

# Human $\alpha/\beta$ Hydrolase Domain Containing 10 (ABHD10) Is Responsible Enzyme for Deglucuronidation of Mycophenolic Acid Acyl-glucuronide in Liver\*<sup>§</sup>

Received for publication, June 13, 2011, and in revised form, January 19, 2012. Published, JBC Papers in Press, January 31, 2012, DOI 10.1074/jbc.M111.271288

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**Background:** The enzyme responsible for deglucuronidation of mycophenolic acid acyl-glucuronide (AcMPAG) in human liver remains unknown.

**Results:** Human  $\alpha/\beta$  hydrolase domain containing 10 (ABHD10) was involved in the deglucuronidation.

**Conclusion:** Human ABHD10 would affect the acyl-glucuronidation of mycophenolic acid.

**Significance:** Human ABHD10 may affect the incidence of mycophenolic acid-induced immunotoxicity, which is associated with AcMPAG.

Mycophenolic acid (MPA), the active metabolite of the immunosuppressant mycophenolate mofetil (MMF), is primarily metabolized by glucuronidation to a phenolic glucuronide (MPAG) and an acyl glucuronide (AcMPAG). It is known that AcMPAG, which may be an immunotoxic metabolite, is deglucuronidated in human liver. However, it has been reported that recombinant  $\beta$ -glucuronidase does not catalyze this reaction. AcMPAG deglucuronidation activity was detected in both human liver cytosol (HLC) and microsomes (HLM). In this study, the enzyme responsible for AcMPAG deglucuronidation was identified by purification from HLC with column chromatographic purification steps. The purified enzyme was identified as  $\alpha/\beta$  hydrolase domain containing 10 (ABHD10) by amino acid sequence analysis. Recombinant ABHD10 expressed in Sf9 cells efficiently deglucuronidated AcMPAG with a  $K_m$  value of  $100.7 \pm 10.2 \mu\text{M}$ , which was similar to those in HLM, HLC, and human liver homogenates (HLH). Immunoblot analysis revealed ABHD10 protein expression in both HLC and HLM. The AcMPAG deglucuronidation by recombinant ABHD10, HLC, and HLH were potently inhibited by  $\text{AgNO}_3$ ,  $\text{CdCl}_2$ ,  $\text{CuCl}_2$ , PMSF, bis-*p*-nitrophenylphosphate, and DTNB. The  $CL_{\text{int}}$  value of AcMPAG formation from MPA, which was catalyzed by human UGT2B7, in HLH was increased by 1.8-fold in the presence of PMSF. Thus, human ABHD10 would affect the formation of AcMPAG, the immunotoxic metabolite.

The immunosuppressant mycophenolate mofetil (MMF),<sup>2</sup> the prodrug of mycophenolic acid (MPA), is widely used for the

prevention or treatment of acute rejection after solid organ transplantation. The metabolic pathway of MMF is shown in Fig. 1. MMF is given orally, absorbed, and hydrolyzed to MPA, the active form, by carboxylesterases (CES) (1). MPA is mainly metabolized by glucuronidation to its inactive phenolic glucuronide (MPAG) (2). In addition, MPA is also metabolized to MPA acyl-glucuronide (AcMPAG), although the production rate is relatively low compared with that of MPAG (3).

The formations of *O*- or *N*-glucuronides by UDP-glucuronosyltransferases (UGTs) are a common pathway in phase II drug metabolism and are considered as a detoxification process because these glucuronides are generally neither active nor reactive and are excreted rapidly from the body (4). However, it has been reported that acyl-glucuronides can bind covalently to proteins and other macromolecules due to their electrophilicity, suggesting that they are associated with the immunogenicity and toxicity (4). Indeed, AcMPAG promotes the release of TNF- $\alpha$  and IL-6, proinflammatory cytokines, from human mononuclear leukocytes (5). In addition, AcMPAG can bind covalently to ATPase/ATP synthase and protein disulfide isomerase, which are essential for the control of the energy and redox state of the cells (6). Thus, AcMPAG might be responsible for some adverse effects of MMF therapy such as leucopenia or gastrointestinal toxicity.

The acyl-glucuronides are susceptible to deglucuronidation in human tissues and plasma. In general, their deglucuronidation is catalyzed by  $\beta$ -glucuronidase, human serum albumin, and esterases (4). Therefore, deglucuronidation of acyl-glucuronides would play a role in the protection against acyl glucuronides-induced toxicity. It has been reported that tissue esterases are responsible for the deglucuronidation of zomepirac acyl-glucuronide in guinea pig and rabbit based on the find-

\* This work was supported in part by Grant-in-aid for Scientific Research (C) from the Japan Society for the Promotion of Science 23590174.

<sup>§</sup> This article contains supplemental Figs. 1–3.

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<sup>2</sup> The abbreviations used are: MMF, mycophenolate mofetil; ABHD10,  $\alpha/\beta$  hydrolase domain containing 10; AcMPAG, mycophenolic acid acyl-glucuronide; BNPP, bis-*p*-nitrophenylphosphate; CES, carboxylesterase; DFP,

diisopropyl fluorophosphate; D-SL, D-saccharic acid 1,4-lactone; DTNB, 5,5'-dithiobis(2-nitrobenzoic acid); eserin, physostigmine sulfate; HLC, human liver cytosol; DMSO, dimethyl sulfoxide; HLH, human liver homogenates; HLM, human liver microsomes; MPA, mycophenolic acid; MPAG, mycophenolic acid phenolic glucuronide; UDPGA, uridine 5'-diphosphate-glucuronic acid; UGT, UDP-glucuronosyltransferase.

