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Identification of a Human Valacyclovirase

BIPHENYL HYDROLASE-LIKE PROTEIN AS VALACYCLOVIR HYDROLASE*

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Valacyclovir is the 5′-valyl ester prodrug of acyclovir, an effective anti-herpetic drug. Systemic availability of acyclovir in humans is three to five times higher when administered orally as the prodrug. The increased bioavailability of valacyclovir is attributed to carrier-mediated intestinal absorption, via the hPEPT1 peptide transporter, followed by the rapid and complete conversion to acyclovir. The one or more human enzymes responsible for *in vivo* activation of the prodrug to the active drug and its conversion sites, however, have not been identified. In this report, we describe the purification, identification, and characterization of a human enzyme that activates valacyclovir to acyclovir. A protein with significant hydrolytic activity toward valacyclovir, the 5′-glycyl ester of acyclovir, and the 5′-valyl ester of zidovudine (AZT), was purified from Caco-2 cells derived from human intestine. Using a non-redundant data base search, the N-terminal 19-amino acid sequence of the purified 27-kDa, basic protein revealed a perfect match within the N terminus of a serine hydrolase, Biphenyl hydrolase-like (BPHL, gi:4757862) protein, previously cloned from human breast carcinoma. Recombinant BPHL exhibited significant hydrolytic activity for both valacyclovir and valganciclovir with specificity constants (k_{cat}/K_m), 420 and 53.2 $mM^{-1}\cdot s^{-1}$, respectively. We conclude that BPHL may be an important enzyme activating valacyclovir and valganciclovir in humans and an important new target for prodrug design.

Prodrugs of therapeutically active agents have been used to improve pharmaceutical, biopharmaceutical, and pharmacokinetic properties of numerous active therapeutic agents. Prodrugs are designed to be inactive until *in vivo* activation to the parent drug, and hence reliable *in vivo* activation of the prodrug is considered critical for their pharmacological activity (1). Identification of the mechanism of *in vivo* activation of prodrugs is important for prodrug design and for investigating clinical applications. Furthermore, design and development of prodrugs for humans has been significantly hampered by the unknown species differences in the activating enzymes. Thus, identification of the one or more prodrug-activating enzymes will significantly aid in the selection of animal models for human drug development.

The participation of peptidases or esterases in prodrug activation will depend on the pro-moiety, its linker to the parent drug, as well as the parent drug. For several prodrugs, their *in vivo* activation mechanism has been studied in more detail. For example, the anti-cancer prodrug, CPT-11 (irinotecan), a carbamate derivative of 7-ethyl-10-hydroxycamptothecin, is converted to its active metabolite, 7-ethyl-10-hydroxycamptothecin by human carboxylesterases. The efficiency of hydrolysis varies depending on isoforms such that carboxylesterase 2 (hCE2)[1] and intestinal carboxylesterase (hiCE) are more efficient activators than human liver carboxylesterase 1 (hCE1) (2–4). The angiotensin-converting enzyme inhibitor temocapril, an ester prodrug, is rapidly hydrolyzed by carboxylesterase to the active temocaprilat. Human brain carboxylesterase (hBr2) has been found to have the highest specific activity toward temocapril, whereas human intestinal carboxylesterase poorly catalyzes the hydrolysis of temocapril (4).

Recently, amino acid ester prodrugs of nucleoside analogs such as valacyclovir (VACV) and valganciclovir (VGCV) have been shown to significantly increase their oral absorption (5–8). The valyl ester prodrug of acyclovir (ACV), valacyclovir, increases the oral bioavailability of ACV 3- to 5-fold (9). The improved oral bioavailability of ACV, when administered as its prodrug, VACV, has been shown to be due to carrier-mediated intestinal absorption of VACV via the human peptide transporter 1 and to the subsequent rapid *in vivo* conversion of VACV to ACV (10–12).

Although the transport of VACV via the intestinal human peptide transporter 1 membrane transporter has been confirmed in several laboratories, the activation step in converting VACV to the active ACV has not been elucidated. Studies have shown that enzymatic hydrolysis of VACV is predominant *in vivo* in rats, primates, and humans (13–15). VACV was shown to be relatively stable in gut lumen while very susceptible to intracellular enzymatic hydrolysis (16). In an

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[1] The abbreviations used are: hCE, human liver carboxylesterase; hiCE, human intestinal carboxylesterase; hBr2, human brain carboxylesterase; BPHL, biphenyl hydrolase-like protein; hVACVase, human valacyclovir hydrolase; rVACVase, rat valacyclovir hydrolase; VACV, L-valyl ester of acyclovir; D-VACV, D-valyl ester of acyclovir; ACV, acyclovir; VGCV, valganciclovir; AZT, zidovudine; Gly-ACV, glycyl ester of acyclovir; Val-AZT, L-valyl ester of AZT; PCMB, *p*-chloromercuribenzoic acid; DFP, diisopropylfluorophosphate; HPLC, high-performance liquid chromatography; HRP, horseradish peroxidase.

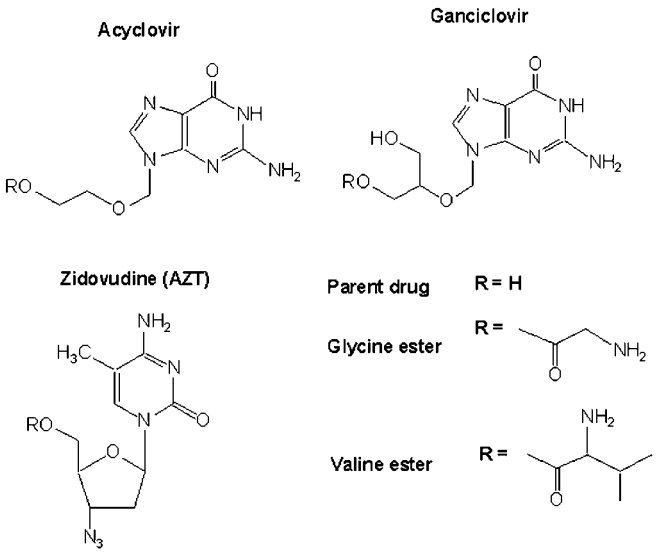

FIG. 1. **Structures of nucleoside analogs.**

attempt to identify a VACV-hydrolyzing enzyme, Burnette *et al.* (17) purified and sequenced several peptide fragments of the major polypeptide from a purified preparation of rat VACV hydrolase, a putative novel protein from rat liver. VACV hydrolase in rat liver (rVACVase) was 29 kDa in size and a basic, monomeric protein that seemed to be associated with mitochondria. However, the identity of this protein was not further determined.

Given the importance of nucleoside analogs in pharmacotherapy, the identification of enzymes responsible for activating this class of prodrugs, particularly in humans, can provide important new targets for the design of more effective therapeutic agents. In this report, we describe the identification of a valacyclovir-hydrolyzing enzyme, human valacyclovirase (hVACVase) from human Caco-2 cells, and identify this protein as being biphenyl hydrolase-like (BPHL, gi:4757862) protein.

