Immunodetection and molecular cloning of a bile-salt-dependent lipase isoform in HepG2 cells

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In this article, we report the nucleotide sequence of the cDNA encoding an isoform of bile-salt-dependent lipase (BSDL) expressed by human hepatoma cells. The BSDL is a 100-kDa glycoprotein normally expressed by the human pancreas. Using a polyclonal antibody raised against an internal peptide located between Ile³²⁷ and Glu³⁵⁰ of the human pancreatic BSDL, we have immunodetected an isoform of human pancreatic BSDL, with an apparent molecular mass of about 62 kDa. This isoform of BSDL was mainly associated with the cytosol of a human hepatoma cell line (HepG2), the remaining protein being found in the microsome fraction. In addition, esterolytic activity on *p*-nitrophenyl hexanoate measured in microsomes and cytosol appeared very low and was weakly stimulated by bile salts, such as taurocholate. In contrast to human pancreatic BSDL, which is secreted as a component of pancreatic juice, this isoform appeared to be retained in the HepG2 cells. Reverse transcription, followed by PCR and amplification, performed on RNA extracted from

HepG2 cells using specific primers hybridizing to the sequence coding for the entire normal human pancreatic BSDL, allowed us to amplify a 1.7-kb transcript that appeared to be 0.5 kb shorter than the transcript of the pancreatic enzyme (2.2 kb). From the sequence of the transcript thus obtained, a protein with a molecular mass of 62 kDa might be predicted, which is in good agreement with the size of the isoform of BSDL immunodetected in HepG2 cells. The N-terminal amino-acid sequence, deduced from the 1.7-kb transcript sequence, matched that of the pancreatic BSDL. However, the C-terminal domain appeared truncated, bearing only a single mucin-like sequence compared with sixteen for the human pancreatic BSDL. The actual intracellular function of this human BSDL hepatoma isoform remains to be elucidated.

Key words: cancer, lipid metabolism, liver.

INTRODUCTION

Bile-salt-dependent lipase (BSDL; EC 3.1.1.-) is produced by pancreatic acinar cells. BSDL has a broad substrate specificity and, as a consequence, has been studied under several names, such as carboxyl ester hydrolase, non-specific lipase, lysophospholipase or bile-salt-dependent cholesterol esterase. It catalyses the hydrolysis *in vitro* of lipid ester substrates such as glycerides and phospholipids. In addition, BSDL is probably the unique enzyme, produced by the pancreas, capable of hydrolysing fat-soluble vitamins and cholesterol esters. It differs from other lipases in the requirement of bile salts to express full activity. The enzyme appears to be a prerequisite for full absorption of cholesterol. The enzyme is released into the duodenum, where it may exert its catalytic function. It has been shown that BSDL may transit through the enterocyte cells and may be recovered in blood plasma where it could have a systemic action on oxidized lipoproteins [1]. It has also been shown that plasma BSDL is able to bind to low-density-lipoprotein particles by a specific interaction with apolipoprotein B-100 [2]. As a consequence, BSDL may be associated with the clearing process of lipoproteins and, thus, with atherosclerosis processes. BSDL is found in the pancreas of numerous species (human, rat, dog, pig and cattle) and also in the milk of primates [3] and carnivores [4]. Its molecular mass differs significantly among these species: from 110 kDa for human to 67 kDa for the rat. Human BSDL is an *N*- and *O*-glycosylated protein with a high proline content. The variability in the molecular mass among species may be explained, in part, by glycosylation and most particularly by *O*-glycosyl-

ation. Putative sites for *O*-glycosylation are located within the C-terminal domain of the protein, which is characterized by the presence of tandem-repeated 'mucin-like' sequences. The human BSDL C-terminal domain is characterized by sixteen repeats, this number varies among species and is reduced to seven, three and four in the ferret, cattle and rat enzymes, respectively. Concomitantly, the number of available *O*-glycosylation sites decreases. It has been shown that the C-terminal domain of the enzyme has an important role, on the one hand for the expression of the catalytic function and, on the other hand, for the intracellular traffic of the newly synthesized protein. The human pancreatic BSDL displays no similarity with the lipase family, except the consensus motif, GX₂SG, located around the serine residue of the active site. Besides its major role in the assimilation of dietary lipids, BSDL appears to be present in a large variety of tissues, where it may function in cholesterol homeostasis [5]. BSDL-like activity has been detected in eosinophil cells [6], steroidogenic cells [7], vascular-endothelium cells [8], human endothelial cells [9], monocytes and macrophages [10]. An enzymic activity with catalytic characteristics very close to those of the pancreatic BSDL has been also described in the rat liver [11]. In humans, BSDL activity is expressed in the fetal liver but does not seem detectable in the normal adult liver [12]. However, an esterase activity resembling that of the human pancreatic BSDL was described [13] in the human HepG2 hepatoma cell line. Data indicate that, compared with pancreatic homogenate activity, the liver activity appears low and moderately dependent on bile salts. To our knowledge, this hepatic BSDL-like activity has never been purified to homogeneity and its structure remains

