result, we attempted to purify P5 from bovine liver mitochondrial extracts using a Sepharose column conjugated with anti-hP5 antibody. As shown in Fig. 1D, two major bands were detected on the silver-stained gel. The bands were excised from the SDS–PAGE gel, in-gel digested, and analysed by direct nano-flow LC-MS/MS. The lower band was identified as P5 and the upper band could not be identified (Fig. 2). Taken together, these results indicate that P5, but not PDI, is localized in the mitochondria.

P5 is Localized, not only in Microsomes, but also in Mitochondria—Since in some types of cells the mitochondrial membrane and the microsomal membrane are very close to each other (*21*), we performed a series of experiments to confirm the mitochondrial localization of P5.

First, we performed cell fractionation experiments using rat liver homogenates. As shown in Fig. 3A, P5 was detected, not only in the microsomal fraction (lane 2), but also in the mitochondrial fraction (lane 1). No bands were detected in the cytosolic fraction (lane 3), suggesting that in the steady state, little or no P5 is present in the cytosol. The fractionation method appeared to be quite successful, since the three marker proteins—AIF for mitochondria, calnexin for microsomes and hsc70 for cytosol—were effectively separated (Fig. 3B). However, the microsomal soluble protein marker, PDI, was detected in both the mitochondrial and microsomal fractions, although the amount in the mitochondrial fraction was negligible compared to that in the microsomal fraction (Fig. 3B).

P5 is Detected in Percoll-Purified Mitochondria—Since the data presented above did not exclude the possibility of contamination, we next attempted to obtain the further purified mitochondrial fraction using percoll-density-gradient centrifugation, which has often been utilized to obtain highly purified mitochondria for proteome analysis (*17*). The results are shown in Fig. 4, in which lane 1 contains the crude mitochondrial fraction, lane 2 contains fraction 1 (contaminating fraction containing microsomes), which was the upper band in the 30% layer and lane 3 contains fraction 2 (purified fraction as described in ref *17*), which was the

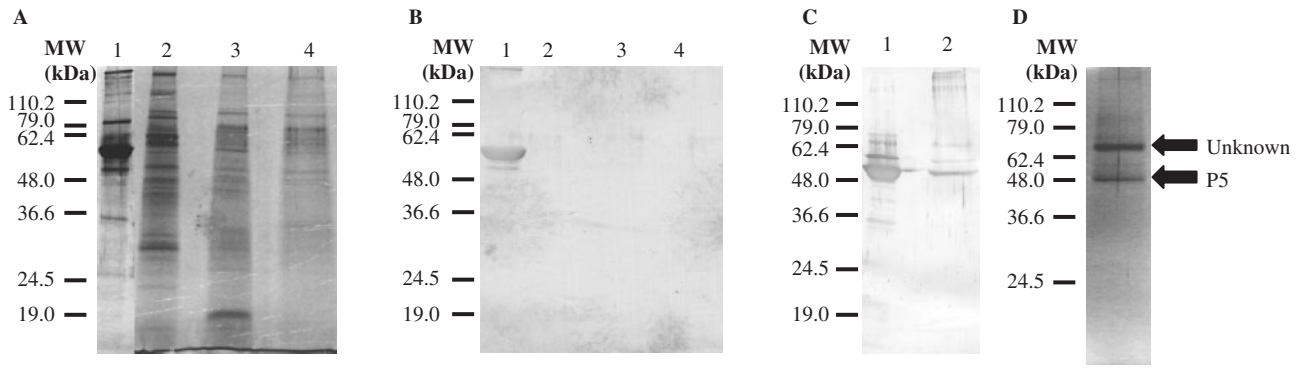

Fig. 1. **Detection of P5 in bovine liver mitochondria**. The bovine liver mitochondrial extract was applied to the scFv 5E-Sepharose column and the resulting fractions were analysed by silver staining (A), and western blotting using anti-bovine PDI antibody (B) and anti-human P5 antibody (C). A and B: lane 1, bovine PDI; lane 2, pass fraction; lane 3, fraction eluted with 100 mM HCl; lane 4, fraction eluted with 10 mM NaOH. C: lane 1, recombinant human P5; lane 2, fraction eluted with 10 mM NaOH. Proteins bound to the anti-human P5 antibody-Sepharose column were examined by silver staining (D). After unbound proteins were thoroughly washed out with 20 mM sodium phosphate buffer (pH 7.4) containing 100 mM NaCl, followed by 100 mM HCl, the bound proteins were eluted with 10 mM NaOH. P5 was identified by LC-MS/MS.