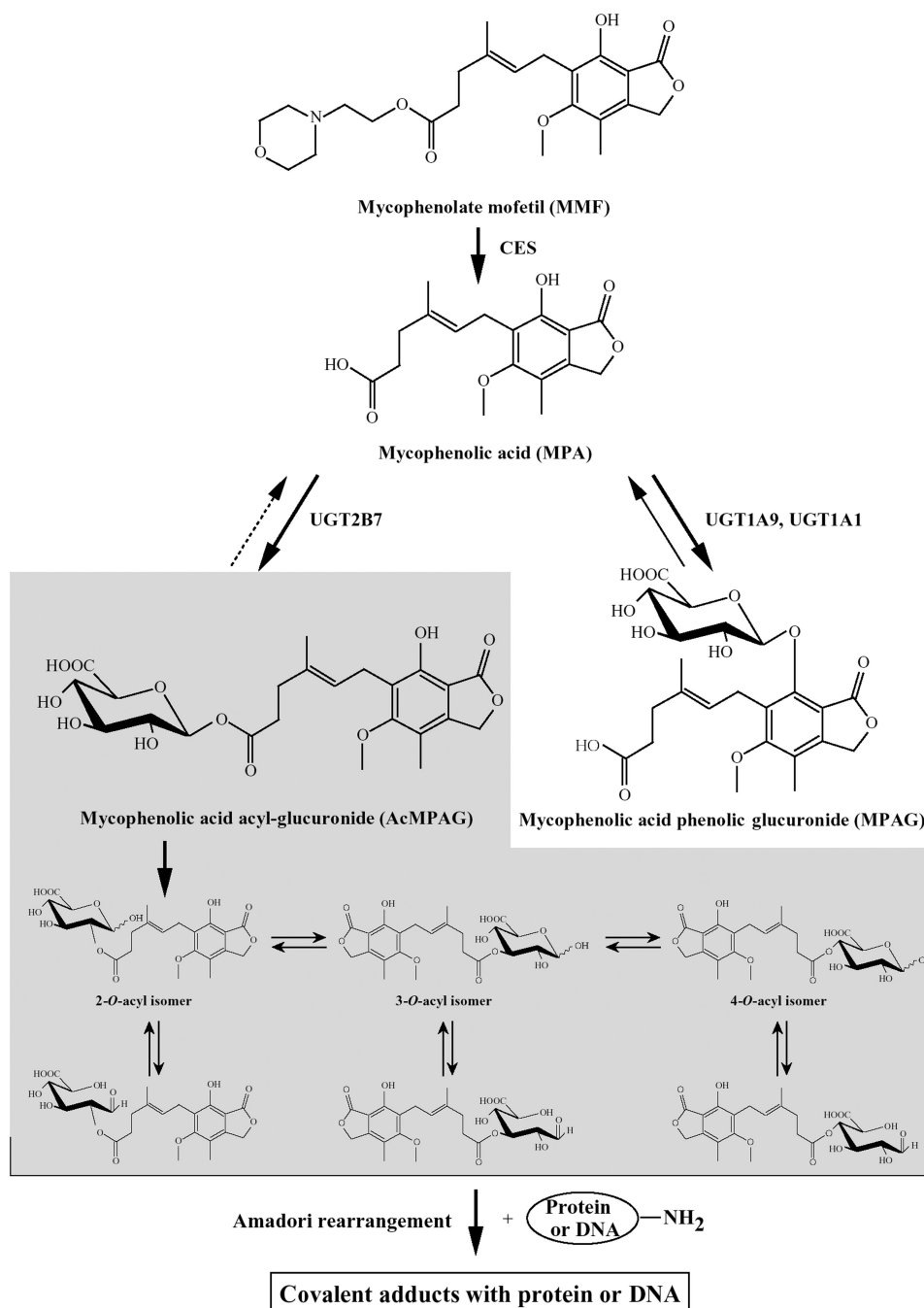


FIGURE 1. Major metabolic pathway of MMF in human.

ing that co-administration of PMSF, a general inhibitor of serine esterases, dramatically decreased the apparent plasma clearance of zomepirac acyl-glucuronide (7). Rowe and Meffin (8) demonstrated that the clearance of clofibrac acid, which is largely excreted as an acyl-glucuronide, was increased by the administration of diisopropyl fluorophosphate (DFP), a serine esterase inhibitor, to rabbits. Recently, it was reported that valproic acid acyl-glucuronide was deglucuronidated by acylpeptide hydrolase (9). As described above, there are some reports about the involvement of tissue esterases in the deglucuronidation of acyl-glucuronide.

In the case of MPA metabolites, it has been reported that  $\beta$ -glucuronidase could exclusively catalyze the MPAG deglucuro-

ronidation, whereas it could not catalyze the AcMPAG deglucuronidation (10). Furthermore, in our preliminary study, human serum albumin could not catalyze the AcMPAG deglucuronidation (data not shown). However, the activity for AcMPAG deglucuronidation was detected in human liver homogenates (HLH) (data not shown). These backgrounds prompted us to investigate whether AcMPAG is deglucuronidated by unknown esterase(s) in human liver. In the present study, we tried to identify and characterize the enzyme responsible for AcMPAG deglucuronidation in human liver. It has been reported that the formation of AcMPAG from MPA in human liver is catalyzed by UGT2B7 (10). If the metabolic potency of AcMPAG deglucuronidation in human liver is relatively high,

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unknown esterase(s) could affect the formation of AcMPAG from MPA. The impact of the identified enzyme on the formation of AcMPAG was also investigated in the present study.

### EXPERIMENTAL PROCEDURES

**Chemicals and Reagents**—MPA, PMSF, DFP, physostigmine sulfate (eserine), disulfiram, silver nitrate ( $\text{AgNO}_3$ ), calcium chloride ( $\text{CaCl}_2$ ), cadmium chloride ( $\text{CdCl}_2$ ), cobaltous chloride ( $\text{CoCl}_2$ ), cupric chloride ( $\text{CuCl}_2$ ), and 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) were purchased from Wako Pure Chemical Industries (Osaka, Japan). AcMPAG and MPAG were purchased from Toronto Research Chemicals (Ontario, Canada). D-Saccharic acid 1,4-lactone (D-SL), bis-*p*-nitrophenylphosphate (BNPP), sodium fluoride (NaF), uridine 5'-diphosphate-glucuronic acid (UDPGA), and alamethicin were obtained from Sigma. Human UGT2B7 Supersomes (recombinant cDNA-expressed UGT enzymes prepared from a baculovirus insect cell system) were purchased from BD Gentest (Woburn, MA). Dulbecco's modified Eagle's medium was from Nissui Pharmaceutical (Tokyo, Japan). Restriction enzymes were from New England Biolabs and Takara Bio (Shiga, Japan). All primers were synthesized commercially at Hokkaido System Sciences (Sapporo, Japan). Other chemicals were of analytical or the highest grade available commercially.

**Enzyme Sources**—For purification of the enzyme(s) responsible for AcMPAG deglucuronidation, human liver cytosol (HLC) was prepared as described previously (11). Human liver samples were supplied by the National Disease Research Interchange (Philadelphia, PA) through the Human and Animal Bridging Research Organization (Chiba, Japan). The use of the human livers was approved by the Ethics Committee of Kanazawa University (Kanazawa, Japan). For other analyses, pooled HLC (prepared from 22 individuals) and pooled human liver microsomes (HLM) (prepared from 50 individuals) purchased from BD Gentest (Woburn, MA) were used. HLH were prepared by homogenizing five human livers in 10 mM Tris-HCl buffer (pH 7.4) containing 20% glycerol and 1 mM EDTA.

**Purification of Enzyme Responsible for AcMPAG Deglucuronidation**—All procedures were carried out at 4 °C. HLC proteins precipitated at between 50 and 70% saturated ammonium sulfate were dissolved in 10 mM Tris-HCl buffer (pH 6.0) containing 1 mM EDTA and then dialyzed against the same buffer to remove the ammonium sulfate. The dialyzed fraction was applied to a CM Sepharose Fast Flow cation exchange column (2.5 × 4.0 cm; GE Healthcare) equilibrated with 10 mM Tris-HCl buffer (pH 6.0) containing 1 mM EDTA. The proteins were eluted by stepwise additions of the equilibration buffer containing 0.1, 0.5, and 1.0 M KCl at 0.5 ml/min. Each eluted fraction (5 ml) was collected, and the protein concentration was monitored by absorbance at 280 nm, and the activity for AcMPAG deglucuronidation was monitored by a method described later. The fractions with enzyme activity (fractions 27 and 28) were pooled and dialyzed against 25 mM bis-Tris-HCl buffer (pH 7.1). The dialyzed fraction was loaded on a Mono P 5/200 GL Chromatofocusing column (GE Healthcare) equilibrated with 25 mM bis-Tris-HCl buffer (pH 7.1). The column was eluted at 0.5 ml/min with 10-fold diluted Polybuffer 74 (GE Healthcare) adjusted to pH 4.0 by HCl. Each eluted fraction (1.0

ml) was collected, and the fractions with AcMPAG deglucuronidation activity (fractions 18–20) were pooled and applied to a Superdex 200 10/300 GL gel filtration column (GE Healthcare) equilibrated with 50 mM potassium phosphate buffer (pH 7.4) containing 150 mM NaCl. Proteins were eluted with the same buffer at 0.25 ml/min. The AcMPAG deglucuronidation activity was determined in each eluted fraction (0.5 ml), and the fractions with enzyme activity (fractions 31 and 32) were pooled. The protein concentrations in the Mono P fractions and the Superdex 200 fractions were determined according to Bradford (12).