Abbreviations used: BSDL, bile-salt-dependent lipase; LDH, lactate dehydrogenase; pAb-IP, polyclonal antibody-internal peptide; RT, reverse transcriptase.

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to be determined. Our preliminary Northern blotting experiments using a BSDL probe showed the presence, in the HepG2 cell line, of mRNA specific for BSDL. By investigating the possible presence of BSDL (or of a related enzyme) in human hepatoma cells, we characterize for the first time a BSDL isoform that is immunologically related to the human pancreatic enzyme.

MATERIALS AND METHODS

Chemicals

Unless otherwise indicated, high-quality chemicals were purchased from Sigma (St Louis, MO, U.S.A.). HepG2 (catalogue number 85011430) and BxPC-3 (catalogue number 93120816) cell lines were from the European Collection of Cell Cultures (Salisbury, Wilts., U.K.). Reverse transcriptase (RT) was from Boehringer Mannheim (Mannheim, Germany). *Taq* polymerase was purchased from Clontech (Palo Alto, CA, U.S.A.) and was a part of the GC-rich PCR kit. The TOPO-TA[®] cloning kit was obtained from Invitrogen (Leek, The Netherlands). [³⁵S]Methionine and cholesteryl [1-¹⁴C]oleate were from NEN (Paris, France). Reticulocyte lysate was purchased from Promega (Madison, WI, U.S.A.). Cell-culture media were from Life Technologies (Gaithersburg, MD, U.S.A.). The agarose-gel extraction kit and miniprep kit were from Qiagen (Chatsworth, CA, U.S.A.).

Northern blotting

Total RNAs were extracted by the method of Chirgwin et al. [14] from human pancreas, from HepG2 cells (a cell line originating from a human hepatocarcinoma) and from BxPC-3 cells (a cell line isolated from a human primary pancreatic adenocarcinoma). In order to detect the presence of mRNA specific for the BSDL in HepG2 cells and to compare the amounts of mRNA specific for the BSDL between tissues tested, RNAs were dotted on to nitrocellulose membrane in decreasing rank amounts from 10 to 0.625 µg/well using a Bio-Rad device (Richmond, CA, U.S.A.). The membranes were prehybridized for 3 h at 42 °C in 50 % formamide/2 × SSC (where 1 × SSC is 150 mM NaCl/15 mM sodium citrate)/1 % SDS/5 × Denhardt's solution (where 1 × Denhardt's solution is 0.02 % Ficoll 400/0.02 % polyvinylpyrrolidone/0.002 % BSA) supplemented with 0.2 mg/ml of heat-denatured herring sperm DNA. A cDNA probe (500 bp) specific for human pancreatic BSDL, obtained as described previously [15], was purified on agarose gel (1 %), excised, radiolabelled with [α -³²P]dCTP to a specific radioactivity of 4 × 10⁸ c.p.m./µg and hybridized overnight at 42 °C with spotted RNA. After extensive washing, blots were autoradiographed for 36 h at -70 °C using an intensifying screen. The same experiment, taken as control, was conducted in parallel with a probe specific for the β -actin mRNA [15]. The amount of mRNA specific for the BSDL, contained in each cell or tissue tested, was estimated by the quantification of the dark intensity of each spot by the National Institutes of Health Image program (Bethesda, MD, U.S.A.). For each cell line and tissue, the relative abundance of specific mRNA was estimated by comparing the slope of the regression line of the dark-intensity function with the amount (in µg) of RNA.

Protein translation *in vitro*

By using a Promega kit, mRNA, isolated from total RNA extracted from HepG2 cells, was translated into protein using the rabbit reticulocyte lysate-translation system from Promega according to the manufacturer's instructions. About 2 µg of RNA was incubated with the rabbit reticulocyte lysate and [³⁵S]-

methionine (10 mCi/ml) in a final volume of 50 µl. After an incubation (0.5 h) at 37 °C, translation products were immunoprecipitated with BSDL antibody (polyclonal antibody-internal peptide, pAb-IP) before being analysed using SDS/PAGE and autoradiography. A control run was performed under the same conditions in the absence of the template mRNA.