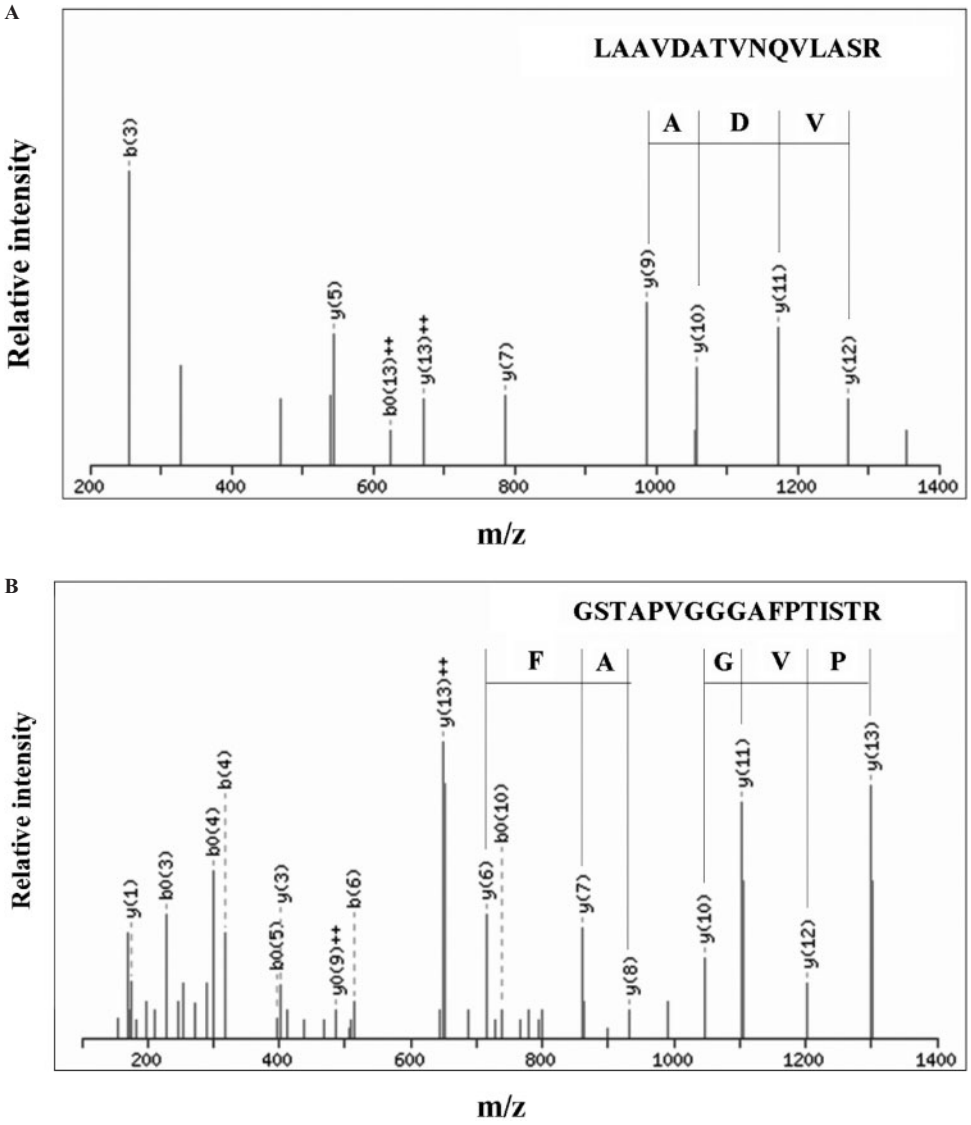

Fig. 2. **MS/MS spectra used to identify P5.** The doubly charged ions at $m/z = 764.42$ (A) and $m/z = 808.43$ (B) separated by LC were fragmented, resulting in the production of ion series containing the C-termini (y series). The sets of ion series yielded the amino-acid sequences LAAVDATVNQVLASR (A) and GSTAPVGGGAFPTISTR (B), corresponding to residues 198–212 and 374–390, respectively, of bovine P5 (gi number: 119903961).