**Gel Electrophoresis**—SDS-PAGE was performed according to the method described previously with slight modifications (13). Proteins were separated on 15% polyacrylamide gel. After electrophoresis, the gels were stained with 0.05% Coomassie Brilliant Blue or a silver staining kit (Cosmo Bio, Tokyo, Japan).

**In-gel Digestion and Mass Spectrometric Identification of Proteins**—In-gel digestion and mass spectrometric identification of proteins were performed using methods reported previously (13). The protein spot was excised from the dried Coomassie Brilliant Blue-stained SDS-polyacrylamide gel using clean scalpels. Identification of proteins was carried out using MASCOT software (Matrix Science, Ltd., London, UK) with the National Center for Biotechnology Information database. These procedures and the amino acid sequence analysis were performed by Pro Phoenix Co. (Hiroshima, Japan).

**Construction of Expression System for Human ABHD10 in Sf9 Cells**—Expression of human ABHD10 using a Bac-to-Bac Baculovirus Expression System (Invitrogen) was carried out according to the manufacturer's protocol. The full-length human ABHD10 cDNA was obtained by RT-PCR using a human liver RNA sample with the following primer sets: ABHD10 sense primer (5'-ACTACGAAGATGGCGGTTGC-3') and antisense primer (5'-CAACTAAACATGTGATAC-TAG-3'). The PCR product was subcloned into pTARGET mammalian expression vector (Promega, Madison, WI). The nucleotide sequences (ABHD10, accession no. NM\_018394.2) were confirmed by DNA sequence analysis (Long-Read Tower DNA sequencer; GE Healthcare). The ABHD10 cDNA in the pTARGET vector was transferred into the pFastBac1 vector using appropriate restriction enzymes. The pFastBac1 vector containing ABHD10 cDNA was transformed into DH10Bac-competent cells, followed by transposition of the inserts into bacmid DNA. Nonrecombinant bacmid DNA (mock) was also prepared by the same procedures.

**Spodoptera frugiperda Sf9 cells** (Invitrogen) were grown in Sf-900 II SFM containing 10% fetal bovine serum at 27 °C. The recombinant bacmid DNA was transfected into Sf9 cells with Cellfectin Reagent (Invitrogen), and the virus was harvested by collecting the cell culture medium at 72-h post-transfection. Cells were harvested routinely 72 h after infection, washed twice with PBS, and stored at –80 °C until use. Cell homogenates were prepared by suspending in TGE buffer (10 mM Tris-HCl (pH 7.4), 20% glycerol, 1 mM EDTA) and by disrupting by freeze-thawing three times. Then, the suspensions were homogenized with a Teflon-glass homogenizer for 10 strokes. The ABHD10 expression was confirmed with immunoblot analysis as described below.

**Immunoblot Analysis**—SDS-PAGE and immunoblot analysis were performed according to Laemmli (14). Enzyme sources (total cell homogenates from Sf9 cells expressing ABHD10 (4  $\mu$ g); HLM and HLC (40  $\mu$ g)) were separated on 10% polyacrylamide gels and electrotransferred onto polyvinylidene difluoride membrane, Immobilon-P (Millipore, Billerica, MA). The membranes were probed with polyclonal mouse anti-human ABHD10 (Abnova, Taipei, Taiwan), and the corresponding fluorescent dye-conjugated second antibody and an Odyssey infrared imaging system (LI-COR Biosciences, Lincoln, NE) were used for detection. The relative expression level was quantified using ImageQuant TL Image Analysis software (GE Healthcare).

**AcMPAG Deglucuronidation Activity**—AcMPAG deglucuronidation activity was determined as follows: a typical incubation mixture (final volume of 0.2 ml) contained 100 mM Tris-HCl (pH 7.4), and various enzyme sources (ammonium sulfate precipitation fraction, 1.0 mg/ml; CM Sepharose fraction, 10  $\mu$ l; Mono P fraction, 20  $\mu$ l; Superdex 200 fraction, 100  $\mu$ l; HLM, 0.4 mg/ml; HLC and HLH, 1.0 mg/ml; Sf9 cell homogenates expressing ABHD10, 0.025 mg/ml). In the preliminary study, we confirmed that the rate of formation of MPA was linear with respect to the protein concentration (<1.5 mg/ml) and incubation time (<90 min). AcMPAG was dissolved in dimethyl sulfoxide (DMSO), and the final concentration of DMSO in the incubation mixture was 1.0%. The reaction was initiated by the addition of 50  $\mu$ M AcMPAG after a 2-min preincubation at 37 °C. After the 60-min incubation at 37 °C, the reaction was terminated by the addition of 20  $\mu$ l of 60% metaphosphoric acid. After removal of the protein by centrifugation at  $9,500 \times g$  for 5 min, a 50- $\mu$ l portion of the supernatant was subjected to HPLC. HPLC analysis was performed using an L-2130 pump (Hitachi, Tokyo, Japan), an L-2200 autosampler (Hitachi), an L-2400 UV detector (Hitachi), and a D-2500 chromatointegrator (Hitachi) equipped with an Inertsil ODS-3 column (5- $\mu$ m particle size, 4.6 mm, inner diameter  $\times$  250 mm; GL Sciences Inc., Tokyo, Japan). The eluent was monitored at 215 nm. The mobile phase was 35% acetonitrile containing 0.1% perchloric acid. The flow rate was 1.0 ml/min. The column temperature was 35 °C. The quantification of MPA was performed by comparing the HPLC peak height with that of an authentic standard. Because AcMPAG is partially deglucuronidated nonenzymatically, the content of MPA in the mixture incubated without the enzyme sources was subtracted from that with the enzyme sources. The activity in each concentration was determined as the mean value in triplicate.

Kinetic analyses of AcMPAG deglucuronidation were performed at ranges of 25–1,000  $\mu$ M. The parameters were estimated from the fitted curves using a computer program (KaleidaGraph; Synergy Software, Reading, PA) designed for nonlinear regression analysis.

**Inhibition Studies on AcMPAG Deglucuronidation Activity**—To clarify the involvement of various esterases in the AcMPAG deglucuronidation, inhibition analysis of its deglucuronidation in human liver was performed by using representative esterase inhibitors. D-SL is a  $\beta$ -glucuronidase inhibitor (15). Organophosphates such as BNPP and DFP are known as general CES inhibitors (16, 17). PMSF is a general serine hydrolase inhibitor

(18). Eserine and NaF are cholinesterase inhibitors (19, 20). Eserine is also a potent arylacetamide deacetylase inhibitor (21). *N*-Ethylmaleimide is a cysteine protease inhibitor (22). Disulfiram was reported to be a monoacylglycerol lipase inhibitor (23). DTNB is an arylesterase inhibitor (24). Because heavy metals are used frequently for esterase inhibition studies, AgNO<sub>3</sub>, CaCl<sub>2</sub>, CdCl<sub>2</sub>, CoCl<sub>2</sub>, and CuCl<sub>2</sub> were also used as inhibitors. BNPP, PMSF, disulfiram, and DTNB were dissolved in DMSO so that the final concentration of DMSO in the incubation mixture was 1.5%. Other inhibitors were dissolved in distilled water. The experimental procedure and condition were the same as above. It was confirmed that 1.5% DMSO did not inhibit AcMPAG deglucuronidation, and the control activity was determined in the presence of 1.5% DMSO.