Antibodies

Polyclonal antibodies were raised in rabbits against a synthetic peptide designed from the sequence of human BSDL located between Ile³²⁷ and Glu³⁵⁰. Its sequence was as follows: NH₂-CIDMPAINKGNGKKVTEEDFYKLVSE-CONH₂. In order to increase the immunogenic response, the synthetic peptide was coupled, via the N-terminally added Cys residue, to keyhole limpet haemocyanin, before being injected in Freund's adjuvant into a rabbit. After the last injection, the rabbit was bled and the serum collected. The IgG fractions were purified on a Protein A-Sepharose column (Pharmacia, Uppsala, Sweden) as directed by the manufacturer. The fractions containing IgG were neutralized and dialysed against a large volume of PBS buffer, pH 7.4. Contaminating antibodies directed against keyhole limpet haemocyanin were removed by immunoaffinity on a haemocyanin-Sepharose column (Sigma). The unbound fractions were dialysed against 5 mM PBS and used as a purified antibody sample (pAb-IP) against BSDL or BSDL isoforms.

PAGE and immunoblotting

SDS/PAGE was performed on a slab gel in 8 % polyacrylamide and 0.1 % SDS, as described by Laemmli [16], using a Bio-Rad Mini Protean II apparatus. After electrophoretic migration, the proteins were stained with Coomassie Brilliant Blue R-250 and destained in ethanol/acetic acid/water (2:3:35, by vol.) or electrotransferred on to nitrocellulose membranes at 18 mA and 4 °C for 18 h. The efficiency of the electrotransfer was checked by staining the transferred proteins with Ponceau S. Replicas were first blocked for 1 h in Tris/HCl buffer (5 mM, pH 8.0) containing 150 mM NaCl and 3 % BSA. The immunodetection was carried out using BSDL internal antipeptide (pAb-IP) as the primary antibody (10 µg/ml). After incubation for 1 h in blocking buffer containing 0.05 % Tween 20, replicas were rinsed and incubated in solution containing alkaline phosphatase-conjugated goat anti-rabbit IgG. After exhaustive washings with PBS supplemented with 0.05 % Tween 20, membranes were developed for 10 min with a mixture of Nitro Blue Tetrazolium and 5-bromo-4-chloro-3-indoyl phosphate (5 × 10⁻⁴ M each) in 0.1 M Tris/HCl buffer (pH 9.5), 0.1 M NaCl and 1 mM MgCl₂.

Cell culture and subcellular fractionation

Human hepatoma cell line HepG2 was grown in Opti-minimal essential medium supplemented with 10 % fetal calf serum, 1 % glutamine, 100 units/ml penicillin, 100 units/ml streptomycin and non-essential amino acids [1 × ; supplied by the manufacturer (Life Sciences) as 10 × and diluted in the Opti-minimal essential medium] in 75-cm² cell-culture dishes maintained at 37 °C in a 95 % air/5 % CO₂ atmosphere. When the cell culture reached about 75–80 % confluence, cells were harvested with 0.25 % trypsin/EDTA and rinsed twice with incomplete PBS buffer (10 mM sodium phosphate, pH 7.0, with 0.15 M NaCl in the absence of Ca²⁺ and Mg²⁺). Cells were sedimented by low-speed centrifugation. Pellets were washed twice with PBS and diluted (ratio 1:5) with growth medium before replating into dishes for large-scale culture. Cell-culture medium was replaced every 2 days. When cells reached approx. 80 % confluence, they were

washed with PBS buffer, scraped with a rubber policeman, sedimented and disrupted with a Potter–Elvehjem homogenizer in a buffer containing 50 mM Tris/HCl, 10 mM EDTA and 0.25 M sucrose (pH 7.4). Subcellular fractionation was performed by serial centrifugations inspired from Jamieson and Palade [17]. The homogenate was cleared by centrifugation at 2000 *g* for 10 min at 4 °C. The supernatant was again centrifuged at 10000 *g* for 30 min in order to remove mitochondria and nuclei. The last supernatant was then centrifuged at 100000 *g* for 2 h to obtain microsomes separated from cytosol. These two subcellular fractions were characterized by their enrichment in lactate dehydrogenase (LDH) [18] and fucosyl transferase [19] activities. For specific experiments, microsomes were suspended in the sucrose buffer and sonicated six times for 5 s each at 4 °C. The insoluble material was again pelleted by centrifugation at 100000 *g* for 1.5 h. The pellet was discarded and the supernatant saved for further experiments. The different subcellular fractions were frozen, when necessary, and stored at –80 °C without any loss of esterolytic activity for at least four weeks.