lower band at the interface between the 30% and 70% layers. As shown in Fig. 4C, the mitochondria were concentrated in fraction 2, since the mitochondrial marker, Grp75, was effectively concentrated in this fraction. It is noteworthy that no Grp75 was detectable in fraction 1, indicating that this was a contaminating fraction, which could not be removed by conventional fractionation. This is consistent with the results in Fig. 4B, showing that PDI was in fraction 1 (lane 2), but was not detectable in fraction 2 (lane 3). From these results, it seems quite plausible that fraction 1 represents contaminating microsomes. As shown in Fig. 4A, P5 was detected, not only in fraction 1 (lane 2), but also in fraction 2 (lane 3), indicating that although P5 was localized in microsomes contaminating the crude mitochondrial fraction, most P5 was localized in the purified mitochondria. Interestingly, the PDI family member ERp57 was detected in both fractions 1 and 2 (Fig. 4D), whereas the PDI family member ERp72 was not detected in fraction 2 (Fig. 4E). Taken together, these data suggest the intriguing possibility that some PDI family proteins are localized, not only in the ER, but also in mitochondria, whereas others are exclusively localized in the ER. The significance of these observations remains to be clarified.

P5 is Detected on the Inner Side of the Inner Mitochondrial Membrane—Next, we performed a protease-sensitivity assay on the percoll-purified mitochondrial fraction. As shown in Fig. 5A, most of the P5 was resistant to externally applied proteinase K (lane 2). As shown in Fig. 5B, Tom20, a mitochondrial outer membrane protein whose large portions are exposed to

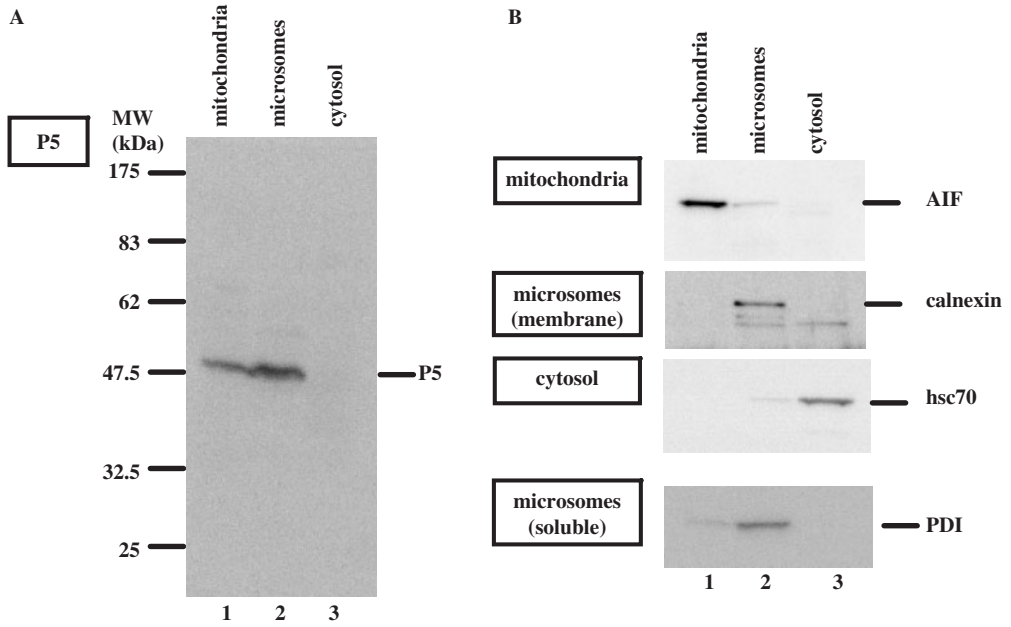

Fig. 3. **Subcellular fractionation of P5 and marker proteins.** Rat liver homogenates were fractionated as described in the EXPERIMENTAL PROCEDURES. and the resulting mitochondrial (lane 1), microsomal (lane 2) and cytosolic (lane 3) fractions were subjected to SDS–PAGE followed by Western blotting using anti-P5 antibody (A) or antibodies against the indicated marker proteins (B).