**Kinetic Analyses of AcMPAG Formation from MPA**—The AcMPAG formation from MPA was determined as follows: a typical incubation mixture (200  $\mu$ l of total volume) contained 100 mM Tris-HCl buffer (pH 7.4), 10 mM MgCl<sub>2</sub>, 2.5 mM UDPGA, 25  $\mu$ g/ml alamethicin, various enzyme sources (HLH, 1.0 mg/ml; UGT2B7 Supersomes, 0.4 mg/ml), and 25–2,000  $\mu$ M MPA. To investigate the effect of the ABHD10 inhibition, 1 mM PMSF was added. The reaction was initiated by the addition of UDPGA following a 2-min preincubation at 37 °C. After incubation at 37 °C for 30 min, the reaction was terminated by the addition of 20  $\mu$ l of 60% metaphosphoric acid. After the removal of the protein by centrifugation at  $9,500 \times g$  for 5 min, a 50- $\mu$ l portion of the sample was subjected to HPLC. The parameters were estimated from the fitted curves using a computer program as described above.

**Statistical Analysis**—Data are expressed as mean  $\pm$  S.D. Statistical significance between two groups was determined by two-tailed Student's *t* test. A value of *p* < 0.05 was considered statistically significant.

## RESULTS

**AcMPAG Deglucuronidation in Human Liver Preparations**—The AcMPAG deglucuronidation activities at 50  $\mu$ M AcMPAG in HLM, HLC, and HLH are shown in Fig. 2. Typical HPLC chromatograms are shown in supplemental Fig. 1. All of liver preparations showed AcMPAG deglucuronidation activity (HLM,  $0.32 \pm 0.01$  nmol/min/mg; HLC,  $0.27 \pm 0.01$  nmol/min/mg; HLH,  $0.18 \pm 0.01$  nmol/min/mg). The activities in all preparations were not inhibited by D-SL but were significantly inhibited by PMSF (Fig. 2). These results suggested that the AcMPAG deglucuronidation would be catalyzed by serine esterase(s), not by  $\beta$ -glucuronidase.

**Purification and Identification of Enzyme Responsible for AcMPAG Deglucuronidation in Human Liver**—The recovery of protein, the AcMPAG deglucuronidation activity, and the protein staining profiles in each step of the purification procedure are shown in Table 1 and Fig. 3A. HLC was first fractionated by the ammonium sulfate precipitation method. The 50–70% ammonium sulfate-saturated fraction with the high AcMPAG deglucuronidation activity was separated using a CM Sepharose cation exchange column. By monitoring the eluent at an absorbance of 280 nm and the AcMPAG deglucuronidation activity, fractions 27 and 28 eluted from the CM Sepharose cation exchange column exhibited the

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highest activities. After the pooled fraction (fractions 27 and 28) was dialyzed, it was loaded on a Mono P chromatofocusing column. Similarly, the active fraction (fractions 18–20) eluted from the Mono P chromatofocusing column was further separated with a Superdex 200 gel filtration column. The fractions 30–34 exhibited the activity with the highest activity in the fractions 31 and 32 (9.54 nmol/min/mg protein in their pooled fraction: Table 1 and Fig. 3B). In these fractions, six proteins were detected by SDS-PAGE and silver staining (Fig. 3A). Among them, the band intensity of only an ~28-kDa protein was correlated with the AcMPAG deglucuronidation activity (Fig. 3B). Accordingly, 55.05-fold purification with a 0.65% yield compared with the initial HLC fraction was achieved (Table 1).

By the amino acid sequence analysis, the 28-kDa protein was identified as ABHD10 precursor (accession no. NP\_060864). As shown in Fig. 4, peptide fragments identical to the ABHD10 sequence are represented in boldface type (ABHD10: 51–70, 224–242, 264–275). Thus, it is suggested that ABHD10 could catalyze the AcMPAG deglucuronidation in human liver.

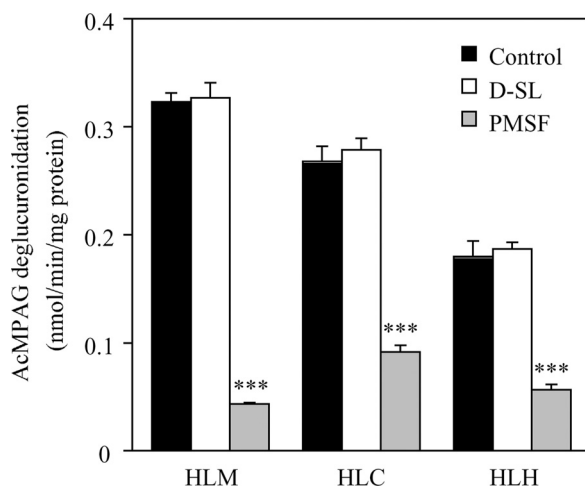
**AcMPAG Deglucuronidation by Recombinant Human ABHD10**—To confirm that human ABHD10 can catalyze the AcMPAG deglucuronidation, homogenates of Sf9 cells expressing human ABHD10 were constructed to use as a recombinant ABHD10. Immunoblot analysis for human ABHD10 protein showed that it was specifically expressed in

Sf9 cells infected with recombinant baculovirus for ABHD10 (Fig. 5A). Two protein bands (28 and 34 kDa) were detected in HLC and recombinant ABHD10, whereas only a 28-kDa protein band was detected in HLM. The AcMPAG deglucuronidation activities were determined at a concentration of 50  $\mu\text{M}$  AcMPAG (Fig. 5B), with the result that recombinant ABHD10 showed the activity ( $15.22 \pm 0.77$  nmol/min/mg protein) that was much higher than that by mock ( $0.03 \pm 0.01$  nmol/min/mg protein). Furthermore, the activity by recombinant ABHD10 was higher than those in HLM and HLC ( $0.32 \pm 0.01$  nmol/min/mg protein and  $0.27 \pm 0.01$  nmol/min/mg protein, respectively), and these activities were correlated with the band intensities of the 28-kDa protein. Thus, these results suggested that human ABHD10 could catalyze the AcMPAG deglucuronidation.

**Kinetic Analyses of AcMPAG Deglucuronidation Activity in Human Liver Preparations and Recombinant ABHD10**—The kinetic parameters of AcMPAG deglucuronidation between recombinant human ABHD10 and human liver preparations were compared (Fig. 6 and Table 2). For recombinant ABHD10, the  $K_m$  and  $V_{max}$  values were  $100.7 \pm 10.2$   $\mu\text{M}$  and  $47.6 \pm 3.9$  nmol/min/mg, respectively, resulting in a  $CL_{int}$  value of  $473.3 \pm 11.9$   $\mu\text{l}/\text{min}/\text{mg}$ . The  $K_m$  values of human liver preparations (HLM,  $144.4 \pm 9.2$   $\mu\text{M}$ ; HLC,  $154.8 \pm 10.6$   $\mu\text{M}$ ; HLH,  $173.7 \pm 9.1$   $\mu\text{M}$ ) were similar to that of recombinant ABHD10 (Table 2), which suggested that human ABHD10 may be the responsible enzyme for AcMPAG deglucuronidation in human liver.