Reverse transcription and PCR amplification

Total RNA (2 µg) was reverse transcribed into cDNA using a reverse-transcriptase system according to the manufacturer's instruction (Promega) with oligo(dT) as primers. The cDNA(–) pool thus obtained was used directly in PCR. For the PCR amplification, primers used were based on the sequence of the 5'- and 3'-terminal regions of the mature human BSDL cDNA [20]. The sequences of these oligonucleotides were: 5'-TTTCGTGAATTCACGCTAAAACCTAATGACTGCAGGCATCTG-3' and 5'-TTTCGTAAGCTTGCGAAGCTGGGCGCCGTGTACACAGAA-3', respectively. These primers included *Eco*RI and *Hind*III overhangs (underlined) for the ligation into the pCR2.1-TOPO vector. After denaturation for 5 min at 94 °C, 35 PCR cycles were performed, with a denaturation step at 94 °C for 2 min, an annealing step at 64 °C for 1 min and an extension step at 68 °C for 3 min. The extension was terminated by a further incubation for 8 min at 68 °C. The PCR reactions were performed with a 2400 Thermocycler (Perkin-Elmer) in a final volume of 50 µl.

Cloning procedure

After separation on a 1 % agarose gel, the PCR transcript was recovered using an agarose-gel extraction kit (Qiagen). The purified transcript was then ligated into the pCR2.1-TOPO vector (Invitrogen) using the TOPO-TA cloning kit as follows: 2 µl of the purified cDNA and plasmid (10 ng) were diluted in 50 mM Tris/HCl, 1 mM EDTA, 1 mM dithiothreitol, 0.1 % Triton X-100 and 100 µg/ml BSA, and incubated for 5 min at room temperature in a final volume of 5 µl. Then, 2 µl of this mixture was immediately laid on 50 µl of competent *Escherichia coli* cells (TOP 10 F' strain) supplemented with 2 µl of β-mercaptoethanol (0.5 M). After incubation for 30 min at 42 °C, cells were cooled quickly at 4 °C for 30 s and diluted with 450 µl of SOC medium (Life Sciences). After agitation for 1 h at 37 °C, the growth medium was spread on prewarmed selective Luria–Bertani plates containing 50 µg/ml ampicillin and treated with 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside and isopropyl β-D-thiogalactoside, to perform blue/white selection. Plates were maintained overnight at 37 °C. The white or light-blue colonies were picked up and cultured overnight in Luria–Bertani medium supplemented with 50 µg/ml ampicillin. Cells were pelleted and plasmid DNA was isolated using a miniprep kit (Qiagen). The presence of the PCR product within the plasmid was controlled by restriction digestion with *Not*I, which

cut at sites present in both PCR primers, and positive plasmids were sequenced.

Enzyme assays and protein determination

Esterase activity was determined by the rate of hydrolysis of a synthetic substrate, *p*-nitrophenyl hexanoate, to chromogenic *p*-nitrophenol. Activity was measured spectrophotometrically at 404 nm and pH 7.4 in a thermostatically controlled cell at 30 °C using a method described previously [21] with a computer-driven Diode Array 8452 A Hewlett-Packard spectrometer. The BSDL activity was expressed as the difference between the esterase activity levels in assays conducted without (control) and with added bile salt. The esterase activity is given in m-units, 1 m-unit corresponding to the release of 1 nmol of *p*-nitrophenol per min.

Cholesterol esterase activity was determined by the rate of hydrolysis of [¹⁴C]oleoyl cholesterol. In a final volume of 250 µl of 50 mM Tris/HCl buffer, pH 7.4, and enzyme source, the reaction was started by adding 10 µl of [¹⁴C]oleoyl cholesterol (final concentration, 10 µM; specific activity approx. 200 d.p.m./pmol) in ethanol solution. The medium was incubated for 3 h at 37 °C under agitation and the assay was stopped by adding 0.83 ml of Dole's solution [22] supplemented with 0.1 mM non-radioactive oleic acid used as a vehicle. Released [¹⁴C]oleic acid was extracted from the medium by partition in a liquid–liquid system [23]. Radioactivity was determined in an LKB-Wallac scintillation counter. Enzyme activity is given as pmol of [¹⁴C]oleic acid released per h per mg of protein. Control experiments were recorded, omitting the enzyme source, and all assays were performed in triplicate at least.

