Senescence marker protein-30 is a unique enzyme that hydrolyzes diisopropyl phosphorofluoridate in the liver

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Abstract **Senescence marker protein-30 (SMP30) was originally identified as a novel protein in the rat liver, the expression of which decreases androgen-independently with aging. We have now characterized a unique property of SMP30, the hydrolysis of diisopropyl phosphorofluoridate (DFP), which is similar to the chemical warfare nerve agents sarine, soman and tabun. Hydrolysis of DFP was stimulated equally well by 1 mM $MgCl_2$, $MnCl_2$ or $CoCl_2$, to a lesser extent by 1 mM $CdCl_2$ but not at all by 1 mM $CaCl_2$. No $^{45}Ca^{2+}$-binding activity was detected for purified SMP30, suggesting that SMP30 is not a calcium-binding protein, as others previously stated. Despite the sequence similarity between SMP30 and a serum paraoxonase (PON), the inability of SMP30 to hydrolyze PON-specific substrates such as paraoxon, dihydrocoumarin, γ-nonalactone, and δ-dodecanolactone indicate that SMP30 is distinct from the PON family. We previously established SMP30 knockout mice and have now tested DFPase activity in their livers. The livers from wild-type mice contained readily detectable DFPase activity, whereas no such enzyme activity was found in livers from SMP30 knockout mice. Moreover, the hepatocytes of SMP30 knockout mice were far more susceptible to DFP-induced cytotoxicity than those from the wild-type. These results indicate that SMP30 is a unique DFP hydrolyzing enzyme in the liver and has an important detoxification effect on DFP. Consequently, a reduction of SMP30 expression might account for the age-associated deterioration of cellular functions and enhanced susceptibility to harmful stimuli in aged tissue.**

Keywords: Diisopropyl phosphorofluoridate; Senescence marker protein-30; Paraoxonase; Luciferin-regenerating enzyme; Hepatocyte; Aging

1. Introduction

By using proteomic analysis of age-associated changes in soluble proteins of the rat liver, we discovered a novel protein then designated it as senescence marker protein-30 (SMP30) [1]. Amounts of SMP30 significantly decrease with aging in an androgen-independent manner [1–3], and the amino acid sequences of this 34-kDa protein are highly conserved among vertebrates [2,4,5]. Recently, several SMP30 homologues were also discovered in non-vertebrates such as *Drosophila* [6,7], *Sarcophaga* [7] and fireflies [8]. By RT-PCR analysis, SMP30 transcripts have been detected in multiple tissues including the liver, kidney, lung, testes and cerebrum [9]. The human SMP30 gene is located in the p11.3–q11.2 segment of the X chromosome [4]. SMP30 regulates Ca^{2+} kinetics involved in the plasma membrane Ca^{2+}-pumping activity of HepG2 and LLC-PK1 cells showing that SMP30 could rescue cells from an apoptotic death induced by a high intracellular Ca^{2+} level ($[Ca^{2+}]_i$) [10,11]. According to a database search, SMP30 has a domain similar to that of bacterial and yeast RNA polymerase [12]. In cultured hepatocytes, SMP30 is distributed in both the cytoplasm and nuclei [12]. Since SMP30 is present in the nuclei, this protein may regulate gene expression such as in transcription.

To clarify the relationships between SMP30's decrease and age-associated organ disorders, we established SMP30 knockout mice [13]. Throughout our experiments in vitro and in vivo, the livers of these knockout animals were far more susceptible to TNF-α- and Fas-mediated apoptosis than those from the wild-type. Interestingly, livers of SMP30 heterozygous mice showed intermediate susceptibility to Fas-induced apoptosis. Thus, SMP30 acts to protect cells from apoptosis. Moreover, histological and biochemical analyses of livers from SMP30 knockout mice showed abnormal accumulations of neutral lipids and phospholipids such as phosphatidylethanolamine, cardiolipin, phosphatidylcholine, phosphatidylserine and sphingomyelin [14]. This abnormal lipid metabolism must increase the tissue's susceptibility or resistance to apoptosis. Such changes of SMP30 expression might, then, account for the deterioration of cellular functions and susceptibility to harmful stimuli in aged tissues.