EXPERIMENTAL PROCEDURES

Materials—Valacyclovir and valganciclovir were provided by GlaxoSmithKline, Inc. (Research Triangle Park, NC) and Hoffman-La Roche, Inc. (Nutley, NJ), respectively. Acyclovir (ACV), zidovudine (AZT), and trifluoroacetic acid were purchased from Sigma Chemical Co. (St. Louis, MO). The amino acid ester prodrugs, D-valyl, glycyl ester of ACV and L-valyl ester AZT were synthesized and identified as previously described (10) (Fig. 1). Bio-Scale DEAE5 column, Bio-Scale CHT2-I column, Bio-Scale S2 column, and Bio-Sil SEC 125–5 column were purchased from Bio-Rad (Hercules, CA). PD-10 columns were from Amersham Biosciences (Piscataway, NJ). Porcine esterase (EC 3.1.1.1, liver), rabbit esterase (EC 3.1.1.1, liver), and chymotrypsin (EC 3.4.22.1, bovine pancreas) were purchased from Sigma. Pefabloc SC, leupeptin, and E-64 were from Roche Applied Science and *p*-chloromercuribenzoic acid (PCMB), diisopropylfluorophosphate (DFP), dithiothreitol, aprotinin, and pepstatin A were from Sigma. Oligonucleotides for the cloning of BPHL were synthesized at the DNA Core Facility at the University of Michigan. DNA extraction and purification kits were purchased from Qiagen Inc. (Valencia, CA). Enzymes for molecular cloning were from Roche Applied Science. Cell culture reagents were obtained from Invitrogen (Rockville, MD), and culture supplies were from Corning (Corning, NY) and Falcon (Lincoln Park, NJ). Other chemicals were either analytical or HPLC grade.

Cells—The human colon carcinoma cell line, Caco-2, was obtained from the American Type Culture Collection (ATCC HTB37, Passage numbers 44–52, Rockville, MD). The cells were routinely maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 1% non-essential amino acids, 1 mM sodium pyruvate, and 1% L-glutamine and penicillin (100 IU/ml)-streptomycin (100 μg/ml). The cells grown in 150-mm tissue culture dishes were split every 5 days at a ratio of 1:5, and all cells were maintained in an atmosphere of 5% CO_2 and 90% relative humidity at 37 °C.

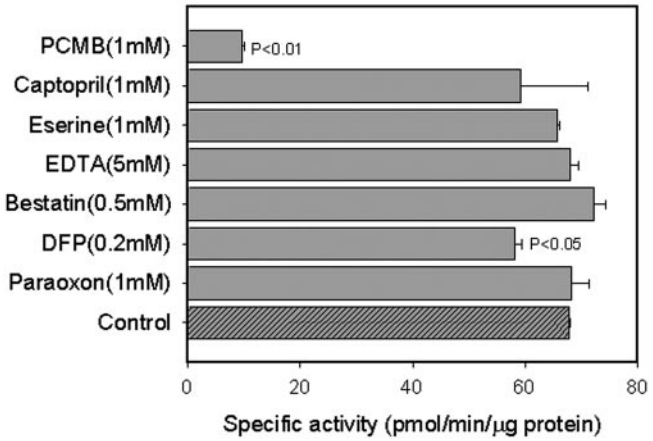

FIG. 2. **Effect of enzyme inhibitors on VACV hydrolysis by Caco-2 cell homogenates (n = 3).**

Prodrug Hydrolysis Assay—The cell homogenates or subcellular fractions were preincubated with 10 mM Tris/HCl buffer (pH 7.4) for 3 min at 37 °C. Substrate was added at 200 μM final concentration to initiate the enzymatic reaction. The reaction was quenched by a volume of 10% ice-cold trifluoroacetic acid in 15 min, and the sample was centrifuged and filtered for HPLC analysis. The effects of enzyme inhibitors, PCMB (1 mM), DFP (0.2 mM), captopril (1 mM), bestatin (0.5 mM), eserine (1 mM), EDTA (5 mM), and paraoxon (1 mM) on VACV hydrolysis by Caco-2 cell were studied by preincubating the cell homogenates with an enzyme inhibitor for 15 min at 37 °C. The HPLC system consisted of a reversed-phase column (Ultrasphere, C-18, 5 μm, 4.6 × 250 mm, Beckman), Waters 515 pump; 996 Photodiode Array UV detector; and WISP model 712 autosampler (Waters, Milford, MA). The remaining prodrugs D/L-valacyclovir (D-VACV, VACV), glycyl ester acyclovir (Gly-ACV), and L-valyl ester AZT (Val-AZT) and production of parent drugs were assayed by HPLC as previously described (10). Briefly, prodrugs and their parent drugs except VGCV were separated and eluted by 2–8% acetonitrile in 40 mM ammonium formate buffer, pH 3.5, at a flow rate of 1 ml/min with detection at 254 nm. VGCV and ganciclovir were eluted in 1% trifluoroacetic acid in water with acetonitrile gradient of 2–16% over 7 min and detected at 254 nm. The enzyme activity was expressed as nanomoles/min/μg of protein based on the production of the parent drug after correcting for any hydrolysis observed with the control.

Purification of Valacyclovir Hydrolase from Caco-2 Cells—The effect of culture time (days) on VACV hydrolysis by Caco-2 cell homogenates was studied with cells grown for 3, 8, 17, and 25 days following confluence. The purification strategy was based on the method described by Burnette *et al.* (17) with some modifications. All buffers contained 0.5 mM EDTA, 1 μg/ml leupeptin, 1 μg/ml pepstatin A, 1 μg/ml E-64, and 2 μg/ml aprotinin; all procedures were performed at 4 °C; and all columns were run using the Biologic HR chromatography system (Bio-Rad, Hercules, CA) with automatic monitoring at A_{280}. For the purification, forty 150-mm culture plates of Caco-2 cells with 14 days culture after confluence were washed with ice-cold phosphate-buffered saline three times, harvested with a cell scraper on ice, and stored at −80 °C until use. Caco-2 cell homogenate was prepared by homogenizing in 10 mM potassium phosphate buffer containing 0.25 M sucrose (pH 7.4). The cell homogenate was centrifuged for 20 min at 25,000 × g to give a pellet (P1) and a supernatant (S1). S1 supernatant was centrifuged for 1 h at 100,000 × g to give a pellet (Mp) containing the microsomal fraction and supernatant (Ms). The P1 pellet was resuspended in 3 volumes of homogenization buffer and frozen at −80 °C. After thawing at room temperature, 4 M NaCl in 10 mM phosphate buffer (pH 7.4) was added into the suspension to make a final concentration of 0.5 M NaCl. The suspension was vortexed vigorously for 30 min and then centrifuged at 100,000 × g for 1 h. The supernatant containing solubilized membrane fraction (MEs) was decanted and kept on ice. An equal volume of 10 mM phosphate buffer (pH 7.4) containing 0.5 M NaCl was then added to the resulting pellet (MEp), and the mixture was again vortexed and centrifuged for 1 h at 100,000 × g. The obtained supernatant and pellet were combined with MEs and MEp, respectively. The MEs fraction was used for further purification.

The MEs fraction was further filtered and concentrated using an Ultrafree-15 centrifuge filter device (Millipore, Bedford, MA). The concentrate was desalted by a PD-10 desalting column equilibrated with 50 mM HEPES, pH 8.0 (buffer A), and then loaded to an anion exchange

TABLE I
Purification of valacyclovir hydrolase from Caco-2 cells

Purification step	Total protein	Specific activity	Total activity	Purification	Yield
	mg	*μmol/min/mg protein*	*μmol/min*	*-fold*	*%*
Homogenate	1465	0.068	99.7	1.0	100
P1 (pellet of 25,000 × centrifugation)	611	0.170	104	2.5	104
MEs (solubilized membrane fraction)	73.0	0.374	27.3	5.5	27.4
DEAE column	4.62	3.76	17.4	55.2	17.4
Hydroxyapatite column	0.83	7.18	6.0	105	5.98
S2 column	0.05	53.2	2.70	781	2.67
Bio-Sil SEC 125–5 column	0.0052[a]	199	1.03	2917	1.03

[a] Protein concentration was estimated by densitometric comparison of the silver-stained proteins of the fraction with the silver-stained calibration band of bovine serum albumin with known concentrations after SDS-PAGE.