The LDH activity was determined as described by Goldberg [18] and protein content was determined with the bicinchoninic test from Pierce (Rockford, IL, U.S.A.) using BSA as standard.

Differential solubilization using the temperature-induced phase separation in Triton X-114

Separation of membrane proteins from soluble proteins was conducted by differential solubilization and temperature-induced phase separation using the non-ionic detergent Triton X-114 according to the three-phase partition method as described by Hooper and Bashir [24]. This protocol allows the partition of protein into glycosylphosphatidylinositol membrane proteins, hydrophilic proteins and transmembrane-polypeptide anchored proteins. Before analysis by SDS/PAGE and immunodetection, the different fractions were concentrated by centrifugation on an Amicon device (molecular-mass cut off, 10000 Da; Amicon, Beverly, MA, U.S.A.).

RESULTS

Northern blotting

By using a probe specific for human pancreatic BSDL transcripts, mRNA hybridizing to this probe (Figure 1, upper left panels) were detected in human pancreatic tissue and in the BxPC-3 cell line, as expected, but also in HepG2 cells, although to a lesser extent. The relative abundance of mRNA, at steady state in cells and tissue tested, was estimated by the slopes obtained from the different dot-blot (Figure 1, lower panel). The amounts of mRNA encoding BSDL were decreased in the following rank order: human pancreatic tissue > BxPC-3 cell line > HepG2 cell line, and their relative abundances, once corrected with the β-actin probe used as a control, were 10.0, 8.22 and 3.8 respectively.

Figure 1 Northern-blot analyses

Analyses were performed on RNA extracted from human pancreas, BxPC-3 and HepG2 cells. Increasing amounts (0.625, 1.25, 2.5, 5 and 10 µg) of RNA were spotted on to nitrocellulose membranes. Specific probes for BSDL (upper left panels) and for β -actin (upper right panels) were used to detect specific mRNA.

Immunological detection of the BSDL immunoreactive form in HepG2 cells

We next asked whether the mRNA detected in HepG2 cells could be translated into BSDL and what was the location of the enzyme within cells. For this purpose, the different subcellular fractions of HepG2 cells were separated from the cell homogenate by sequential centrifugation. For these fractions, compared with LDH and fucosyl transferase activities present in cell homogenate and taken as 100%, more than 60% of activity levels were currently recovered. Specific activities were increased by 2.4-fold for LDH and 2.7-fold for fucosyl transferase in the cytosol and microsomes, respectively. The same amounts of proteins from cytosolic and microsome fractions were then resolved on SDS/PAGE, transferred on to nitrocellulose and immunodetected (Figure 2) using pAb-IP as a primary antibody. Only one band, corresponding to a protein with an apparent molecular mass of about 62–65 kDa, was detected in the cytosolic fraction, whereas a weak signal was recovered in the microsome fraction. Some minor bands were stained in the low-molecular-mass region and could have resulted from degradation. The coloured bands, corresponding to the immunodetected BSDL isoform present in both fractions, were scanned and their area was quantified with the National Institutes of Health Image program. The stained bands represented 81 and 19% for the cytosolic and microsomal fractions, respectively. These data suggested that a 62-kDa,

Figure 2 Immunodetection of the BSDL isoform contained in HepG2-cell-line subcellular fractions

After differential centrifugation of cell homogenates, proteins contained in microsomes (A) and in cytosol (B) were resolved by SDS/PAGE, electrotransferred on to nitrocellulose and probed with pAb-IP antibodies. Lane 1, HepG2-cell microsomes; lanes 2 and 3, human pancreatic BSDL; lane 4, HepG2-cell cytosol.

Table 1 Effects of different bile salts on the BSDL isoform activity

The cytosolic fraction (about 2.8 mg of protein/ml) obtained from HepG2 homogenate was used as an enzymic source. The microsomal activity was assayed on the supernatant fraction obtained after sonication and centrifugation (about 0.75 mg of protein/ml) of the basal microsomal suspension. The activity was measured using *p*-nitrophenyl hexanoate as substrate in the presence of 10 mM bile salt. Results are the means $\pm$ S.D. of assays conducted in triplicate.