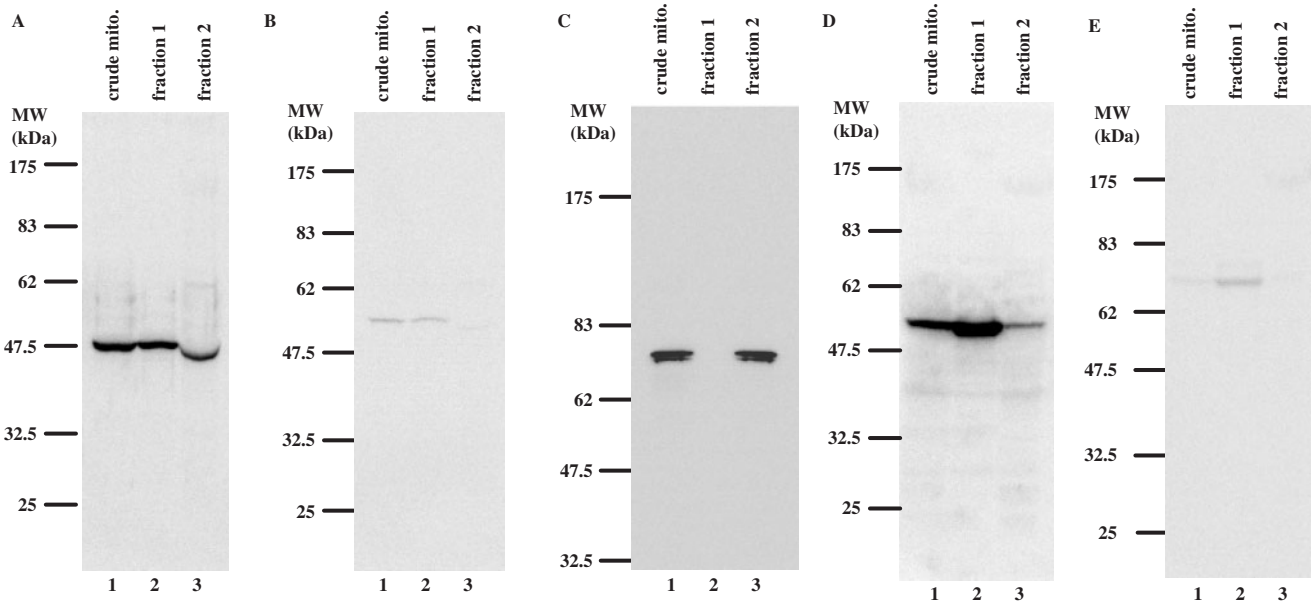

Fig. 4. **Assessment of percoll-purified mitochondria.** Crude mitochondria were purified by percoll-step-gradient ultracentrifugation as described in the EXPERIMENTAL PROCEDURES. Western blotting was performed with antibodies against P5 (A), PDI (B), Grp75 (C), ERp57 (D) and ERp72 (E). Lane 1, crude mitochondrial fraction; lane 2, fraction 1, which is enriched in microsomes; lane 3, fraction2, which is enriched in pure mitochondria.

the cytosol, was effectively degraded, while AIF, an integral membrane protein whose large portions are exposed to the intermembrane space of the mitochondrial inner membrane (*22*), was not significantly degraded, so it is unlikely that the mitochondrial outer membrane was severely damaged. These results indicate that P5 is localized in the interior side of the mitochondrial outer membrane. In the presence of the detergent Triton X-100, P5 was digested to a size of ~35 kDa (Fig. 5A, lane 3), possibly reflecting a domain structure of P5, that is similar to that of yPDI (*23*).

Next we performed the following experiments on the percoll-purified mitochondrial fraction after hypotonic treatment (Fig. 6). With this treatment only the outer