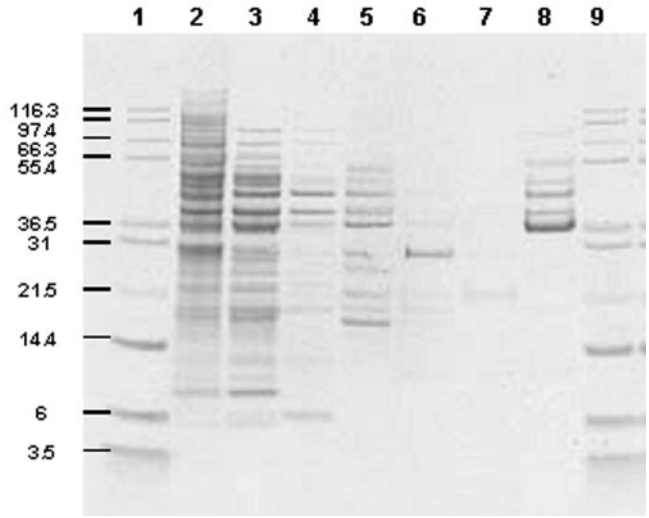

FIG. 3. **Purification of valacyclovir hydrolase from Caco-2 cells.** Active fractions after each step were analyzed by 10% SDS-PAGE as described by Laemmli (19). *Lane 2*, MEs (solubilized membrane fraction); *lane 3*, DEAE column; *lane 4*, hydroxyapatite column; *lane 5*, S2 column; *lane 6*, Bio-Sil SEC 125; *lane 7*, Bio-Sil SEC 125 column; *lane 8*, Bio-Sil 125 column. *Lanes 7* and *8* correspond to inactive fractions after Bio-Sil SEC 125 column chromatography; *lanes 1* and *9* are size markers. The molecular mass of protein markers is in kDa.

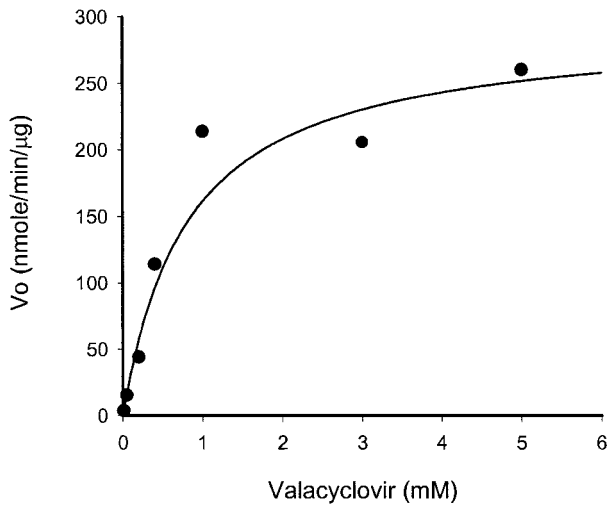

FIG. 4. **V_0 *versus* [S] plot of VACV hydrolysis by hVACVase.** Initial velocity of VACV hydrolysis by the purified hVACVase was determined by incubating hVACVase (213 ng/ml) in 50 mM HEPES buffer, pH 7.4, with VACV of various concentrations ranging from 0.01 to 5 mM at 37 °C for 15 min. The Michaelis-Menten equation was fitted to the data by non-linear least-squares regression using SigmaPlot 8.0 to estimate kinetic parameters.

column, Bio-Scale DEAE5 column (10 × 64 mm, Bio-Rad) equilibrated with buffer A. The sample was eluted with 15 ml of buffer A followed by a gradient from 0 to 0.5 M NaCl (total 50 ml) at a flow rate of 1.5 ml/min. The active fractions were pooled, concentrated, and desalted using a PD-10 equilibrated with 10 mM potassium phosphate buffer, pH 7.5 (buffer B), as described above and loaded onto a Bio-Scale ceramic hydroxyapatite, Type I column (CHT2, 7 × 52 mm, Bio-Rad). The column was equilibrated in buffer B, and the protein was eluted with a 24-ml linear gradient from 10 to 250 mM potassium phosphate buffer (pH 7.5) at a flow rate of 1 ml/min. The active fractions were pooled, concentrated, passed through PD-10 column to exchange buffer B into 50 mM HEPES, pH 6.8 (buffer C), and then loaded onto a cation exchange Bio-Scale S2 column (7 × 52 mm, Bio-Rad) equilibrated with buffer C. After washing with 6 ml of buffer C, the elution was performed with a salt gradient from 0 to 0.2 M NaCl at 1 ml/min for 10 min, followed by 6 ml of 0.2 M NaCl, and then a 0.2 to 0.5 M NaCl gradient (14 ml), followed by 6 ml of 0.5 M NaCl. To neutralize pH, 1-ml fractions were collected in tubes containing 40 μl of 1 M HEPES buffer (pH 8.0). The active fractions were pooled, concentrated, and centrifuged at 14,000 rpm for 5 min, and 100 μl of supernatant was loaded onto a column of Bio-Sil SEC 125-5 (300 × 7.8 mm, Bio-Rad) for gel filtration chromatography in 50 mM HEPES, pH 7.4, 0.1 M NaCl (buffer D) at 0.5 ml/min. Active fractions were stored at −80 °C until use. All purification steps were examined by 10% SDS-PAGE with Mark 12™ unstained protein standard marker (Invitrogen, Carlsbad, CA) for the estimation of the molecular weight as described by Laemmli (19). The VACV hydrolytic activity in each fraction was measured as described above, and the protein concentration was determined based on the Bradford method (20) using a Bio-Rad protein assay kit with bovine serum albumin as a standard.

Characterization of Valacyclovir Hydrolase Purified from Caco-2 Cells (hVACVase)—Hydrolysis of prodrugs, VACV as well as D-VACV, Val-AZT, and Gly-ACV by the purified hVACVase, was studied as described above. Kinetic measurements were carried out in 50 mM HEPES buffer, pH 7.4, at 37 °C with 213 ng/ml of hVACVase within the linear hydrolysis range at various concentrations of VACV ranging from 0.01 to 5 mM. Kinetic parameters for hVACVase were determined by fitting the Michaelis-Menten equation to the initial velocities by the non-linear least square regression module in Sigma Plot 8.0 software.

N-terminal Amino Acid Sequence Analysis and Homology Search—The purified protein was subjected to SDS-PAGE and then electrotransferred onto a Mini ProBlott™ membrane (Applied Biosystems, Foster City, CA) following standard methods (21), and the transferred protein was visualized with Coomassie Brilliant Blue. The band having an apparent molecular mass of 27 kDa from the active fraction was excised and subjected to N-terminal sequencing and analyzed by the Procise™ protein sequencing system (Applied Biosystems, Foster City, CA) at the Protein Structure Core Facility, the University of Michigan. The obtained N-terminal 19-amino acid sequence was used as a query for the homology search by BLAST (basic local alignment search tool) against the non-redundant (nr) protein data base (22).