Bile salt	Activity (m-units/mg of cell protein)	
	Cytosol	Microsomes
None	57.8 $\pm$ 3.5	115.7 $\pm$ 4.1
Taurocholate	90.5 $\pm$ 0.3	129.4 $\pm$ 3.9
Cholate	55.0 $\pm$ 0.7	84.2 $\pm$ 1.2
Glycocholate	49.1 $\pm$ 1.2	63.8 $\pm$ 0.9
Taurodeoxycholate	32.0 $\pm$ 1.3	Not detectable

mainly cytosolic, immunoreactive form of BSDL was expressed by hepatocarcinoma HepG2 cells.

We next attempted to determine whether this isoform of BSDL could be secreted. Therefore, HepG2 cells were allowed to secrete for 6 h in fresh medium. Then, proteins present in the cell-free medium were concentrated, submitted to SDS/PAGE, electrotransferred and probed with the pAb-IP antibody. No material corresponding to the 62-kDa isoform of BSDL could be detected, even after transfer of an overloaded gel (results not shown).

BSDL activity

In agreement with results reported by Winkler et al. [13], it appeared that BSDL activity levels of HepG2 cells were low in homogenates as well as in fractions obtained after sequential centrifugation. They presented a great variability between different preparations, although cells were always harvested and treated under identical conditions. By comparison with estero-lytic activity levels assayed in the absence of bile salts, the esterase activity measured in the presence of bile salts was recovered mainly in the cytosol, whereas some activity was detected in microsomes, which corroborates the immunodetection shown

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1      gcg aag ctg ggc gcc gtg tac aca gaa ggt ggg ttc gtg gaa ggc gtc aat aag aag ctc
1      ala lys leu gly ala val tyr thr glu gly phe val glu gly val asn lys lys leu

61     ggc ctc ctg ggt gac tct gtg gac atc ttc aag ggc atc ccc ttc gca gct ccc acc aag
21     gly leu leu gly asp ser val asp ile phe lys gly ile pro phe ala ala pro thr lys

121    gcc ctg gaa aat cct cag cca cat cct ggc tgg caa ggg acc ctg aag gcc aag aac ttc
41     ala leu glu asn pro gln pro his pro gly trp gln gly thr leu lys ala lys asn phe

181    aag aag aga tgc ctg cag gcc acc atc acc cag gac agc acc tac ggg gat gaa gac tgc
61     lys lys arg cys leu gln ala thr ile thr gln asp ser thr tyr gly asp glu asp cys

241    ctg tac ctc aac att tgg gtg ccc cag ggc agg aag caa gtc tcc cgg gac ctg ccc gtt
81     leu tyr leu asn ile trp val pro gln gly arg lys gln val ser arg asp leu pro val

301    atg atc tgg atc tat gga ggc gcc ttc ctc atg ggg tcc ggc cat ggg gcc aac ttc ctc
101    met ile trp ile tyr gly gly ala phe leu met gly ser gly his gly ala asn phe leu

361    aac aac tac ctg tat gac ggc gag gag atc gcc aca cgc gga aac gtc atc gtg gtc acc
121    asn asn tyr leu tyr asp gly glu glu ile ala thr arg gly asn val ile val val thr

421    ttc aac tac cgt gtc ggc ccc ctt ggg ttc ctc agc act ggg gac gcc aat ctg cca ggt
141    phe asn tyr arg val gly pro leu gly phe leu ser thr gly asp ala asn leu pro gly

481    aac tat ggt ctt cgg gat cag cac atg gcc att gct tgg gtg aag agg aat atc ggc gcc
161    ser tyr gly leu arg asp gln his met ala ile ala trp val lys arg asn ile ala ala

541    ttc ggg ggg gac ccc aac aac atc acg ctc ttc ggg gag tct gct gga ggt gcc agc gtc
181    phe gly gly asp pro asn asn ile thr leu phe gly glu ser ala gly gly ala ser val

601    tct ctg cag acc ctc tcc ccc tac aac aag ggc ctc atc cgg cga gcc atc agc cag agc
201    ser leu gln thr leu ser pro tyr asn lys gly leu ile arg arg ala ile ser gln ser

661    ggc gtg gcc ctg agt ccc tgg gtc atc cag aaa aac cca ctc ttc tgg gcc aaa aag gtg
221    gly val ala leu ser pro trp val ile gln lys asn pro leu phe trp ala lys lys val

721    gct gag aag gtg ggt tgc cct gtg ggt gat gcc gcc agg atg gcc cag tgt ctg aag gtt
241    ala glu lys val gly cys pro val gly asp ala arg met ala gln cys leu lys val