In 1999, Billecke et al. [15] purified from mouse livers a novel soluble enzyme capable of hydrolyzing diisopropyl phosphorofluoridate (DFP) and similar to the nerve-paralyzing agents isopropyl methylphosphonofluoridate (sarine), ethyl-*N*-dimethyl phosphoramidocyanidate (tabun) and pinacolyl methylphoshonofluoridate (soman). Sequencing the partial peptide

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Abbreviations: DFP, diisopropyl phosphorofluoridate; LRE, luciferin-regenerating enzyme; PLC, phospholipase C; PLD, phospholipase D; PON, paraoxonase; SDS–PAGE, SDS–polyacrylamide gel electrophoresis; SMP30, senescence marker protein-30

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fragments of the purified mouse liver DFPase showed an almost identical amino acid sequence to that of mouse SMP30. However, the DFPase activity of the cell lysate expressing recombinant SMP30 was too low for definitive identification.

In this study, we purified SMP30 from rat livers and confirmed its DFPase activity. Moreover, by using our previously established SMP30 knockout mice, we clearly showed that SMP30 is a unique enzyme that hydrolyzes DFP in the liver.

2. Materials and methods

2.1. Materials and chemicals

Phenyl acetate, paraoxon, dihydrocoumarin, γ-nonalactone and δ-dodecanolactone were purchased from Sigma–Aldrich (St. Louis, MO, USA). DEAE-Sephacel, Phenyl Sepharose CL-4B and Sephacryl S-200 HR were purchased from Amersham Biosciences (Piscataway, NJ, USA). DFP and other reagents were purchased from Wako Pure Chemical (Osaka, Japan).

2.2. Animals

Male Wister rats, 3–9 months of age, were obtained from the Animal Facility at Tokyo Metropolitan Institute of Gerontology. SMP30 knockout mice were generated by gene targeting [13]. In this study, we used male SMP30 knockout (SMP30Y/−) mice and male wild-type (SMP30Y/+) mice at an age of 3 months. Heterozygous male mice do not exist, because the SMP30 gene is located on the X chromosome. Mice were maintained on 12 h light/dark cycles in a controlled environment and fed ad libitum. The Animal Care and Use Committee of Tokyo Metropolitan Institute of Gerontology approved the protocol of animal experiments performed in the present study.

2.3. Purification of rat SMP30

All purification procedures were carried out at 0–4 °C unless otherwise stated. Rat livers (20 g) were homogenized with 8 vol. of ice-cold homogenization buffer (10 mM Tris–HCl, pH 8.0, 1 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM phenyl-methanesulfonyl fluoride), in a Polytron homogenizer. The homogenate was centrifuged at $7000 \times g$ for 30 min. The supernatant was fractionated by ammonium sulfate precipitation, and the fraction precipitated from 45% to 70% saturation was suspended in buffer A (10 mM Tris–HCl, pH 8.0, 1 mM $MgCl_2$), and thoroughly dialyzed against the same buffer. Insoluble materials were removed by centrifugation at $10\,000 \times g$ for 20 min, and the supernatant was applied to a DEAE-Sephacel column (1.4×25 cm) pre-equilibrated with buffer A. Elution was performed with a linear gradient of 0–0.35 M NaCl in 400 ml of buffer A. The fractions containing SMP30 were determined by dot-blot analysis using mouse anti-recombinant human SMP30 monoclonal antibody. The SMP30 fractions were pooled and brought to 45% saturation in ammonium sulfate, then dialyzed against the buffer A saturated with 45% ammonium sulfate. Insoluble materials were removed by centrifugation and the supernatant was applied to a Phenyl Sepharose CL-4B (1.0×13 cm) pre-equilibrated with buffer A saturated with 45% ammonium sulfate. Elution was performed with a linear gradient of 45 to 0% ammonium sulfate in 80 ml of buffer A. The SMP30 fractions were pooled and concentrated by ammonium sulfate precipitation. The concentrated solution was subjected to gel filtration on Sephacryl S-200 HR (1.0×47 cm) equilibrated with buffer A containing 0.2 M NaCl. Each SMP30 fraction was stored at −70 °C until use.