Expression of Biphenyl Hydrolase-like Protein in Escherichia coli—The N-terminal 19-amino acid sequence showed an exact match with the N-terminal region of biphenyl hydrolase-like protein (BPHL) (gi: 4757862) (23). The 844-bp DNA fragment containing the complete coding sequence for BPHL (cDNA of BPHL was kindly provided by Dr. López-Otín) (23) with *Nde*I and *Hin*dIII sites at the 5′ and 3′ ends, respectively, was amplified by PCR using primers 5′-CAT GTG TGC ATA TGC CCA GGA ATC-3′ and 5′-GAG TGA AGC TTT CCT TGT AGG AAG TCT TCT GCT AAC TTG TTG AAT TCA TCT GCA AAA CGC-3′. The PCR using *Pwo* DNA polymerase (Roche Applied Science) was performed for 40 cycles of 94 °C for 30 s, primer annealing for 30 s at 53 °C, extension at 68 °C for 1 min, and final extension at 68 °C for 7 min. The PCR product was phosphorylated with T4 polynucleotide kinase, ligated into pBluescript-SK (+) (Stratagene), which was treated

TABLE II
Prodrug hydrolysis in a Caco-2 cell homogenate and a solubilized membrane fraction

Cell homogenate or the MEs fraction was preincubated in 10 mM Tris-HCl (pH 7.4) at 37°C for 3 min. The prodrug was then added to a final concentration of 200 μM. After 15-min incubation, the production of ACV and AZT was measured as described under "Experimental Procedures." The results are means ± S.E. of three experiments.

Prodrugs	Specific activity	
	Cell homogenate	MEs fraction
	mol/min/mg protein	
VACV	68.1 ± 2.2	571 ± 11.9
D-VACV	9.0 ± 0.2	21.6 ± 0.1
Val-AZT	134 ± 0.8	605 ± 6.9
Gly-ACV	79.4 ± 0.7	389 ± 3.6

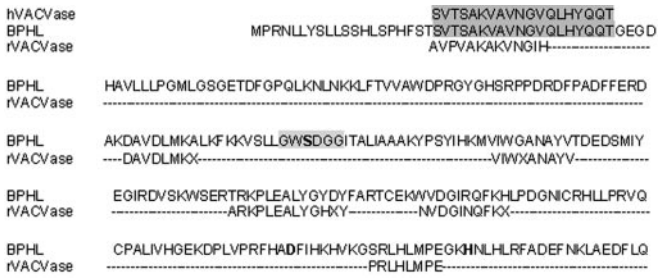

FIG. 5. **Sequence alignment of BPHL with hVACVase and rVACVase.** Sequence alignment was performed by using ClustalW (Blosum matrix, gap opening penalty 10, and gap extension penalty 0.2) at the Pôle Bio-Informatique Lyonnais site (pbil.univ-lyon1.fr) and manually adjusted (29). N-terminal sequence of hVACVase matched with BPHL is *boxed* in *gray*. The putative catalytic triad of BPHL, Ser^{122}-Asp^{227}-His^{255} (26) is in *boldface*. The serine hydrolase consensus motif is *boxed* in *light gray*. The order of six peptide sequences of the major polypeptide from a purified preparation of rVACVase was manually arranged based on its sequence similarity to BPHL. hVACVase (valacyclovir hydrolase from Caco-2 cells); BPHL (biphenyl hydrolase-like; gi:4757862) (23); rVACVase (valacyclovir hydrolase from rat liver) (17).

with *Sma*I (New England BioLabs) and shrimp alkaline phosphatase, and the resulting plasmid (pBS-BPHL) was transformed into *E. coli* strain, DH5α (Invitrogen). The BPHL cDNA was excised with *Nde*I and *Hin*dIII from a selected positive clone and ligated into pET29b (Invitrogen) to make pET-BPHL for recombinant protein expression. The positive clones of pET-BPHL were confirmed by PCR and restriction enzyme digestion and were subjected to DNA sequencing at the Protein Core Facility of the University of Michigan.

The pET-BPHL vector was transformed into *E. coli* strain BL21(DE3), and the transformants were grown overnight in LB broth containing 30 μg of kanamycin/ml. The culture was diluted to 1:20 with the same medium and grown to an A_{600} of 0.8–1. BPHL protein expression was induced by 1 mM (final) isopropyl-1-thio-β-D-galactopyranoside at 25 °C for 6 h. Cells were collected by centrifugation (5000 rpm, 20 min, JA 10) and lysed with a 1/50 volume of B-PER II (Pierce, Rockford, IL) containing 0.5 mM EDTA, 1 μg/ml leupeptin, 1 μg/ml pepstatin A, 1 μg/ml E-64, and 2 μg/ml aprotinin. After centrifugation (27,000 rpm for 20 min at 4 °C), BPHL was purified from the supernatant by anion exchange, hydroxyapatite, and gel filtration column chromatography, and the relative purity of the fractions containing BPHL was judged by 4–20% SDS-PAGE stained with Gelcode® blue stain reagent (Pierce). After filtration through 45 μm, the supernatant was loaded onto a DEAE anion exchange column in 50 mM Tris buffer, pH 7.4 (buffer E), and the flow through was pooled, concentrated, and passed through a PD-10 column to exchange buffer to 10 mM phosphate buffer, pH 7.5 (buffer B). This concentrate was applied to a hydroxyapatite column and was eluted by a 10–250 mM potassium phosphate buffer gradient as for the purification of hVACVase. The BPHL-containing fractions were collected, concentrated, loaded onto a gel filtration column, and eluted by buffer B. The identity of BPHL was confirmed by N-terminal sequencing of the first five residues. The purified BPHL was concentrated and stored at −80 °C until use. The polyclonal anti-serum against BPHL was produced according to the standard method (21) by immunizing rabbits with 500 μg of the purified recombinant BPHL (Rockland Inc., Gilbertsville, PA).

Characterization of BPHL—The hydrolytic activity of BPHL on

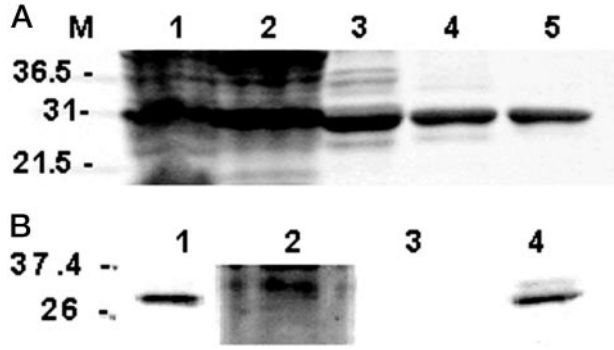

FIG. 6. ***A*, purification of recombinant BPHL.** Recombinant BPHL was purified from the soluble fraction of cell lysate as described under "Experimental Procedures." Each purification step was subjected to 4–20% SDS-PAGE. *M*, molecular size marker; *lane 1*, cell lysate; *lane 2*, soluble fraction of cell lysate; *lane 3*, DEAE column; *lane 4*, hydroxyapatite column; *lane 5*, gel filtration column. The molecular mass of protein markers is indicated in kilodaltons. *B*, Western blot analysis of BPHL in Caco-2 cells. 10 μg of protein was subjected to SDS-PAGE and transferred to a Hybond-P polyvinylidene fluoride transfer membrane (Amersham Biosciences) and immunoblotted as described under "Experimental Procedures" following a standard protocol (21). *Lane 1*, 1:5000 anti-serum and 1:5000 goat anti-rabbit antibody-HRP (Promega); *lane 2*, 1:5000 pre-immune serum and 1:2500 goat anti-rabbit antibody-HRP; *lane 3*, 1:2500 goat anti-rabbit antibody-HRP alone; *lane 4*, 1:5000 anti-serum and 1:2500 goat anti-rabbit antibody-HRP.