781    act gat ccc cga gcc ctg acg ctg gcc tat aag gtg ccg ctg gca ggc ctg tac ccc
261    thr asp pro arg ala leu thr leu ala tyr lys val pro leu ala gly leu glu tyr pro

841    atg ctg cac tat gtg ggc ttc gtc cct gtc att gat gga gac ttc atc ccc gct gac ccg
281    met leu his tyr val gly phe val pro val ile asp gly asp phe ile pro ala asp pro

901    atc aac ctg tac gcc aac gcc gcc gac atc gac tat ata gca ggc acc aac aac atg gac
301    ile asn leu tyr ala asn ala ala asp ile asp tyr ile ala gly thr asn asn met asp

961    ggc cac atc ttc gcc agc atc gac atg cct gcc atc aac aag ggc aac aag aaa gtc acg
321    gly his ile phe ala ser ile asp met pro ala ile asn lys gly asn lys lys val thr

1021   gag gag gac ttc tac aag ctg gtc agt gag ttc aca atc acc aag ggg ctc aga ggc gcc
341   glu glu asp phe tyr lys leu val ser glu phe thr ile thr lys gly leu arg gly ala

1081   aag acg acc ttt gat gtc tac acc gag tcc tgg gcc cag gac cca tcc cag aat aag
361   lys thr phe asp val tyr thr glu ser trp ala gln asp pro ser gln glu asn lys

1141   aag aag act gtg gtg gac ttt gag acc gat gtc ctc ttc ctg gtg ccc acc gag att gcc
381   lys lys thr val val asp phe glu thr asp val leu phe leu val pro thr glu ile ala

1201   cta gcc cag cac aga gcc aat gcc aag agt gcc aag acc tac gcc tac ctg ttt tcc cat
401   leu ala gln his arg ala asn ala lys ser ala lys thr tyr ala tyr leu phe ser his

1261   ccc tct cgg atg ccc gtc tac ccc aaa tgg gtg ggg gcc gac cat gca gat gac att cag
421   pro ser arg met pro val tyr pro lys trp val gly ala asp his ala asp asp ile gln

1321   tac gtt ttc ggg aag ccc ttc gcc acc ccc acg ggc tac cgg ccc caa gac agg aca gtc
441   tyr val phe gly lys pro phe ala thr pro thr gly tyr arg pro gln asp arg thr val

1381   tct aag gcc atg atc gcc tac tgg acc aac ttt gcc aaa aca ggg gac ccc aac atg ggc
461   ser lys ala met ile ala tyr trp thr asn phe ala lys thr gly asp pro asn met gly

1441   gac tcg gct gtg ccc aca cac tgg gaa ccc tac act acg gaa aac agc ggc tac ctg gag
481   asp ser ala val pro thr his trp glu pro tyr thr thr glu asn ser gly tyr leu glu

1501   atc acc aag aag atg ggc agc agc tcc atg aag cgg agc ctg aga acc aac ttc ctg cgc
501   ile thr lys lys met gly ser ser ser met lys arg ser leu arg thr asn phe leu arg

1561   tac tgg acc ctc acc tat ctg gcg ctg ccc aca gtg acc gac cag gag gcc acc cct gtg
521   tyr trp thr leu thr tyr leu ala leu pro thr val thr asp gln glu ala thr pro val

1621   ccc ccc aca gat gac tcc aag gaa gct cag atg cct gca gtc att agg ttt tag
541   pro pro thr asp asp ser lys glu ala gln met pro ala val ile arg phe END

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Figure 3 Sequence of the RT-PCR transcript of the human BSDL isoform from HepG2 cells

Nucleotide sequence and predicted amino acid sequence of human BSDL isoform. By RT-PCR amplification, a 1671-bp cDNA was isolated from HepG2 cells, as described in the Materials and methods section, coding for 557 amino acid residues. The underlined amino acids are involved in the single tandem-repeat sequence. The asterisk indicates the Gly → Asp mutation.

in Figure 2. Of the total BSDL activity (1876 ± 86 m-units; specific activity, 46 ± 11 m-units/mg of cell protein) measured in whole homogenate, about 58% was detected in the cytosol (765 ± 121 m-units; specific activity, 51 ± 7 m-units/mg) and only 8% (109 ± 21 m-units; specific activity, 10 ± 2.4 m-units/mg) was detected within the microsome fraction. BSDL has been described

previously as a carboxylic ester hydrolase [25]. Therefore, BSDL activity, as determined with the non-specific substrate *p*-nitrophenyl hexanoate, may be altered by numerous carboxylic ester hydrolases present in liver cells [26]. To circumvent this issue, we used radiolabelled cholesteryl oleate as a more specific substrate. In this case, activity levels were reduced to 5.1 ± 0.8 and