2.4. Protein determination and SDS–polyacrylamide gel electrophoresis

The protein content of fractions from the column chromatographic separations was monitored by measuring the absorbance at 280 nm. The protein concentration in samples was determined by the BCA protein assay (Pierce Biotechnology, Inc., Rockford, IL, USA) using bovine serum albumin as a standard. SDS–PAGE was performed on vertical slab gels (1 mm × 9 cm) containing 14% (w/v) acrylamide and 0.25% (w/v) *N*,*N*′-methylenebisacrylamide, by the method of Laemmli [16]. The proteins were visualized by Quick CBB (Wako Pure Chemical, Osaka, Japan).

2.5. DFPase activity

DFPase activity was measured as described by Billecke et al. [15]. Briefly, the reaction cuvette contained 5.74 mM DFP in 2 mM HEPES, pH 8.0, 1 mM $MgCl_2$, 0.004% phenol red in a total volume of 1 ml. Reactions were initiated by adding the enzyme and the acid production rate was followed by monitoring increased absorbance at 422 nm at 25 °C. A calibration curve was constructed using a range of HCl concentrations, and an equation relating slope (the change in optical density per unit time) to acid production was derived: 1.900×10^6 (ΔOD/Δtime)/sample volume (μl) = nmol/ml. The slope of the absorbance trace was determined over 5–10 min to measure the rate of acid production. For determination of divalent cations' requirement for DFPase activity, 1 mM $MgCl_2$, $CaCl_2$, $MnCl_2$, $CoCl_2$ and $CdCl_2$ were used.

For determination of DFPase activity in the liver, livers were removed from mice and homogenized with ice-cold homogenization buffer (10 mM Tris–HCl, pH 8.0, and 1 mM phenyl-methanesulfonyl fluoride) for 30 s at high speed with a Polytron homogenizer. The homogenate was centrifuged at $10\,000 \times g$ for 10 min. The protein concentration of crude extract was adjusted to 5 mg/ml. The reaction was initiated by the addition of crude extract (0.2 mg) in the presence of 1 mM $MgCl_2$.

2.6. Arylesterase activity

Arylesterase activity was determined as described by Gan et al. [17]. Briefly, the cuvette contained 1 mM phenylacetate in 20 mM Tris–HCl, pH 8.0, and 1 mM $CaCl_2$ in a total volume of 1 ml. The reaction was initiated by adding enzyme, and the increase in absorbance at 270 nm was recorded. The amount of phenol was calculated from the molar difference extinction coefficient, 1310 $M^{-1}\,cm^{-1}$. Blanks without enzyme were used to correct for spontaneous hydrolysis of phenylacetate. For determination of divalent cations' requirement for arylesterase activity, 1 mM $CaCl_2$, $MgCl_2$, $MnCl_2$, $CoCl_2$ and $CdCl_2$ were used.

2.7. PON activity

Paraoxonase (PON) activity was measured with 1 mM paraoxon in a total volume of 1 ml. Enzymatic activity was measured in 50 mM Tris–HCl, pH 8.0. The reaction was initiated by the addition of enzyme, and the increase in absorbance at 412 nm was recorded. The amount of *p*-nitrophenol was calculated from the molar difference extinction coefficients 17 100 $M^{-1}\,cm^{-1}$.

2.8. Other enzyme activities

Hydrolysis of dihydrocoumarin was monitored by the increase in UV absorbance at 270 nm. In a typical experiment, a cuvette contained 1 mM substrate in 50 mM Tris–HCl, pH 8.0, in a total volume of 1 ml. The molar difference extinction coefficients used to calculate the rate of hydrolysis were 1295 $M^{-1}\,cm^{-1}$ [18].