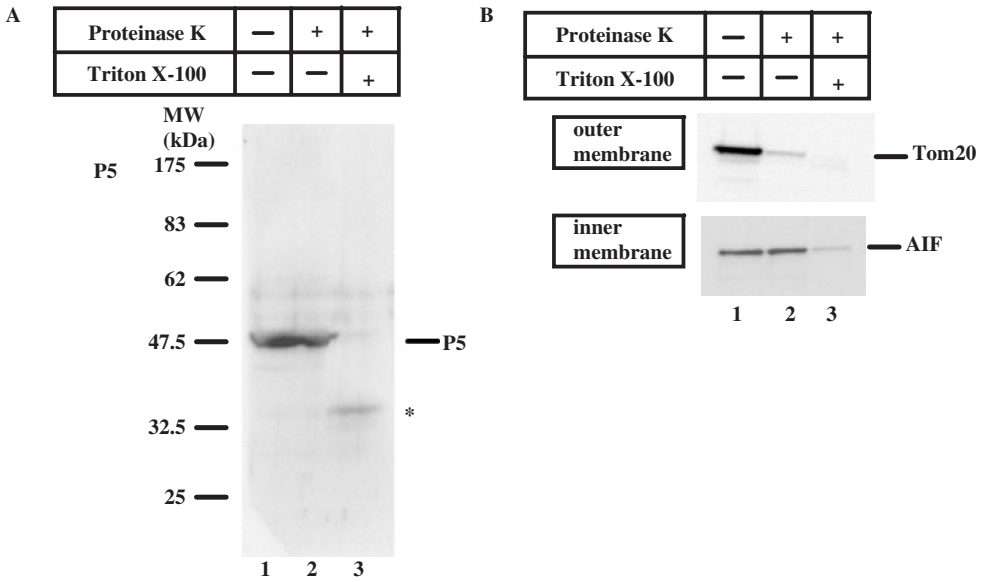

Fig. 5. **Proteinase sensitivity of P5 in percoll-purified mitochondria.** Percoll-purified mitochondria were treated with proteinase K (25 μg/ml) in the presence or absence of 1% (v/v) Triton X-100, as described in the EXPERIMENTAL PROCEDURES and analysed by Western blotting with antibodies against P5 (A). Tom20 (B upper), or AIF (B lower). Lane 1, untreated; lane 2, proteinase K treatment; lane 3, proteinase K and Triton X-100 treatment. Asterisk indicatesputative degradation product of P5.

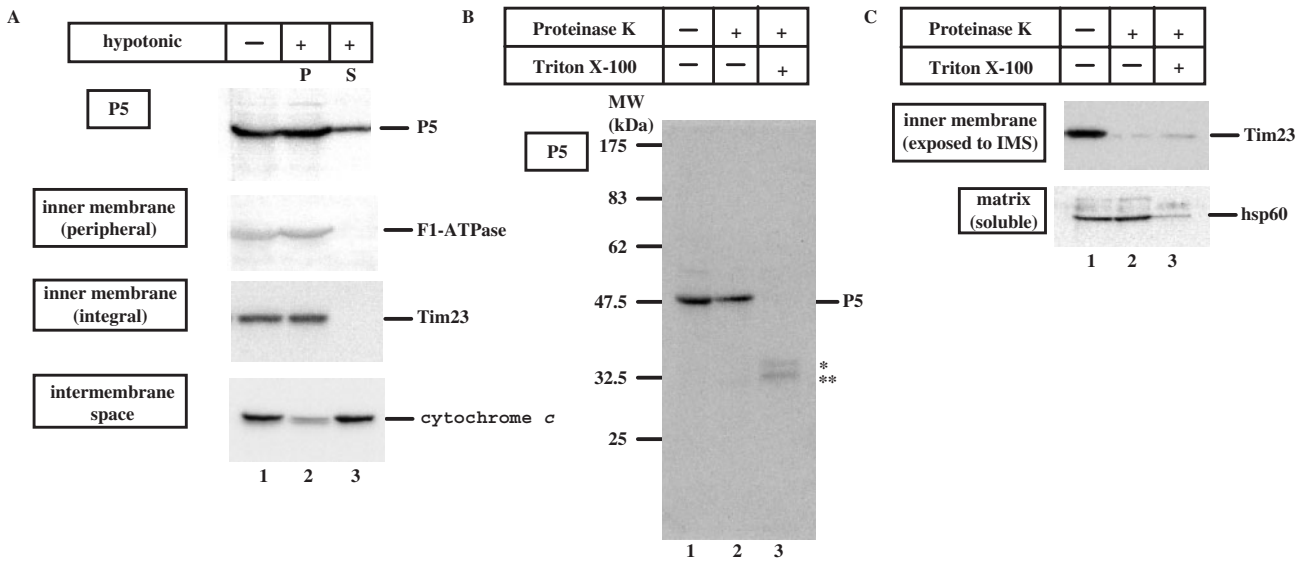

Fig. 6. **Proteinase sensitivity of P5 using purified, hypotonically treated mitochondria.** Percoll-purified mitochondria were incubated in hypotonic buffer to prepare 'mitoplasts'. Hypotonically treated mitochondria were centrifuged to separate pellet and supernatant fractions. Each fraction was applied to SDS–PAGE and analysed by Western blotting with antibodies against P5, F1-ATPase, Tim23 or cytochrome *c* (A). Lane 1, untreated; lane 2, the pellet (P) after treatment; lane 3, the supernatant (S) after treatment. The mitoplasts were treated as described in the legend to Fig. 5 and analysed by Western blotting with antibodies against P5 (B), Tim23 (C, upper panel) or hsp60 (C, lower panel). Lane 1, untreated; lane 2, treated with proteinase K; lane 3, treated with proteinase K and Triton X-100. Single Asterisk, Double Asterisk indicates putative degradation products of P5.