VACV, D-VACV, and VGCV was measured in 10 mM potassium phosphate buffer, pH 7.5, at 37 °C. The effect of pH on BPHL activity against VACV was examined at from pH 5.8 to 8 using 10 mM phosphate buffer and from pH 8 to 9 using 50 mM Tris-HCl. The effect of enzyme inhibitors was investigated using Pefabloc SC, E-64, and PCMB by preincubating BPHL with an inhibitor at 3, 30, and 300 μM final concentrations for 5 min and with dithiothreitol at 300 μM.

Determination of Kinetic Parameters of BPHL—The kinetic parameters of BPHL for VACV and VGCV hydrolysis were determined as follows. Kinetic measurements were carried out in 10 mM potassium phosphate buffer, pH 7.4, 37 ± 0.5 °C. Kinetic parameters were calculated from initial velocity data at substrate concentrations ranging from 0.04 to 6 mM. The reaction was initiated by adding 100 ng/ml recombinant BPHL to the preincubated substrate solution, with aliquots taken at 2 min intervals up to 10 min, after which the reaction was terminated by adding trifluoroacetic acid to a final concentration of 5% (v/v). Initial velocities were calculated from the linear time course for the product formation. The Michaelis-Menten equation was fitted to the data by the non-linear least-square regression analysis in Sigma plot 8.0. The k_{cat} value was calculated from $V_{max} = k_{cat}$·[enzyme] based on the 31-kDa molecular mass of BPHL.

Western Blot Analysis of BPHL in Caco-2 Cells—The rabbit anti-BPHL serum was used for Western blot analysis of BPHL in Caco-2 cells. Caco-2 cell homogenate was prepared as for the protein purification. The cell homogenate was incubated with 2× loading buffer for 5 min at 90 °C, and then 10 μg of protein was subjected to 4–20% SDS-PAGE. The protein was transferred to Hybond-P polyvinylidene fluoride transfer membrane (Amersham Biosciences) for 1 h at 100 V. The membrane was blocked in 1% bovine serum albumin Tris-buffered saline tween-20 (TBS-T) overnight at 4 °C and incubated with anti-BPHL polyclonal serum (1:5000) in 1% bovine serum albumin TBS-T for 1 h, washed five times with PBS for 5 min each, and then incubated with horseradish peroxidase-conjugated secondary antibody (Promega, Madison, WI) for 1 h at room temperature. The membrane was washed for 3 h with TBS-T exchanged every 30 min, and an enhanced chemiluminescence system ECL Plus (Amersham Biosciences) was used to detect the expression of BPHL. Immunoblotting using secondary antibody only or pre-immune serum instead of anti-serum served as controls.

RESULTS

Purification of Valacyclovir Hydrolase from Caco-2 Cells—Substantial hydrolysis of VACV was observed in Caco-2 cells. VACV hydrolysis was also detected in HeLa cell homogenate; however, Caco-2 cells exhibited about 2-fold higher VACV hydrolytic activity (data not shown). In both cell homogenates, the

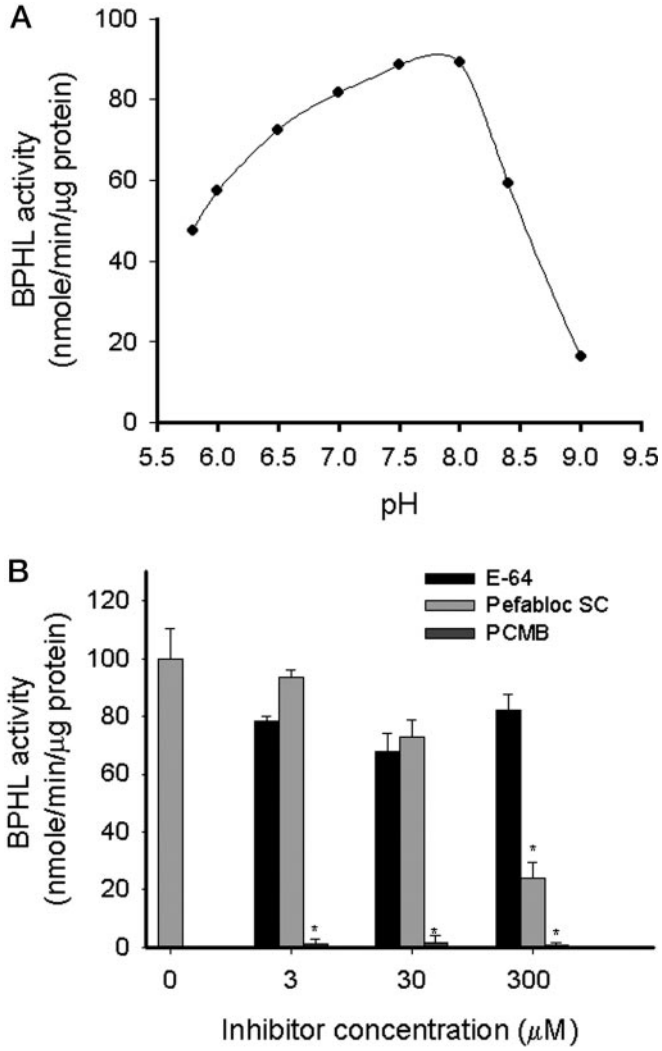

FIG. 7. **Hydrolysis of VACV was studied by incubating BPHL (0.1 μg/ml) with VACV (200 μM final) under several conditions as described under "Experimental Procedures."** *A*, effect of pH on BPHL activity. The VACV hydrolysis by BPHL was studied in buffers in the pH range 5.8–9. Phosphate buffer (10 mM) was used for pH 5.8–8, and 50 mM Tris-HCl was used from pH 8.4 to 9 ($n = 3$). *B*, effect of enzyme inhibitors on BPHL activity. The VACV hydrolysis by BPHL was studied by preincubating with the enzyme inhibitor for 5 min. *, $p < 0.01$ ($n = 3$).

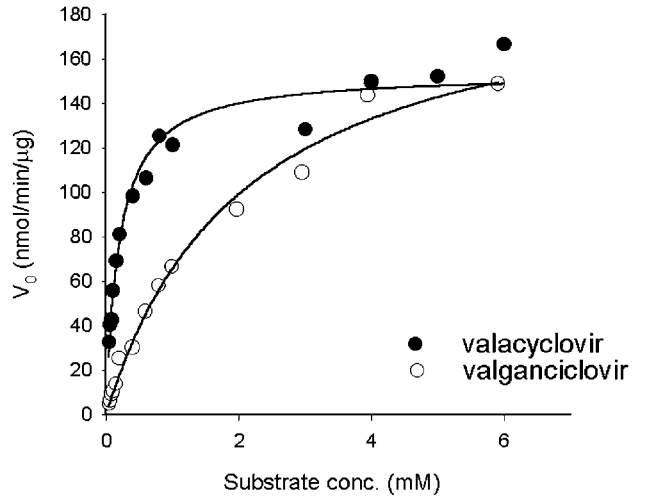

FIG. 8. **V_0 *versus* [S] plot of VACV and VGCV hydrolysis by BPHL.** Initial velocity of VACV and VGVC hydrolysis by BPHL was determined as described under "Experimental Procedures." The averages of at least three determinations are presented.

hydrolysis of D-VACV was not significant. The VACV hydrolysis by Caco-2 cell homogenate increased as the culture days increased until 14 days of culture. Therefore, Caco-2 cells were harvested after an additional 14–16 days of culture following confluence when the highest VACV hydrolysis by the cell homogenate was observed. The VACV hydrolysis in Caco-2 cell homogenate was higher at pH 7.4 and pH 8 than at pH 7 or below (data not shown). The hydrolysis of VACV was not inhibited by 5 mM EDTA, 1 μg/ml leupeptin, 1 μg/ml pepstatin A, 1 μg/ml E-64, and 2 μg/ml aprotinin, nor was it inhibited by the peptidase inhibitors captopril (1 mM) and bestatin (0.5 mM) or the esterase inhibitors eserine (1 mM) and paraoxon (1 mM). This indicates that VACV hydrolysis by typical peptidases and esterases may not be significant. VACV hydrolysis was significantly inhibited by irreversible serine hydrolase inhibitors, DFP (0.2 mM) and Pefabloc SC (1 mg/ml), and almost completely inhibited by a free thiol group modifier, PCMB (1 mM) (Fig. 2). Similar to the previous report (17), VACV hydrolytic activity was enriched in the solubilized membrane fraction (MEs), and specific VACV hydrolysis activity was significantly less in other subcellular fractions (data not shown). Therefore, the MEs fraction was used for further biochemical purification.