**Effects of Chemical Inhibitors on AcMPAG Deglucuronidation in Human Liver Preparations and Recombinant ABHD10**—To investigate whether ABHD10 is the principal enzyme for AcMPAG deglucuronidation in human liver, the effects of various inhibitors on AcMPAG deglucuronidation by recombinant ABHD10 and human liver preparations were analyzed (Fig. 7). The AcMPAG deglucuronidation activity by recombinant ABHD10 was efficiently inhibited by BNPP, PMSF, and DTNB in a concentration-dependent manner (Fig. 7A). In addition, this activity was potently inhibited by 0.1 and 1 mM  $\text{AgNO}_3$  and 1 mM  $\text{CdCl}_2$  and  $\text{CuCl}_2$ . Although the inhibitory characteristic in HLM was slightly different from that in recombinant ABHD10 (Fig. 7B), those in HLC and HLH were similar to that in recombinant ABHD10 (Fig. 7, C and D). These results also implied that ABHD10 is the major esterase responsible for the AcMPAG deglucuronidation in human liver.

**Kinetic Analyses of AcMPAG Formation from MPA in Human Liver Preparations and Recombinant UGT2B7**—To investigate the impact of AcMPAG deglucuronidation by ABHD10 on the formation of AcMPAG from MPA, the



**FIGURE 2. AcMPAG deglucuronidation in human liver preparations and the effects of inhibitors on their activities.** Activity for AcMPAG deglucuronidation was measured by quantitative analysis of MPA by HPLC. Black, white, and gray columns indicate the activities with 0.5% DMSO (control), 10 mM D-SL, and 1 mM PMSF, respectively. Human liver preparations (HLM, 0.4 mg/ml; HLC and HLH, 1.0 mg/ml) were incubated with 50  $\mu\text{M}$  AcMPAG for 60 min. Each column represents the mean  $\pm$  S.D. of triplicate determinations. \*\*\*,  $p < 0.001$  compared with control.

**TABLE 1**  
Purification of the enzyme involved in AcMPAG deglucuronidation in HLC

Step	Volume	Total protein	Total activity <sup>a</sup>	Specific activity <sup>a</sup>	Purification	Recovered activity
	<i>ml</i>	<i>mg</i>	<i>nmol/min</i>	<i>nmol/min/mg</i>	<i>fold</i>	<i>%</i>
Cytosol	20.0	763.6	132.4	0.17	1.0	100.0
50–70% $(\text{NH}_4)_2\text{SO}_4$	15.0	457.1	88.4	0.19	1.1	66.8
CM Sepharose	10.0	80.3	19.3	0.24	1.4	14.6
Mono P	3.0	1.3	3.8	3.03	17.5	2.9
Superdex 200	1.0	0.1	0.9	9.54	55.1	0.7

<sup>a</sup> Activity for AcMPAG deglucuronidation measured by quantitative analysis of MPA by HPLC.

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1 MAVARLAAVA AWVPCRSWGW AAVFPGPHRG LSVLLARIPO RAPRWLPACR  
**QK****TSL****SFL****LN**R **DDL****PN****L****AY****KK** LKGKSPGIIF IPGYLSYMNG TKALAIIEFC 100  
 KSLGHACIRF DYSGVGSDDG NSEESTLGKW RKDVLSDIIDD LADGPQIILV  
 SSLGGWLMH AAIARPEKVV ALIGVATAAD TLVTKFNQLP VELKKEVEMK 200  
 GVWSMPSKYS EEGVYINVQYS FIK**EA****EH****H****CL** **LH****S****P****I****P****V****N****C**P IRLHLGMMKDD  
 IVPWHTSMQV ADR**VL****ST****D****V**D **V****IL****R****K****S****D****H****R** MREKADIQLL VYTIDDLIDK 300  
 LSTIVN 306

FIGURE 4. **Amino acid sequence of human ABHD10.** Three peptide fragments of the purified enzyme identical to ABHD10 (accession no. NP\_060864) are represented in boldface type. The putative transit peptide of ABHD10 is underlined.

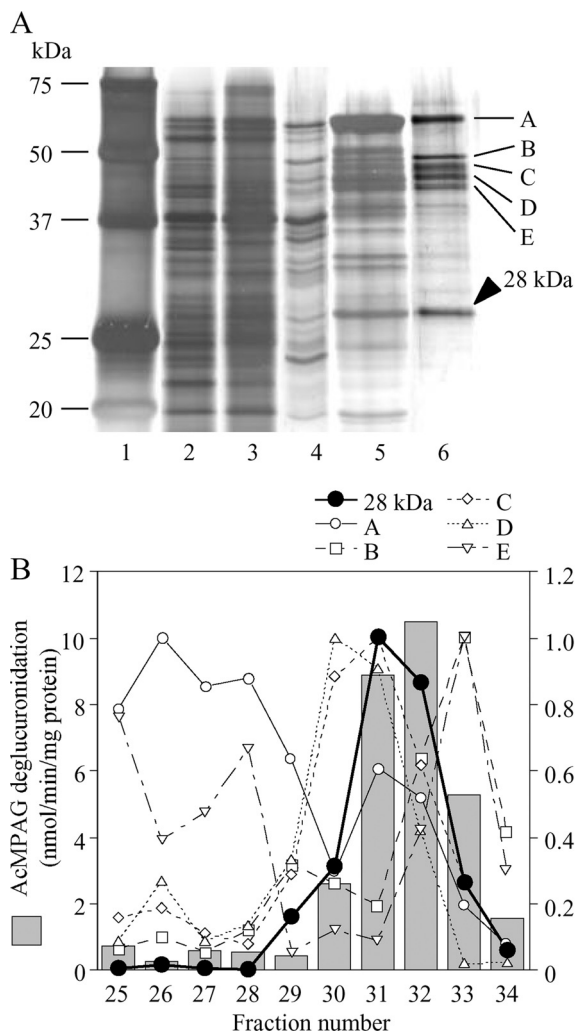


FIGURE 3. **Purification of the enzyme responsible for AcMPAG deglucuronidation.** *A*, protein profiles of HLC fractionated by ammonium sulfate and column chromatographies. The proteins were separated by SDS-PAGE. After electrophoresis, proteins were visualized by silver staining: lane 1, molecular weight standard; lane 2, HLC (10  $\mu$ g of protein); lane 3, 50–70% ammonium sulfate precipitated fraction (10  $\mu$ g of protein); lane 4, CM Sepharose fraction (5  $\mu$ g of protein); lane 5, Mono P fraction (2  $\mu$ g of protein); lane 6, Superdex 200 fraction (1  $\mu$ g of protein; pooled sample of fractions 29–33 in *B*). The arrowhead indicates the 28-kDa band correlated with the activity for AcMPAG deglucuronidation. *B*, activity for AcMPAG deglucuronidation in the fractions eluted from Superdex 200 and relative intensities of the six protein (A–E and 28 kDa) bands shown in Fig. 2*A*. The activities of each fraction are indicated with columns. Relative intensities of the six protein bands are indicated by filled circle (28 kDa), open circle (A), square (B), diamond (C), triangle (D), and inverted triangle (E), respectively. To determine the activity for AcMPAG deglucuronidation, 100  $\mu$ l of the fractions were incubated with 50  $\mu$ M AcMPAG for 60 min. Activity for AcMPAG deglucuronidation was measured by quantitative analysis of MPA by HPLC. The intensities of each protein band were represented as relative to the highest intensity value in each protein band.