mitochondrial membrane is disrupted and the inner membrane is swollen, resulting in a 'mitoplast'. First we examined whether the outer membrane indeed disrupted or not. After the hypotonic treatment the aliquots were separated to mitoplast and supernatant fractions by a brief centrifugation, and applied to SDS-PAGE and analysed by Western blotting. As shown in Fig. 6A, most fractions of P5 remained in the mitoplast fraction. However, a smaller but significant amount was released into the supernatant fraction. For controls most fractions of F1-ATPase, a peripheral mitochondrial inner membrane protein on the matrix side, and Tim23, a mitochondrial inner membrane protein, remained in the mitoplast fraction, while a

large fraction of cytochrome *c* was released into the supernatant fraction. Thus these results indicate that P5 is mainly localized to the mitoplast fraction with a minor fraction being in the intermembrane space. Next we performed the same protease-sensitivity assay with this mitoplasts as described in Fig. 5. As shown in Fig. 6B, the large portions of P5 were resistant to the proteinase (compare lanes 1 and 2). The outer membrane was successfully disrupted, since Tim23, an integral inner membrane protein whose amino-terminal portion is exposed to the intermembrane space, was effectively digested by the proteinase treatment (Fig. 6C, top panel, lane 2), whereas the integrity of the inner membrane was retained because the soluble matrix protein, hsp60, was almost completely resistant to the proteinase (Fig. 6C, bottom panel, lane 2). These results indicate that large portions of P5 are either localized to the interior side of the mitochondrial inner membrane or deeply embedded in the inner membrane, with a lower portion being in the intermembrane space as a soluble protein.

We hypothesized that P5 should be localized in the mitochondrial matrix as a soluble protein, since it contains no apparent transmembrane domain long enough to cross the biological membrane and no known membrane-associating motifs. To validate this prediction, we examined the salt- and alkali-sensitivities of P5. For the salt-sensitivity assay, the mitoplast fraction prepared as above was subjected to sonication and centrifugation to separate the pellet and supernatant (Fig. 7, lanes 2 and 3). The resulting pellet fraction was treated with buffer containing 1 M NaCl. As shown in Fig. 7A (lanes 4 and 5), although some portions of P5 were salt-sensitive, significant portions remained salt-resistant, possibly reflecting the existence of at least two distinct binding forms to the inner membrane. This was quite similar to the results obtained for F1-ATPase, a peripheral membrane protein in the inner membrane (Fig. 7B, lanes 4 and 5). It was quite distinct from the results obtained for Tim23 (Fig. 7C, lanes 4 and 5). For the alkali-sensitivity assay, the mitoplast pellet fraction was treated with 0.1 M Na_2CO_3 (pH 11.5). As shown in Fig. 7A (lanes 6 and 7), most of P5 was in the supernatant fraction, indicating that some portions of P5 behave like a peripheral membrane protein. This was also quite similar to the results obtained for F1-ATPase (Fig. 7B, lanes 6 and 7) and distinct from the results obtained for Tim23 (Fig. 7C, lanes 6 and 7). These dual properties of P5 as both a soluble and a peripheral membrane protein are not necessarily a common feature of mitochondrial matrix proteins, because hsp60, a typical matrix soluble protein, is mostly collected in the supernatant following sonication and ultracentrifugation (Fig. 7D, lanes 2 and 3).

Taken together, these results led us to conclude that P5 is localized in mitochondria both as a soluble matrix protein and as a peripheral membrane protein on the matrix side of the inner membrane, with a lower portion being in the intermembrane space as a soluble protein.

P5 is Observed in Mitochondria by Immuno-Electron Microscopy—The localization of P5 was examined by immuno-electron microscopy using ultra-cryotomy and immuno-gold staining. Ultra-thin sections of mouse liver were labelled by an immuno-gold method. As shown in Fig. 8A, gold particles indicating the presence of P5 were located in mitochondria as well as in the lumenal space of the ER. The outer membrane of the mitochondria was not stained by gold particles. In control experiments using IgG, gold particles were barely detectable in any part of the cells (Fig. 8B). These observations correspond well with the above biochemical data and confirm the presence of P5 in mitochondria as well as in the ER.