Valacyclovir hydrolase was enriched from Caco-2 cell homogenate 2917-fold (Table I) through successive purification steps. Following the last purification step a major band with a molecular mass of 27 kDa (determined by gel filtration chromatography) was present in the active fractions (Fig. 3, *lane 6*), whereas it was absent in inactive fractions (*lanes 7* and *8*). This 27-kDa band was tentatively named human valacyclovirase, hVACVase, to distinguish it from rat liver valacyclovirase (rVACVase). This hVACVase did not bind to a DEAE anion exchange column at pH 8, suggesting that hVACVase remains positively charged at pH 8. The enrichment of VACV hydrolytic activity in the solubilized membrane fraction and a high pI value are consistent with those observed for rVACVase (17).

Characterization of hVACVase—The hydrolysis of VACV by the purified hVACVase was concentration-dependent up to 223 ng/ml hVACVase at 200 μM VACV and linear at least for 15 min. The K_m and V_{max} for VACV was 0.59 mM and 280 nmol/min/μg of protein, respectively (Fig. 4). The k_{cat} was 126 s^{-1} calculated based on the estimated molecular mass of hVACVase, 27 kDa. Val-AZT (specific activity of 225 nmol/min/μg) and Gly-ACV (specific activity of 178 nmol/min/μg) were also substrates of hVACVase. hVACVase exhibited stereoselectivity by hydrolyzing D-VACV at a 20-fold slower rate than VACV (specific activity of 8.4 *versus* 199 nmol/min/μg), whereas hydrolysis of other L-amino acid isomer ester prodrugs was comparable to that of VACV. The hydrolytic activity for Val-AZT and Gly-ACV was also enriched in the solubilized membrane fraction (MEs) similar to VACV hydrolysis (Table II). This suggests that hVACVase may be a primary enzyme for hydrolysis of Val-AZT and Gly-ACV in Caco-2 cells as well. hVACVase and rVACVase share similar characteristics in that both are basic, monomeric proteins of low molecular mass, 27 and 29.7 kDa, respectively. The specificity constant (k_{cat}/K_m) of hVACVase for VACV was 213 $mM^{-1}\cdot s^{-1}$ (at 37 °C), whereas that of rVACVase was 58 $mM^{-1}\cdot s^{-1}$ (at 25 °C). The enzymes, porcine esterase (EC 3.1.1.1), rabbit esterase (EC 3.1.1.1), and bovine chymotrypsin (EC 3.4.22.1) did not hydrolyze VACV when 3 μg of each enzyme was incubated with 200 μM (final) of VACV for 15 min.

Expression and Purification of BPHL—The N-terminal 19-amino acid sequence of the purified hVACVase was determined: Ser-Val-Thr-Ser-Ala-Lys-Val-Ala-Val-Asn-Gly-Val-

TABLE III
Kinetic parameters for BPHL

Kinetic parameters were calculated by fitting the Michaelis-Menten equation to the initial velocity data by using the non-linear least-square regression analysis module in SigmaPlot 8.0. The rate of hydrolysis was measured by incubating BPHL (100 ng/ml) with various concentrations of prodrug in 10 mM phosphate buffer (pH 7.4) at 37 °C. The initial velocity was determined during the linear time course at a substrate concentration ranging from 0.04 to 6 mM. Values are means ± S.E. of at least three independent determinations.

	K_m	V_{max}	k_{cat}	k_{cat}/K_m
	mM	*nmol/min/μg protein*	s^{-1}	$mM^{-1}\ s^{-1}$
VACV	0.19 ± 0.02	152 ± 4.7	78.7 ± 2.44	420
VGCV	1.90 ± 0.24	197 ± 11.1	101 ± 5.72	53.2

Gln-Leu-His-Tyr-Gln-Gln-Thr. BLAST search against the non-redundant (nr) data base using the obtained peptide sequence as a query has led to an exact match with the N terminus of BPHL (gi:4757862) (Fig. 5). It was also observed that there was significant homology between sequences of several peptide fragments previously reported for the major polypeptide from a purified preparation of rVACVase and those of BPHL (Fig. 5); BPHL had been previously cloned from breast cancer tissue (23). Subsequently, BPHL was expressed in *E. coli* and purified from the soluble fraction of the bacterial lysate to apparent homogeneity (Fig. 6*A*). The recombinant BPHL with a C-terminal six-histidine tag was functional. However, an attempt to purify the recombinant His-tagged BPHL with a single step nickel-affinity column was not successful due to the difficulty in removing a major contaminant protein of about 40 kDa. Thus, BPHL was purified by anion exchange, hydroxyapatite, and gel filtration column chromatography. BPHL did not bind to the DEAE anion exchange column at pH 7.4, suggesting that BPHL is a basic protein at neutral pH similar to VACVases with a theoretical pI value greater than 8 (17).

Characterization of BPHL—The purified recombinant BPHL catalyzed the hydrolysis of VACV to ACV and L-valine, providing further support that BPHL is hVACVase. The hydrolysis of VACV (final, 200 μM) by BPHL was proportional to the BPHL concentration up to 110 ng/ml. Enzymatic hydrolysis of BPHL at 37 °C was not significantly different from that at 25 °C (4.14 nmol/min at 25 °C, whereas 4.97 nmol/min at 37 °C). The chemical hydrolysis of VACV at both temperatures was negligible compared with its enzymatic hydrolysis (0.04 nmol/min at 25 °C and 0.11 nmol/min at 37 °C). The activity of BPHL was dependent on pH with the highest activity observed in the range pH 7.4–8 with about 54 and 18% of that activity at pH 5.8 and pH 9, respectively (Fig. 7*A*).

Hydrolysis of VAVC by BPHL was inhibited by Pefabloc SC, a serine hydrolase inhibitor at 300 μM by 75% and by the free-thiol modifier, PCMB, at 3 μM by 99% (Fig. 7*B*). BPHL activity was not significantly inhibited by the cysteine hydrolase inhibitor, E-64, or by dithiothreitol at 300 μM (data not shown). These results are consistent with the pattern of inhibition of VACV-hydrolyzing activity in Caco-2 cell homogenates, where only the serine hydrolase inhibitors DFP and Pefabloc SC, or PCMB had significant inhibitory effect (Fig. 2), and are in agreement with the previously reported results of complete abolition of BPHL function by DFP (23). Although the inhibition by a serine hydrolase inhibitor suggests that BPHL is a serine hydrolase, the significant effect of PCMB also indicates that cysteine residues of BPHL may have a role in the catalytic function of BPHL. Although cysteines may be important in BPHL function, BPHL is not likely a cysteine hydrolase, because a cysteine hydrolase inhibitor, E-64, did not show significant inhibitory effect. In addition to the N-terminal sequence identity, the similarity of the characteristics of BPHL to that of the purified hVACVase provides further support for identity of BPHL as the hVACVase.