activities of the AcMPAG formation from MPA in HLH and UGT2B7 Supersomes were measured with 1 mM PMSF (Fig. 8). Typical HPLC chromatograms are shown in supplemental Fig. 2. In the preliminary study, it was confirmed that UGT2B7 Supersomes showed extremely low deglucuronidation activity ( $0.04 \pm 0.00$  nmol/min/mg at 50  $\mu$ M AcMPAG). In HLH, the  $K_m$  value was significantly decreased but the  $V_{max}$  value was not changed by 1 mM PMSF, resulting in a 1.8-fold increase in the  $CL_{int}$  value (Table 3). In contrast, the

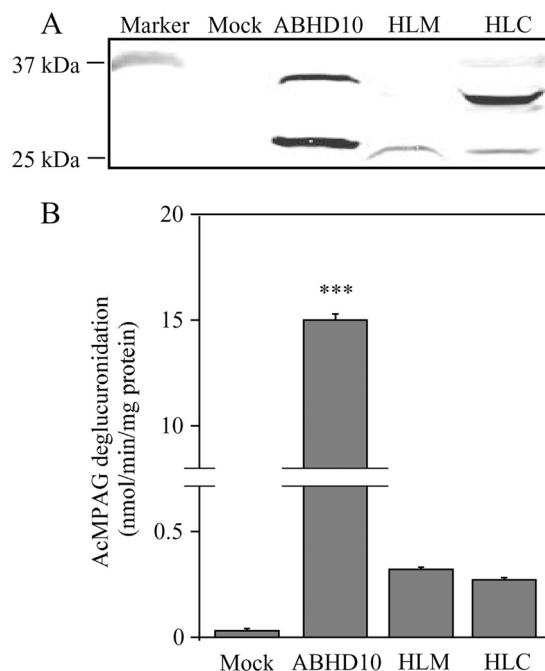


FIGURE 5. **AcMPAG deglucuronidation by recombinant human ABHD10.** *A*, immunoblot analysis of recombinant human ABHD10 expressed in Sf9 cells, HLM, and HLC. Total cell homogenates from Sf9 cells expressing ABHD10 (4  $\mu$ g), HLM and HLC (40  $\mu$ g) were separated by electrophoresis using 10% SDS-polyacrylamide gel. ABHD10 protein was detected by mouse anti-human ABHD10 polyclonal antibody. *B*, activity for AcMPAG deglucuronidation by recombinant ABHD10, HLM, and HLC measured by quantitative analysis of MPA by HPLC. The homogenates of Sf9 cells expressing ABHD10, HLM, and HLC were incubated with 50  $\mu$ M AcMPAG for 60 min. Each column represents the mean  $\pm$  S.D. of triplicate determinations. \*\*\*,  $p < 0.001$  compared with mock.

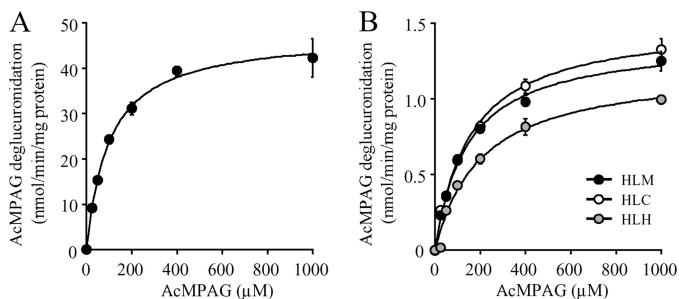
AcMPAG formation from MPA by recombinant UGT2B7 was not inhibited by PMSF. These results suggested that ABHD10 was important in the formation of AcMPAG in human liver.

## DISCUSSION

MPA is metabolized by glucuronidation to AcMPAG as well as MPAG, and it has been suggested that AcMPAG is an immunotoxic metabolite that plays a role in MMF-induced adverse effects (25). Therefore, the AcMPAG deglucuronidation may be a detoxification pathway. In the present study, we first found that AcMPAG was deglucuronidated by human ABHD10.

AcMPAG was deglucuronidated efficiently in all human liver preparations, HLM, HLC, and HLH (Fig. 2). The deglucuronidation in these preparations was inhibited by PMSF, a general inhibitor of serine esterases, but was not inhibited by D-SL, a

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**FIGURE 6. Kinetic analyses of AcMPAG deglucuronidation.** Kinetic analyses of AcMPAG deglucuronidation by recombinant human ABHD10 expressed in Sf9 cells (A) and human liver preparations (HLM, HLC, and HLH) (B). Recombinant ABHD10 protein (0.025 mg/ml) or human liver preparations (HLM, 0.4 mg/ml; HLC and HLH, 1.0 mg/ml) were incubated with AcMPAG for 60 min. Activity for AcMPAG deglucuronidation was measured by quantitative analysis of MPA by HPLC. Each data point represents the mean  $\pm$  S.D. of triplicate determinations.

**TABLE 2**

**Kinetic parameters of AcMPAG deglucuronidation in human liver preparations and recombinant ABHD10**

Data are mean  $\pm$  S.D. ( $n = 3$ ). Activity for AcMPAG deglucuronidation was measured by quantitative analysis of MPA by HPLC.

Enzyme source	$K_m$ $\mu\text{M}$	$V_{\text{max}}$ $\text{nmol}/\text{min}/\text{mg}$	$CL_{\text{int}}$ $\mu\text{l}/\text{min}/\text{mg}$
HLM	$144.4 \pm 9.2$	$1.4 \pm 0.1$	$9.7 \pm 0.5$
HLC	$154.8 \pm 10.6$	$1.5 \pm 0.1$	$9.8 \pm 0.3$
HLH	$173.7 \pm 9.1$	$1.2 \pm 0.0$	$6.7 \pm 0.4$
Recombinant ABHD10	$100.7 \pm 10.2$	$47.6 \pm 3.9$	$473.3 \pm 11.9$