Hydrolysis activities of γ-nonalactone and δ-dodecanolactone were followed by a colorimetric assay with phenol red [18].

2.9. Isolation and culture of mouse hepatocytes

Mouse hepatocytes were isolated by the collagenase perfusion method as described [19]. Briefly, each liver was perfused in situ through the vena cava with EGTA solution containing 0.5 mM EGTA, 5 mM glucose, 136 mM NaCl, 5.3 mM KCl, 0.3 mM Na_2HPO_4, 0.4 mM KH_2PO_4, and 10 mM HEPES, pH 7.2, at a rate of 15 ml/min. After that perfusion, the first solution was replaced with a solution containing 0.5 mg/ml collagenase, 0.05 mg/ml trypsin inhibitor, 4.5 mM $CaCl_2$, 136 mM $NaCl_2$, 5.3 mM KCl, 0.3 mM NaH_2PO_4, 0.4 mM KH_2PO_4, and 10 mM HEPES, pH 7.4. After the second perfusion, the livers were removed and filtered through nylon mesh (100 μm) and then washed three times with Hanks' Balanced Salt Solution and twice with Williams medium E to remove non-parenchymal cells. Final hepatocyte preparations were suspended at 1.25×10^5 cell/ml in Williams medium E containing 0.1 mg/ml aprotinin and 10^{-9} M dexamethasone supplemented with 5% fetal calf serum and then placed into culture plates coated with rat type I collagen (Sigma–Aldrich). Cells were cultured at 37 °C under 5% CO_2 in air for 3 h to allow attachment to the wells, then the medium was replaced with serum-free Williams medium E containing 0.1 mg/ml aprotinin and 10^{-9} M dexamethasone. Cell viability was determined by trypan blue dye exclusion before plating.

Table 1
Summary of purification and yields of SMP30 from the rat liver

	Total protein (mg)	Total DFPase activity (nmol/min)	Specific activity (nmol/min/mg)	Overall purification (fold)
Soluble fraction	1830	55900	31	1
DEAE-Sephacel	140	19100	136	4
Phenyl Sepharose CL-4B	7.84	4100	523	17
Sephacryl S-200 HR	0.84	1270	1512	49

Wister rat livers (20 g) were used for preparing the liver soluble fraction. The purification steps and DFPase activity measurement are described in Section 2.

2.10. DFP-induced cytotoxicity assay

Mouse hepatocytes were incubated with various concentrations of DFP for 24 h at 37 °C. Then, cell viability was determined by the XTT assay using the Cell Proliferation Kit II (XTT) (Roche Diagnostics, Mannheim, Germany). Briefly, the medium was replaced with phenol red-free Williams medium E containing 0.1 mg/ml aprotinin, 10^{-9} M dexamethasone, and XTT solution (0.3 mg/ml). After incubation for 4 h at 37 °C, absorbance at 492 nm was measured with a Multiscanner Autoreader (Emax, Molecular Devices, Sunnyvale, CA, USA).

3. Results

3.1. Purification of rat SMP30

The purification protocol and yield of SMP30 from rat livers are summarized in Table 1. Electrophoretic profiles of each fraction appear in Fig. 1. The purity of the SMP30 fraction obtained by Sephacryl S-200 HR column chromatography was demonstrated by the single band it yielded with an apparent *Mr* of 34000 estimated by SDS–PAGE.

3.2. DFPase activity of purified SMP30

Using the purified rat SMP30, we measured DFPase activity under standard assay conditions in the presence of 1 mM $MgCl_2$. The results indicated that purified SMP30 hydrolyzed DFP with specific activity of 998 nmol/min/mg of protein in a dose-dependent manner between 0 and 40 μg of protein (Fig. 2A). The time course of DFP hydrolysis during the first 10 min was linear, i.e., no lag phase occurred (data not shown). Without $MgCl_2$ present, SMP30 did not produce DFPase activity; however, at a concentration of 1 mM, Mg^{2+} effectively incited DFPase activity (Fig. 2B), and that amount was then used in all subsequent experiments here.