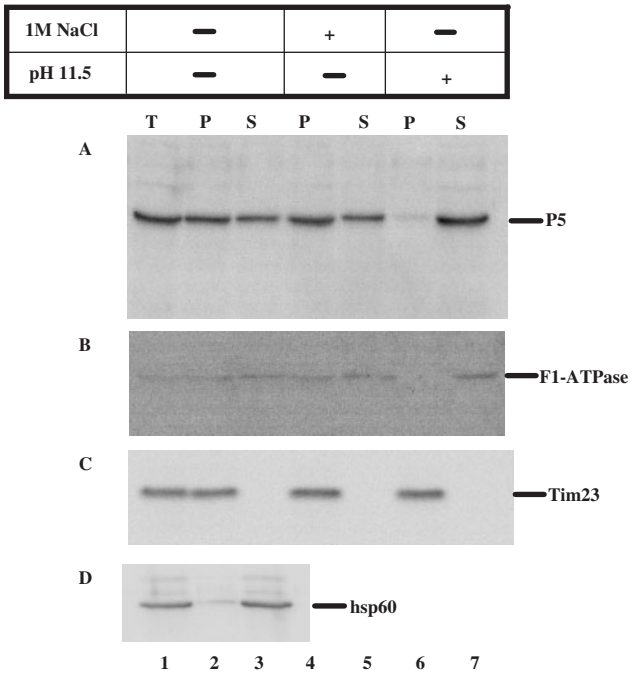

Fig. 7. **P5 behaves as both a soluble and a peripheral membrane protein.** Mitoplasts prepared as described in the EXPERIMENTAL PROCEDURES were sonicated to disrupt them into vesicles. The vesicles were subjected to ultracentrifugation. The resulting pellet was treated with 1 M NaCl or 0.1 M Na_2CO_3 (pH 11.5). Following incubation, the reaction mixtures were again subjected to ultracentrifugation. The pellets and supernatants from each stage and treatment were analysed by Western blotting with antibodies against P5 (A), F1-ATPase (B), Tim23 (C) or hsp60 (D). Lanes 1, vesicle fraction before centrifugation; lane 2, pellet (P) before treatment; lane 3, supernatant (S) from first centrifugation; lane 4, pellet (P) after salt treatment; lane 5, supernatant (S) after salt treatment; lane 6, pellet (P) after alkali treatment; lane 7, supernatant (S) after alkali treatment.

DISCUSSION

It has been thought that most PDI family proteins localize only in the ER. However recently, PDI and ERp57 have been found in the nucleoli (*6*) and PDI and P5 have been reported to be secreted to the cell surface (*24–26*). To investigate the novel localization of PDI family proteins in the cell, we have screened for CGHC-containing proteins in organelles other than the ER using the scFv 5E (*8*), which recognizes the highly conserved active site (CGHC) in PDI family proteins. We have found for the first time that P5 is present in bovine mitochondria. This result has been confirmed by

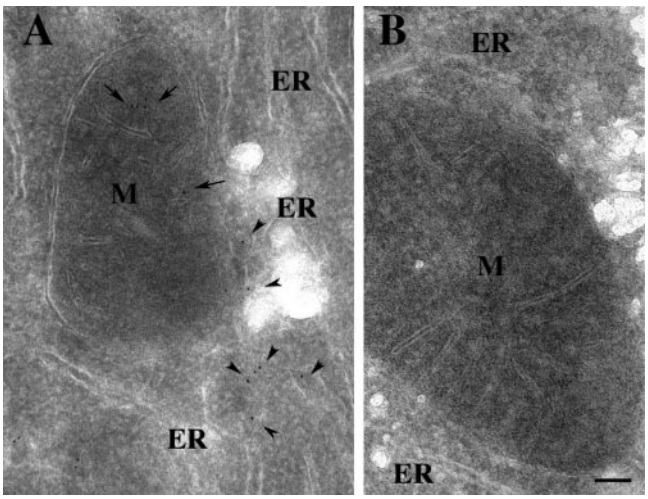

Fig. 8. **Detection of P5 by immuno-gold staining of ultra-thin cryosections of mouse hepatocytes.** Frozen sections were incubated with rabbit anti-P5 polyclonal antibody (A) and non-immunized rabbit IgG (B) at the same concentration, followed by incubation with anti-rabbit IgG coupled to gold particles. Gold particles (5 nm in diameter) indicating the presence of P5 in mitochondria and the ER are shown by arrows and arrowheads, respectively. M, mitochondria; ER, endoplasmic reticulum; bar, 100 nm.