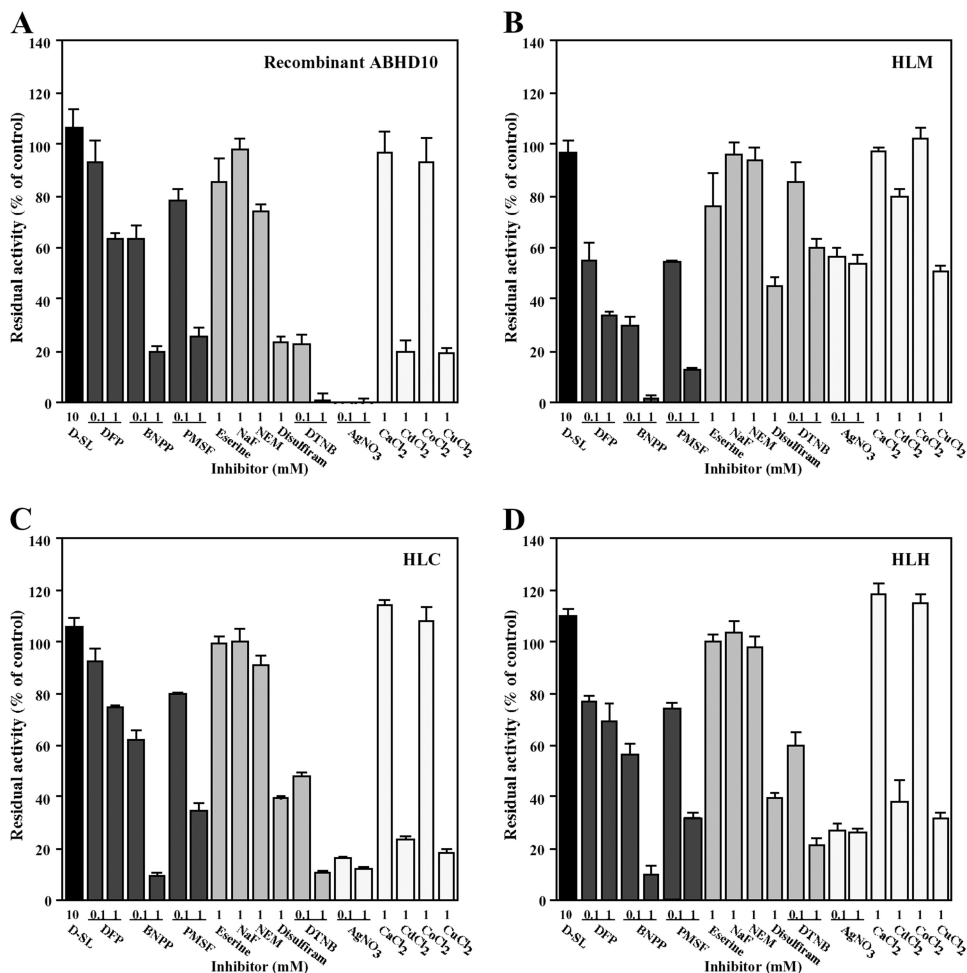
$\beta$ -glucuronidase inhibitor, which result was consistent with a previous report that AcMPAG was not deglucuronidated by  $\beta$ -glucuronidase (10). However, when we confirm the deglucuronidation by incubating 50  $\mu\text{M}$  AcMPAG with 100 units of recombinant  $\beta$ -glucuronidase from *Helix pomatica* (Sigma) for 1 h, a large amount of AcMPAG ( $82.9 \pm 9.5\%$ ) was deglucuronidated to MPA (data not shown). Our observation was different from the previous report (10), but the involvement of  $\beta$ -glucuronidase in human liver could be excluded from the fact that the AcMPAG deglucuronidation in human liver preparations was not inhibited by D-SL (Fig. 2). HLM and HLC showed similar AcMPAG deglucuronidation activity ( $0.32 \pm 0.01$  nmol/min/mg and  $0.27 \pm 0.01$  nmol/min/mg at 50  $\mu\text{M}$  AcMPAG, respectively) (Fig. 2). Suzuki *et al.* (9) found that acylpeptide hydrolase catalyzes the deglucuronidation of valproic acid acylglucuronide, but its involvement in AcMPAG deglucuronidation could be excluded because the acylpeptide hydrolase activity in HLC is much higher than that in HLM. The contribution of HLC to the AcMPAG deglucuronidation would be higher than that of HLM because liver S9 contains 5-fold more cytosolic protein than microsomal protein (13). Therefore, in the present study, the enzyme catalyzing the AcMPAG deglucuronidation was purified from HLC, and ABHD10 was identified by amino acid sequence analysis (Figs. 3 and 4). The subsequent study using recombinant ABHD10 expressed in Sf9 cells revealed that AcMPAG was deglucuronidated efficiently by ABHD10 (Fig. 5B). In the process of purification using Superdex 200, several proteins were detected in the active fractions (Fig. 3A). Among them, a 60-kDa protein (*band A* in Fig. 3A) was identified as CES1A1 by amino acid sequence analysis (data not shown). However, it was confirmed that recombinant

CES1A1, constructed in our previous study (26), could not catalyze the AcMPAG deglucuronidation (data not shown). The other proteins detected in SDS-PAGE (*bands B–E* in Fig. 3A) were also not correlated with the activities for AcMPAG deglucuronidation (Fig. 3B).

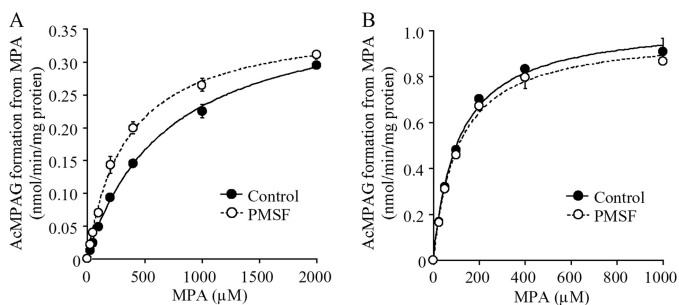
The ABHD gene family is a small group belonging to the  $\alpha/\beta$  hydrolase superfamily, and it contains  $\alpha/\beta$  hydrolase fold (27). In human, 17 ABHD genes have been identified, but most of them have not been annotated functionally. Mouse Abhd4 was proved to be involved in an alternative synthesis pathway of *N*-acyl ethanolamines (28). Mutations in the human ABHD5 gene, which is also known as CGI-58 (comparative gene identification-58), would be associated with Chanarin-Dorfman syndrome, an autosomal recessive disorder of neutral lipid metabolism (29). However, the involvement of ABHD enzymes in drug metabolism as well as the function of ABHD10 has never been clarified.

In the immunoblot analysis, two proteins of different molecular weight, 28- and 34-kDa proteins, were detected in HLC and recombinant ABHD10, although only the 28-kDa protein was detected in HLM (Fig. 5A). Generally, the signal peptide on the N-terminal end is suggested to play an important role in the localization of protein in mammalian cells (30). According to the National Center for Biotechnology Information data base (accession no. Q9NUJ1), 52 amino acids on the N-terminal end and the residual 254 amino acids of human ABHD10 precursor are regarded as the transit peptide and mature chain, respectively. In general, the transit peptide is a signal peptide consisting of 6–100 amino acids, and the precursor protein is cut off after translocation to the target organelle (31). Therefore, the 34- and 28-kDa proteins might correspond to a precursor form containing a transit peptide and a mature form, respectively. However, we could not find the experimental evidence about transit peptide of human ABHD10. In addition, the peptide fragment in the amino acid sequence analysis of 28-kDa protein was overlapped with the putative transit peptide (Fig. 4). Therefore, further studies are needed to clarify the mechanism of ABHD10 cleavage. Among esterases, CES is also known to be expressed in both HLM and HLC (32, 33). Although it was suggested that CES1A precursor proteins become CES1A mature proteins by cleavage at the signal peptide in the N-terminal end after translocation to the endoplasmic reticulum (33, 34), the mechanism of intracellular translocation remains unknown due to the expression in HLC (13). Similarly, a difference in the ABHD10 protein expression profiles between HLM and HLC was observed (Fig. 5A), but the expression level of mature protein (28-kDa protein) coincided with the AcMPAG deglucuronidation activity. In addition, the precursor protein (34-kDa protein) was not purified as the enzyme catalyzing AcMPAG deglucuronidation in the present study. These observations implied that only the mature protein has the ability for AcMPAG deglucuronidation.