Kinetic Parameters of BPHL—In addition to VACV, BPHL also catalyzed the hydrolytic activation of VGCV to GCV and L-valine (Fig. 8). Kinetic parameters of BPHL for VACV and VGCV were determined and are shown in Table III. BPHL exhibited higher affinity for VACV than VGCV (K_m = 0.19 *versus* 1.90 mM, respectively). The V_{max} of BPHL for VACV and VGCV was 152 and 197 nmol/min/μg of protein, whereas the turnover number was 79 and 101 s^{-1}, respectively. The large difference in the K_m values resulted in VACV being ~8-fold more specific substrate for BPHL than VGCV, which was suggested by the specificity constants (k_{cat}/K_m) that were 420 for VACV and 53.2 $mM^{-1}\cdot s^{-1}$ for VGCV, respectively. VGCV exists as a mixture of two diastereomers (24) and VGCV differs from VACV by a hydroxymethyl group (-CH_2OH) in place of a hydrogen in VACV (Fig. 1). A likely explanation for the observed differences in activities against these two substrates, therefore, is that the presence of the hydroxymethyl group reduces the affinity for BPHL. The hydrolysis results did not show BPHL to have a preference for either diastereomeric form of VGCV. In addition, the hydrolytic activity for VGCV paralleled that for VACV in anion exchange column and hydroxyapatite column fractions (data not shown), suggesting that the same enzyme was responsible for the hydrolysis of VGCV as well as VACV in Caco-2 cells. BPHL exhibited stereoselectivity such that it hydrolyzed D-VACV at a much lower rate (specific activity, 28.1 ± 4.26 nmol/min/μg of protein with 1 mM D-VACV) than L-VACV (98 ± 17 nmol/min/μg of protein with 0.4 mM VACV). This is in agreement with the observed stereoselectivity of the biochemically purified hVACVase and rVACVase (17).

BPHL Expression in Caco-2 Cells—mRNA and protein expression of BPHL in Caco-2 cells was investigated by RT-PCR and Western blot analysis. A single band around 850 bp, close to the size of the BPHL cDNA, was obtained by RT-PCR with 1 μg of total RNA from Caco-2 cells using the primer set used for BPHL cDNA amplification (data not shown). As shown in Fig. 6*B*, the protein expression of BPHL in Caco-2 cells was detected as a 30-kDa band by Western blot (*lanes 1* and *4*), and its molecular weight corresponded to that of recombinant BPHL. The BPHL band was absent in the control immunoblots (*lanes 2* and *3*). The similar molecular weights of recombinant BPHL and BPHL in Caco-2 cells suggests the absence of substantial post-translational modification of BPHL, as has been previously suggested by Western blot analysis of BPHL using protein extract of human liver (23).

DISCUSSION

The N-terminal sequence (19 amino acids) of hVACVase is identical to a N-terminal region of BPHL spanning Ser^{21} to Thr^{39} and is about 50% identical (7 out of 13 amino acids) to the N terminus of rVACVase. In addition, the six peptide fragment sequences of the major polypeptide from a purified preparation of rVACVase (17) showed significant sequence similarities with sequences contained within BPHL. Interestingly, both hVACVase and rVACVase are missing an N-terminal 20-amino acid sequence present in BPHL (Fig. 5). This is probably not the result of simple protein degradation, because the first amino acid of both VACVases corresponded to the same amino

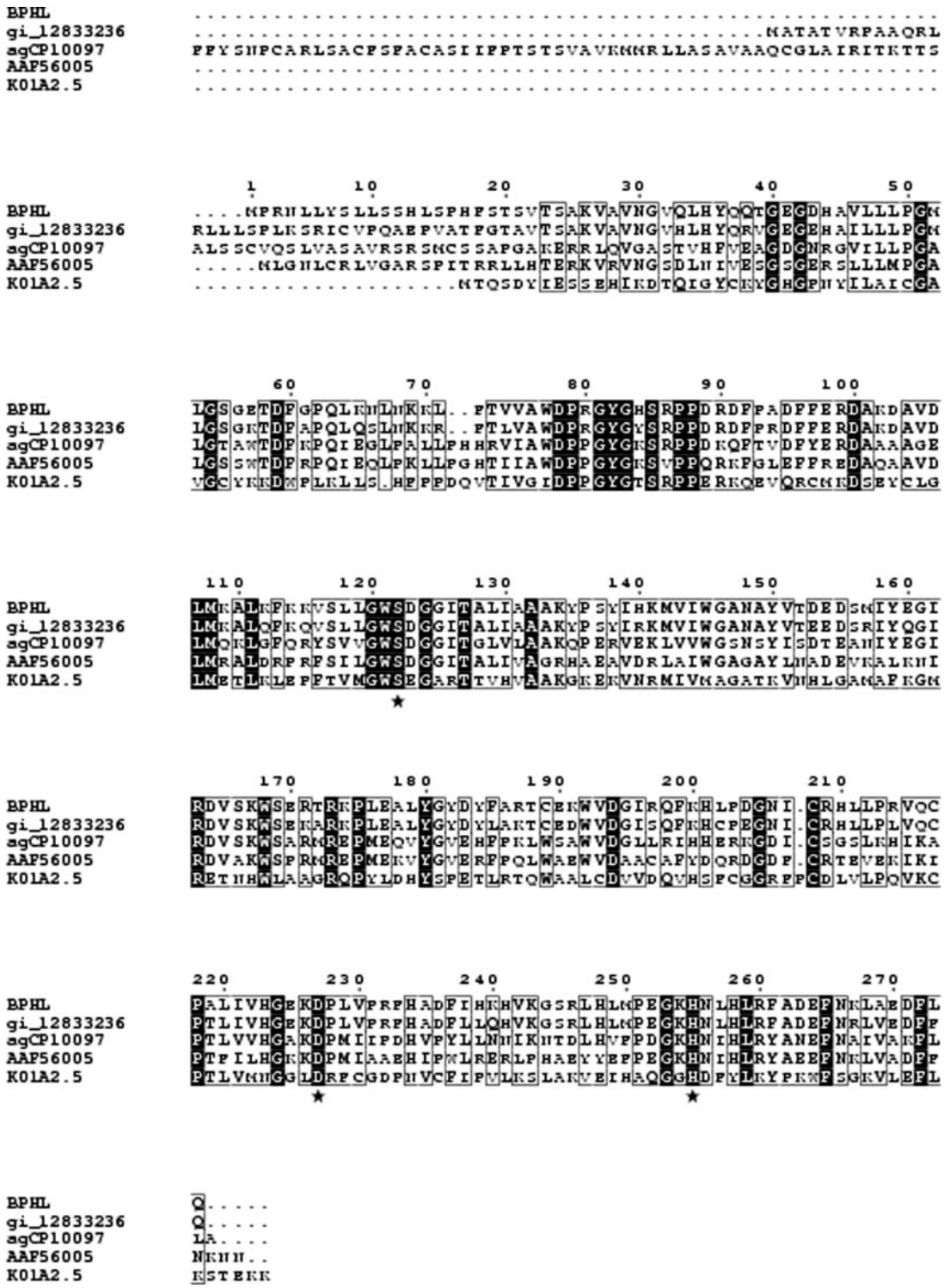

FIG. 9. **Sequence alignment between BPHL and homologous sequences.** Multiple alignments were performed by Multalin (Blosum62, gap opening 12, and gap extension 2) at the Institut National de la Recherche Agronomique server (prodes.toulouse.inra.fr/multalin/multalin.html) (30), and alignment is presented by ESPscript 2.0 (18). *Asterisks* indicate residues comprising the putative catalytic triad of BPHL. Identical residues are in *dark shading*, and similar residues are *boxed*. *BPHL*, biphenyl hydrolase-like (gi:4757862); *gi_12833236*, unnamed protein product (*Mus musculus*); *agCP10097*, hypothetical protein (*A. gambiae* str. PEST, gi:21295261); AAF56005, hypothetical protein (*D. melanogaster*, gi:7300864); *K01A2.5*, hypothetical protein K01A2.5 (*C. elegans*, gi:3811366).

acid, Ser-21 of BPHL in the sequence alignment. In addition, neither sequence begins with methionine, the translation-initiating amino acid of eukaryotic proteins, which indicates the possibility of proteolytic cleavage of the N terminus of VACVases after the translation. The absence of the N-terminal 20-amino acid peptide in the purified protein, combined with the VACV activity enrichment in the solubilized membrane fraction, suggests that the N terminus of BPHL might be an organelle targeting sequence that is removed after protein translocation to the subcellular location. Subcellular localization studies of BPHL are currently underway.