protease-sensitivity assays using percoll-purified rat liver mitochondria, and by electron microscopic observation. These results prove that the scFv fragment of 5E is truly a powerful tool for detecting and screening for CGHC-carrying proteins. Enzymatic treatments of purified bovine mitochondrial P5 with *O*-glycosidase (Roche, Basel, Switzerland) and alkaline phosphatase (TAKARA BIO INC., Otsu, Japan), as well as the deduced amino-acid sequence, demonstrate that bovine mitochondrial P5 does not undergo any *O*-glycosylation, *N*-glycosylation or phosphorylation (data not shown). Recently, it has been suggested that ER proteins such as PDI and BiP/Grp78 also localize in the mitochondria (*7*, *27*). However, the molecular weight of the mitochondrial protein identified as PDI in that report is clearly smaller than that of PDI and the protein does not cross-react with monoclonal antibody against PDI (*7*). Here, we find that PDI does not localize in mitochondria and that P5 localizes in the mitochondrial matrix and the intermembrane space. We also find that ERp57, but not ERp72, localizes in mitochondria. Our results raise the interesting question of how these proteins are transported to the mitochondria. Most mitochondrial proteins are nuclear-encoded, synthesized as preproteins in the cytosol, and imported into mitochondria via translocation complexes in the outer and inner mitochondrial membranes (*28–30*). The protein translocation machinery in the outer mitochondrial membrane includes two surface receptors, Tom20 and Tom70 (*31*, *32*). Tom20 recognizes classical N-terminal mitochondrial targeting sequences, which form positively charged amphipathic helices (*31*, *33*). In contrast, the Tom70 receptor mediates the import of the multi-transmembrane carrier proteins of the inner mitochondrial membrane (*32*, *33*) as well as multi-transmembrane proteins and peripheral membrane protein of outer membrane (*34*, *35*). How P5 is imported into mitochondria is unknown, with respect of the transport pathway, the mitochondrial targeting signal, and so on. One possibility is that a fraction of P5 is released from signal recognition particle and redirected and imported into mitochondria *via* Tom70-dependent pathway, such as the case of P4501B1 (*36*). Of course, further studies are needed.

It is known that P5 has oxidative, reductive and chaperone activities (*5*, *37*); however, the physiological significance of P5 in the cell has not been extensively characterized. Although P5 belongs to the PDI family of proteins, its isomerase activity is weaker than that of PDI (*5*) because it lacks the lysine residue at the end of the CGHC motif (*8*). However P5 possesses a function which PDI does not have: expression of P5 in the embryonic midline has been reported to be required for the establishment of left/right asymmetries during ontogeny (*38*). Thus, P5 may have functions other than as an isomerase and chaperone in mitochondria.

We suggest three possibilities for the physiological significance of P5 in mitochondria. (i) P5 might function as an oxidoreductase in the mitochondrial matrix or in the intermembrane space. In contrast to the ER, it has been thought that disulphide bonds are not intentionally formed in mitochondria, which are maintained in a reductive state. Interestingly, it has recently been reported that disulphide bridges are formed between Mia40 and newly imported proteins in the intermembrane space (*39*). Thus, it is possible that a protein with oxidative function is also present in the mitochondrial matrix. Based on these observations, we speculate that P5 functions as an oxidoreductase and controls the redox state of the mitochondrial matrix. It is not excluded that P5 has some role(s) in the intermembrane space, for example, together with the Mia40 system. (ii) P5 might function as an assembly factor for mitochondrial inner membrane proteins. Although the result is preliminary, we have found that P5 binds to F1-ATP synthase α and β subunits, which are localized in the mitochondrial matrix (data not shown) using an hP5 affinity column. Since P5 has chaperone activity independent of its redox activity (*8*), it may interact with F1-ATP synthase α and β subunits to settle these subunits in the membrane and it likely contributes to the assembly of F1-ATP synthase. (iii) P5 may have an unidentified activity independent of its oxidoreductase or chaperone activities. These possibilities are not mutually exclusive and further study is required to elucidate the function of P5.

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