Since the activity of SMP30 seemed to entail an absolute requirement for Mg^{2+}, other divalent cations were also tested. At a

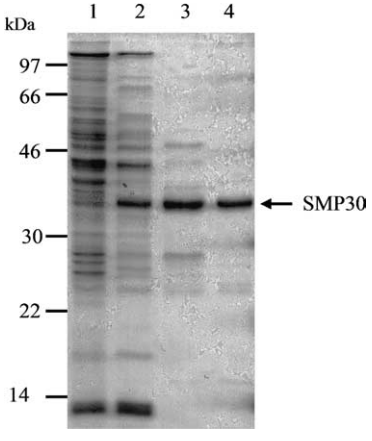

Fig. 1. SDS–PAGE profile of protein samples from each purification step. Aliquots of material from each step of the purification were subjected to SDS–PAGE, and the gel was stained with Quick CBB. Lane 1, liver soluble fraction (9 μg of protein); lane 2, DEAE-Sephacel pool (6 μg of protein); lane 3, Phenyl Sepharose CL-4B pool (1 μg of protein); lane 4, Sephacryl S-200 HR pool (0.8 μg of protein).

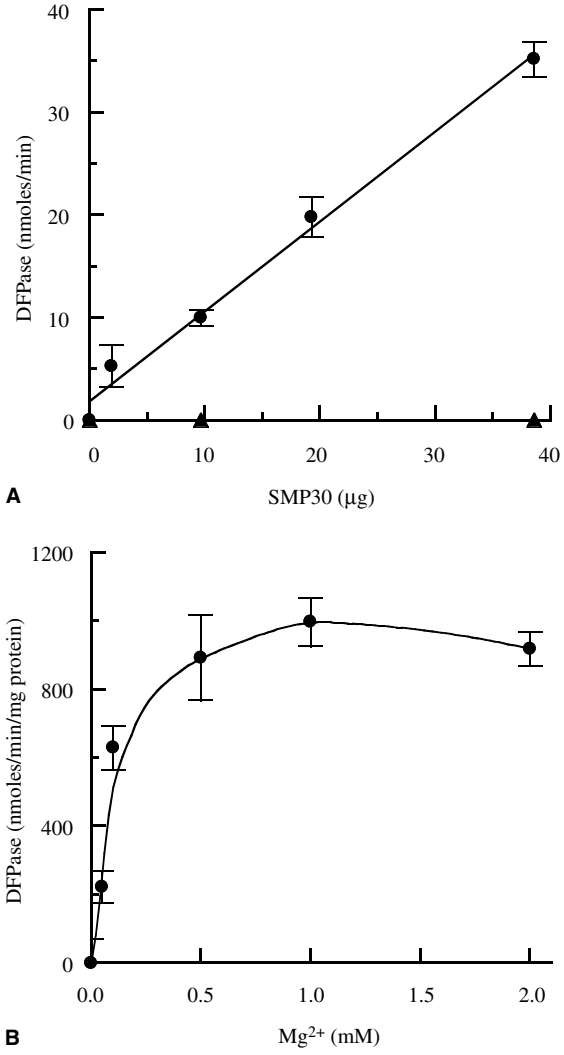

Fig. 2. DFPase activity of SMP30. (A) Dose-response curve of DFPase activity stimulated by SMP30. Rat SMP30 was purified as described in Section 2. DFPase activity was determined by using increasing doses of SMP30 in the presence (closed circle) or absence (closed triangle) of 1mM $MgCl_2$. (B) Effect of Mg^{2+} on the hydrolysis of DFP by SMP30. DFPase activity was determined by applying several concentrations of $MgCl_2$ in the presence of SMP30 (40 μg of protein). Values are expressed as an average of DFPase activity ± S.E.M. of three independent experiments.