The potential diversity of substrates of hVACVase is suggested by its comparable activity on Val-AZT as well as Gly-ACV. Whereas ACV is a synthetic purine analog, AZT is a

pyrimidine analog, and BPHL has activity on both VGCV and VACV. Moreover, in our preliminary study, BPHL has activity on amino acid ester prodrugs of other nucleoside analogs such as the valyl and phenylalanyl esters of floxuridine (25). Thus, BPHL may serve as a molecular target for prodrug design, for which reliable *in vivo* activation to the parent drug is critical.

The biphenyl hydrolase-like (BPHL, biphenyl hydrolase-related protein) originally cloned from a breast carcinoma cDNA library (23), is a novel serine hydrolase whose substrate specificity and function are yet to be determined. BPHL has the serine hydrolase consensus motif, G*X*S*X*G and a putative catalytic triad composed of Ser^{122}-Asp^{227}-His^{255} (23, 26). Sequence similarities with BPHL were found mainly with sequences from microorganisms such as serine hydrolases encoded by *bphD* (2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoate hydrolase) genes of different bacterial strains. However, BPHL failed to show significant activity on biphenyls (23). BPHL was reported to hydrolyze *p*-nitrophenyl butyrate, a common serine hydrolase substrate; however, natural substrates seem to be distant from *p*-nitrophenyl butyrate, because BPHL exhibited considerably lower specific activity toward *p*-nitrophenyl butyrate than other serine hydrolases such as human carboxylesterase and human milk bile-salt activated lipase (23). The distant relationship of BPHL with other serine hydrolases is further suggested based on structural characteristics and the chromosomal localization of its gene, *bphl* (27).

With the recent growth of databases, several eukaryotic homologs of BPHL have been found (Fig. 9). For example, over 85% of sequence identity was observed with two unnamed mouse proteins (gi:12833236 and gi:26347073) and with two mouse proteins described as "similar to RIKEN cDNA 2010012D11 gene" (gi:19263620 and gi:18606328). The partial peptide sequences of the major polypeptide from a purified preparation of rVACVase showed high sequence identity with sequences contained within these proteins as well. Significant sequence identities were also found in a hypothetical protein of *Anopheles gambiae* strain PEST (gi:21295261; 44% identity) and two sequences from *Drosophila melanogaster* (gi:7300864 and gi:21430884; 45% identity). Borderline homology was detected with a putative α/β hydrolase from Heterodera glycines (gi:13445759; 25% identity) and a hypothetical protein K01A2.5 from *Caenorhabditis elegans* (gi:3811366; 25% identity). The homology search, to date, suggests that BPHL is a relatively unique enzyme in eukaryotes with unknown endogenous function.

In addition to sequence homology between BPHL and rVACVase, BPHL and rVACVase share similar characteristics in that both are basic, monomeric proteins of low molecular mass, 31 and 29.7 kDa, respectively. The similar biochemical characteristics between BPHL and rVACVase combined with the sequence homology suggests that rVACVase and the putative mouse proteins are likely to be isoforms of BPHL in these different species, suggesting that they may serve as animal models for drug development, providing further initiative for defining these unnamed proteins and their role in drug metabolism and delivery.

The kinetic parameters K_m and k_{cat} of the purified hVACVase for VACV hydrolysis were 0.58 mM and 126 s^{-1} and that of recombinant BPHL were 0.19 mM and 79 s^{-1}, respectively. The differences are less than an order of magnitude in the kinetic parameters between the purified hVACVase and the recombinant BPHL and could be attributed to several factors such as the fact that BPHL was expressed in *E. coli* where post-translational modifications are absent and that the recombinant BPHL has an additional 20 amino acids at the N terminus and a six-histidine tag at the C terminus that are absent in the purified hVACVase. The recombinant BPHL is clearly functional despite these factors; however, we cannot rule out the possible effects of these differences on the determination of kinetic parameters.

While BPHL has relatively high K_m values, it exhibits a catalytic efficiency comparable to that of carboxylesterase against some widely used synthetic substrates (28). For example, the k_{cat}/K_m of human liver carboxylesterase (hCE-1) for 2-naphthyl acetate is 551 mM^{-1} s^{-1}, whereas that of BPHL for VACV is 420 mM^{-1} s^{-1}. Moreover, compared with the catalytic efficiency of the carboxylesterases hCE-1 and hCE-2 for pharmaceutical esters, which range from 0.006 mM^{-1} s^{-1} for meperdine to 5.2 mM^{-1} s^{-1} for heroin (modified from Ref. 4), BPHL shows fairly high catalytic efficiencies toward VACV and VGCV. Therefore, despite the relatively high K_m of BPHL, BPHL appears to be capable of efficient hydrolysis of VACV *in vivo*. For example, transport studies of VACV in Caco-2 cells by Han *et al.* (10) demonstrated that more than 90% of the drug in the receiver compartment was ACV rather than the prodrug, indicating extensive intracellular VACV hydrolysis during cellular transport. This result suggests that one or more VACV-hydrolyzing enzymes, including BPHL in Caco-2 cells, are efficient at hydrolyzing VACV during mucosal cell transport.

Our results, including distinct peaks of activity from the chromatography separation and the rather unique amino acid sequence of BPHL, suggest that BPHL is at least one of the primary enzymes for effecting VACV hydrolysis *in vivo*. Nonetheless, the possibility of the presence of one or more other enzymes *in vivo* that could potentially serve to hydrolyze VACV along with BPHL cannot be ruled out.

In conclusion, we have described the purification of a valacyclovir-hydrolyzing enzyme from human cells (hVACVase), its characterization as an enzyme capable of significant hydrolysis of VACV and VGCV, and its identity with the biphenyl hydrolase-like (BPHL) protein that had been previously cloned from human breast cancer tissue. Evidences for the identification were obtained from the identity of the directly sequenced N-terminal peptide from the purified hVACVase with the deduced peptide sequences from the cloned cDNA of BPHL (23) and the significant hydrolytic activity of recombinant BPHL for VACV and VGCV. This is the first report to identify a human enzyme capable of significant hydrolytic activation of the amino acid ester nucleoside prodrugs, valacyclovir and valganciclovir, and to identify this enzyme as the novel serine hydrolase, BPHL. The high expression of BPHL in the normal human intestine, liver, and kidney (23) suggests an important role for BPHL in the activation of VACV and VGCV in human tissues.

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Identification of a Human Valacyclovirase: BIPHENYL HYDROLASE-LIKE PROTEIN AS VALACYCLOVIR HYDROLASE

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