Human ABHD10 has the nucleophile-His-acid catalytic triad (Ser<sup>152</sup>, Asp<sup>249</sup>, and His<sup>279</sup>) as does CES (36), suggesting that ABHD10 could hydrolyze compounds with ester or amide bound by the same catalytic mechanism as CES. Among phenolic, amine, and acyl glucuronides, only acyl glucuronides contain ester bound. In fact, we found that the recombinant



**FIGURE 7. Effects of chemical inhibitors on AcMPAG deglucuronidation.** Activity for AcMPAG deglucuronidation by recombinant human ABHD10 expressed in Sf9 cells (A), HLM (B), HLC (C), and HLH (D) at 50  $\mu\text{M}$  AcMPAG was measured by quantitative analysis of MPA by HPLC. The control activity values were 9.97 (A), 0.32 (B), 0.19 (C), and 0.17 (D) nmol/min/mg protein, respectively. Each column represents the mean  $\pm$  S.D. of triplicate determinations. NEM, N-ethylmaleimide.



**FIGURE 8. Kinetic analyses of the AcMPAG formation from MPA.** Kinetic analyses of the AcMPAG formation from MPA in HLH (A) and UGT2B7 Supersomes (B) in the presence (open circle) or absence (filled circle) of 1 mM PMSF. HLH (1.0 mg/ml) or UGT2B7 Supersomes (0.4 mg/ml) were incubated with MPA for 30 min. Activity for AcMPAG formation from MPA was measured by quantitative analysis of AcMPAG by HPLC. Control groups were treated with 0.5% DMSO. Each data point represents the mean  $\pm$  S.D. of triplicate determinations.

human ABHD10 could catalyze the deglucuronidation of AcMPAG, but could not catalyze the deglucuronidation of MPAG, a phenolic glucuronide of MPA (data not shown). Thus, human ABHD10 is unlikely to catalyze phenolic and amine glucuronides. Two glucosides, mycophenolic acid phenolic glucoside and mycophenolic acid acyl-glucoside,

are also known as the minor metabolites of MPA (10). Because mycophenolic acid acyl-glucoside contains ester bound, it would be interesting to analyze the involvement of human ABHD10 in its hydrolysis.

To evaluate the involvement of ABHD10 in AcMPAG deglucuronidation, kinetic analysis was performed using human liver preparations and recombinant ABHD10. As shown in Fig. 6 and Table 2, the  $K_m$ ,  $V_{max}$  and  $CL_{int}$  values were similar among human liver preparations, and their  $K_m$  values were similar to that by the recombinant ABHD10 ( $100.7 \pm 10.2 \mu\text{M}$ ). Thus, it was conceivable that ABHD10 was responsible for the AcMPAG deglucuronidation in human liver. To further confirm the involvement of ABHD10 in the AcMPAG deglucuronidation, inhibition analyses using various chemical inhibitors were performed (Fig. 7). ABHD10 is a serine esterase, therefore the AcMPAG deglucuronidations in human liver preparations and recombinant ABHD10 were inhibited by serine esterase inhibitors such as BNPP and PMSF in a concentration-dependent manner. DFP is also known as a serine esterase inhibitor, but the AcMPAG deglucuronidations were not inhibited strongly by DFP. The present study used various inhibitors, but their inhibitory effects in HLC, HLH, and recombinant ABHD10 were a little different from that in HLM.



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**TABLE 3**

**Kinetic parameters of AcMPAG formation in HLH and recombinant UGT2B7**

Data are mean  $\pm$  S.D. ( $n = 3$ ; \*\*,  $p < 0.01$  and \*\*\*,  $p < 0.001$  compared with control (DMSO)). The activity for AcMPAG formation from MPA was measured by quantitative analysis of AcMPAG by HPLC.

Enzyme source	$K_m$ $\mu\text{M}$	$V_{\text{max}}$ $\text{nmol/min/mg}$	$CL_{\text{int}}$ $\mu\text{l/min/mg}$	Increased ratio of $CL_{\text{int}}$ <i>Fold</i>
<b>HLH</b>				
Control	623.0 $\pm$ 18.6	0.39 $\pm$ 0.01	0.57 $\pm$ 0.02	
PMSF	352.2 $\pm$ 35.2***	0.37 $\pm$ 0.01	1.04 $\pm$ 0.11**	1.84
<b>Recombinant UGT2B7</b>				
Control	109.9 $\pm$ 7.1	1.04 $\pm$ 0.05	9.46 $\pm$ 0.16	
PMSF	107.6 $\pm$ 1.6	0.99 $\pm$ 0.03	9.18 $\pm$ 0.34	0.97

Our previous study (37) found that the inhibitory effects of some drugs on the CES enzyme activity were different between microsomes and cytosol in human liver and jejunum. These differences may be due to the characteristic difference between membrane binding and soluble proteins. Collectively, we thought that ABHD10 is the major esterase responsible for the AcMPAG deglucuronidation in human liver.

In a previous report (10), the  $CL_{\text{int}}$  value of the AcMPAG formation from MPA in HLM was calculated to be 0.38  $\mu\text{l/min/mg}$ . In contrast, the  $CL_{\text{int}}$  values of the AcMPAG deglucuronidation in HLM (9.67  $\pm$  0.46  $\mu\text{l/min/mg}$ ) as well as HLC (9.81  $\pm$  0.28  $\mu\text{l/min/mg}$ ) and HLH (6.72  $\pm$  0.43  $\mu\text{l/min/mg}$ ) were calculated to be much higher than that of the AcMPAG formation from MPA, which suggests that the ABHD10 enzyme activity could attenuate AcMPAG formation in human liver. To evaluate the impact of AcMPAG deglucuronidation by ABHD10 on the formation of AcMPAG, we investigated whether the inhibition of ABHD10 could affect the AcMPAG formation from MPA in human liver (Fig. 8 and Table 3). In HLH, the  $K_m$  value was decreased significantly by treatment with PMSF, resulting in a 1.8-fold increase of the  $CL_{\text{int}}$  value. In contrast to the AcMPAG formation, the  $CL_{\text{int}}$  value of the MPAG formation from MPA in HLH was not changed by PMSF (data not shown). This result was consistent with the finding that human ABHD10 could catalyze the AcMPAG deglucuronidation but not the MPAG deglucuronidation. Although it was considered that UDP, which is produced by the glucuronidation of MPA, could affect the AcMPAG deglucuronidation activity, we confirmed no effect of UDP on the activity (supplemental Fig. 3). The  $K_m$  value of the activity for AcMPAG formation from MPA in HLH (623.0  $\pm$  18.6  $\mu\text{M}$ ) was much higher than that of the activity for AcMPAG deglucuronidation (173.7  $\pm$  9.1  $\mu\text{M}$ ). This difference might cause the change of  $K_m$  value of AcMPAG formation in HLM by PMSF, although we can hardly provide the proper explanation why the  $V_{\text{max}}$  value was not changed. Because AcMPAG was reported to be associated with immunogenicity and toxicity (5, 6), ABHD10 may play an important role in the detoxification of MPA-induced immunotoxicity.

In conclusion, we found that human ABHD10 was involved in the AcMPAG deglucuronidation in human liver, which could be important in AcMPAG-related adverse effects. The present study is the first report about the contribution of human ABHD10 to the metabolism of a therapeutic drug.

*Acknowledgment*—We acknowledge Brent Bell for reviewing the manuscript.

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