Table 2
Effect of divalent cations on DFPase activity

Divalent cation	Activity (%)
$MgCl_2$	100
$MnCl_2$	100
$CoCl_2$	102
$CdCl_2$	38
$CaCl_2$	0

DFPase activity was measured in the presence of each divalent cation at a concentration of 1 mM. The enzyme activity was expressed as the percentage of $MgCl_2$ with a specific activity of 341 nmol/min/mg of protein. The results represent the average of three independent experiments.

final concentration of 1 mM in the standard assay mixture, Mg^{2+}, Mn^{2+}, and Co^{2+} ions were equally as active as Mg^{2+} in stimulating SMP30 to hydrolyze DFP (100%); however, the Cd^{2+} ion was considerably less effective (38%) (Table 2). In the presence of Ca^{2+} ion, no DFPase activity was observed.

3.3. Substrate specificity of SMP30

According to a NCBI Conserved Domain Search using the GenBank protein database, the rat SMP30 domain (103 amino acids, residues 138–240) bears close sequence similarity to that of rat serum PON1 (100 amino acids, residues 208–307). Mammalian PON has three distinct isozymes: PON1, PON2 and PON3; and these hydrolyze toxic organophosphates, carbamates and aromatic esters [20]. The rat SMP30 domain showed 65% similarity to the PON1 domain (Fig. 3). To determine the specificity of SMP30 for PON substrates, we tested such substrates as phenylacetate, paraoxon, dihydrocoumarin, γ-nonalactone, and δ-dodecanolactone (Table 3). SMP30 successfully hydrolyzed phenylacetate with a specific activity of 527 nmol/min/mg of protein in the presence of 1 mM $CdCl_2$, but failed to hydrolyze paraoxon, dihydrocoumarin, γ-nonalactone or δ-dodecanolactone.

3.4. DFPase activity in livers from SMP30 knockout (SMP30Y/−) mice

To ascertain the ability of SMP30 to hydrolyze DFP, we compared DFPase activity in the livers from SMP30Y/+ and SMP30Y/− mice. Livers from SMP30Y/+ mice had 32 ± 5 nmol/min/mg protein ($n = 5$) of DFPase activity, whereas livers from SMP30Y/− mice had no detectable DFPase activity. Thus, SMP30 is a unique enzyme that, when present in the liver, effectively hydrolyzes its content of DFP.

3.5. DFP-induced cytotoxicity toward hepatocytes of SMP30Y/− mice

To investigate the detoxification effect of SMP30 on DFP-induced lysis, hepatocytes from SMP30Y/+ and SMP30Y/− mice were treated with increasing concentrations of DFP. The extent of cell death was determined by XTT assay (Fig. 4). Exposure to 0.01 mM DFP had no effect on SMP30Y/+ hepatocytes (100% survival); however, significantly fewer SMP30Y/− hepatocytes remained viable (about 65% survival) after this treatment. Moreover, exposure to a higher concentration of DFP (1 mM) decreased the viability of hepatocytes from both SMP30Y/+ and SMP30Y/− mice. These results clearly showed that hepatocytes without SMP30 are far more

Table 3
PON substrate specificity of SMP30

Substrate	Specific activity (nmol/min/mg of protein)
DFP	998 ± 71
Phenyl acetate	527 ± 15
Paraoxon	ND[a]
Dihydrocoumarin	ND[a]
γ-Nonalactone	ND[a]
δ-Dodecanolactone	ND[a]

Hydrolyzing activity in each substrate was measured as described in Section 2. The results represent the average ± S.E.M. of three independent experiments.
[a] ND, no detectable enzyme activity.

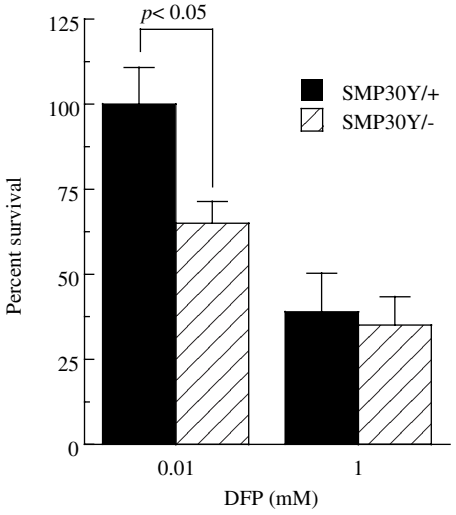

Fig. 4. DFP-induced cell death. SMP30Y/+ and SMP30Y/− hepatocytes were incubated for 24 h in medium containing 0.01 mM and 1 mM DFP. Percent survival was calculated relative to treatment with 0.01 mM DFP in SMP30Y/+ hepatocytes by XTT assay. Values are expressed as an average percentage of survival ± S.E.M. of four independent experiments.

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SMP30  138 FPDHSVKKYFDQVDISNGLDWSLDHKIFYYIDSLSYTVDAFDYDLPTGQISNRRT
PON1   208 YSPDKVRVVADGFDFANGIGISLDGKYVYIAELLAHKIHV--YE---KHANWTLT

SMP30  193 VYKMEKDEQIPDGMCID-VEGKLWVACY-NGGRVIRLDPETGKRLQTVKL  240
PON1   258 PLKVLSFDTLVDNISVDPVTGDLWVGCHPNGMRIFFYDSENPPGSEVLRI  307
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Fig. 3. Sequence similarities of the rat SMP30 domain (103 amino acids, residues 138 to 240) to a rat PON1 domain (100 amino acids, residues 208 to 307). Identical residues are shown with black boxes, and similar residues are shaded. The PON1 domain shows 65% similarity with the SMP30 domain. SMP30 : GenBank Accession No. CAA48786; PON1: GenBank Accession No. XP_342640.

susceptible to DFP-induced cytotoxicity than their SMP30Y/+ counterparts.

4. Discussion

In the present work, SMP30 from the rat liver was purified to homogeneity and established as having potent DFPase activity in the presence of $MgCl_2$. Moreover, by analyzing this effect in SMP30 knockout mice, we proved that SMP30 is a unique enzyme with the distinctive ability to hydrolyze DFP in the liver.

Billecke et al. [15] reported that the mouse liver contains a soluble enzyme that hydrolyzes organophosphates such as DFP, sarine, tabun and soman and noted that enzyme's similarity to SMP30. However, because the enzyme activity in their cell lysates expressing recombinant SMP30 was quite low, they could not definitively identify it as DFPase. To determine whether SMP30 actually has DFPase activity, we isolated SMP30 from rat livers and purified the soluble fraction about 49-fold with about 2% recovery (Table 1). The elution profile on a Sephacryl S-200 HR showed a coincident single peak at a position characteristic of SMP30 and DFP hydrolyzing activity (data not shown). These results confirmed that SMP30 has DFPase activity.

Little et al. [21] described an enzyme in the liver's soluble fraction and noted some DFPase activity in the absence of metal ions. However, our purified SMP30 had no DFPase activity unless a divalent cation was present. In fact, 1 mM $MgCl_2$, $MnCl_2$ and $CoCl_2$ all stimulated DFPase activity to the same extent, and 1 mM $CdCl_2$ did so to a lesser degree, but 1 mM $CaCl_2$ had no such effect. Possibly, even a slight amount of endogenous divalent cation such as Mg^{2+}, Mn^{2+}, Co^{2+} and/or Cd^{2+} in their soluble liver fraction was sufficient to stimulate DFPase activity.

Yamaguchi and Sugii [22] reported that SMP30 (regucalcin) is a calcium-binding protein. Yet we could not detect any DFPase activity at all in the presence of $CaCl_2$. To resolve this discrepancy, we examined the calcium-binding activity of SMP30 on a nitrocellulose membrane using $^{45}Ca^{2+}$ and autoradiography as described by Maruyama et al. [23]. Although calmodulin clearly bound to ^{45}Ca, purified SMP30, even a large amount (40 μg), completely failed to bind (data not shown). However, the possibility remained that calcium-binding activity dissipated in the course of SDS–PAGE and transfer to the nitrocellulose membrane. To eliminate that possibility, we conducted equilibrium dialysis using $^{45}Ca^{2+}$ and a microdialyzer, essentially the same procedure described by Yamaguchi and Sugii [22]. However, even then, the purified SMP30 had no calcium-binding activity at all (data not shown). Therefore, SMP30 does not seem to be a calcium-binding protein.

The rat SMP30 domain has sequence similarity to a rat serum PON1 domain (Fig. 3). PON family members, which consist of PON1, PON2 and PON3, act as important guardians against cellular damage from toxic agents, such as organophosphates, oxidized lipids in the plasma low density lipoprotein, and bacterial endotoxins [20]. In particular, like SMP30, PON1 also hydrolyzes DFP in the presence of $CaCl_2$ [24]. However, testing the substrate specificity of SMP30 revealed a distinct difference between the two, because SMP30 did not hydrolyze any of several PON substrates, i.e., paraoxon, dihydrocoumarin, γ-nonalactone, and δ-dodecanolactone (Table 3). Thus, SMP30 is distinct from the PON family.

Our previously established SMP30 knockout mice [13] enabled us to compare DFPase activity in their livers to that in SMP30-replete wild-type mice. Accordingly, livers from these wild-type mice had readily detectable DFPase activity, whereas in liver from SMP30 knockout mice did not. Moreover, in vitro, SMP30 knockout hepatocytes were highly susceptible to DFP-induced cytotoxicity. These results indicated that SMP30 is a unique DFP hydrolyzing enzyme with the important effect of neutralizing DFP toxicity in the liver. In support, our previous findings revealed that the livers of SMP30 knockout mouse were highly susceptible to TNF-α and Fas-mediated apoptosis [13]. Collectively, these outcomes denote that a reduction of SMP30 expression might account for the age-associated deterioration of cellular functions and enhanced susceptibility to harmful stimuli in aged tissue.

Mammalian SMP30 shows high levels of homology with deduced amino acid sequences for luciferin-regenerating enzyme (LRE) in fireflies [25]. LRE is a single polypeptide with a molecular mass of 38 kDa and is capable of converting oxyluciferin to 2-cyano-6-hydroxybenzothiazole that, in turn, is converted into luciferin in the presence of D-cysteine. Because of their similarity, we examined whether LRE has DFPase activity and whether SMP30 has LRE activity. Testing with recombinant LRE located DFPase activity with approximately half the specific activity of SMP30 (data not shown). However, SMP30 had no detectable LRE activity. These results strongly suggested that DFPase activity is a common function for SMP30 and that LRE activity has evolved as a new function of converting oxyluciferin into 2-cyano-6hydroxybenzothiazole specifically in fireflies.

From the results reported in this paper, we conclude that SMP30 is a unique enzyme, whose ability to hydrolyze DFP protects the liver from its toxic effects. However, the physiological substrate remains unclear, because DFP is an artificial chemical compound. Recently, we found abnormal accumulations of such phospholipids as phosphatidylethanolamine, cardiolipin, phosphatidylcholine, phosphatidylserine and sphingomyelin in the livers of SMP30 knockout mice [14]. Because the chemical structure of phospholipids includes organophosphate, we tested directly if SMP30 has phospholipid catabolic activity like that of phospholipase C (PLC) and phospholipase D (PLD). However, we did not detect notable PLC or PLD activity even when a large amount of purified rat SMP30 was used (data not shown). The future identification of SMP30's physiological substrate bears a strong potential for increasing our understanding of this interesting and important molecule.

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