1426	gac ccc aac atg ggc gac tcg gct gtg ccc aca cac tgg gaa ccc tac act acg gaa aac gac ccc aac atg ggc gac tcg gct gtg ccc aca cac tgg gaa ccc tac act acg gaa aac
1486	agc ggc tac ctg gag atc acc aag aag atg ggc agc agc tcc atg aag cgg agc ctg aga agc ggc tac ctg gag atc acc aag aag atg ggc agc agc tcc atg aag cgg agc ctg aga
1546	acc aac ttc ctg cgc tac tgg acc ctc acc tat ctg gcg ctg ccc aca gtg acc gac cag acc aac ttc ctg cgc tac tgg acc ctc acc tat ctg gcg ctg ccc aca gtg acc gac cag
1	2
1606	gag gcc acc cct gtg ccc ccc aca ggg gac tcc gag gcc act ccc gtg ccc ccc acg ggt gag gcc acc cct gtg ccc ccc aca --- --- --- --- --- --- --- --- --- ---
3	4
1666	gac tcc gag acc gcc ccc gtg ccg ccc acg ggt gac tcc ggg gcc ccc ccc gtg ccg ccc --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
5	6
1726	acg ggt gac tcc ggg gcc ccc ccc gtg ccg ccc acg ggt gac tcc ggg gcc ccc ccc gtg --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
7	8
1786	ccg ccc acg ggt gac tcc ggg gcc ccc ccc gtg ccg ccc acg ggt gac tcc ggg gcc ccc --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
9	10
1846	ccc gtg ccg ccc acg ggt gac tcc ggg gcc ccc ccc gtg ccg ccc acg ggt gac tcc ggc --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
11	
1906	gcc ccc ccc gtg ccg ccc acg ggt gac gcc ggg ccc ccc ccc gtg ccg ccc acg ggt gac --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
12	13
1966	tcc ggc gcc ccc ccc gtg ccg ccc acg ggt gac tcc ggg gcc ccc ccc gtg acc ccc acg --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
14	15
2026	ggt gac tcc gag acc gcc ccc gtg ccg ccc acg ggt gac tcc ggg gcc ccc cct gtg ccc --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
16	
2086	ccc acg ggt gac tct gag gct gcc cct gtg ccc ccc aca gat gac tcc aag gaa gct cag --- --- --- --- --- --- --- --- --- --- --- --- --- gat gac tcc aag gaa gct cag
2146	atg cct gca gtc att agg ttt tag atg cct gca gtc att agg ttt tag

Figure 4 Comparison of the sequences of the cDNA fragments coding for the C-termini of native human pancreatic BSDL (upper line) and the HepG2 isoform (lower line)

The dashed line indicates missing nucleotides. The bold numbers show the positions (first nucleotide) of the different repeated sequences present in human pancreatic BSDL. Nucleotides are numbered with respect to the first nucleotide encoding for the beginning of the mature BSDL.

1.3 ± 0.2 pmol/h per mg of cell protein in cytosol and microsomes, respectively. These activities, representing 80 and 20% respectively of the total homogenate activity, corroborate the partitioning of BSDL protein between cytosol and microsomes (see Figure 2).

In all cases, maximum activity was obtained after addition of 10 mM sodium taurocholate. Preliminary studies have shown that other detergents, such as non-ionic detergent (1-*O*-n-octyl β -D-glucopyranoside) or zwitterionic detergent (CHAPS), induced no significant stimulation of esterase activity.

Effects of bile salts on the activity of the BSDL immunoreactive form

The effects of different bile salts on hepatoma BSDL activity were analysed further, and the results are summarized in Table 1.

Bile-salt concentrations from 2 up to 80 mM were tested. Under our experimental conditions and in the absence of bile salts, the basal esterolytic activity showed significant high levels, probably due to the contribution of numerous esterases, present in hepatic cells, that are capable of hydrolysing *p*-nitrophenyl hexanoate. Compared with the assay conducted without added bile salt, only sodium taurocholate increased the cytosolic activity, by about 56%. The maximum activity was obtained after addition of 10 mM bile salt (final concentration). No effect was recorded with other bile salts tested. In the microsomal fraction, no significant activation was detected. However, when the BSDL isoform, present in the latter fraction, was partially purified by sonication and centrifugation, the specific activity of the enzyme was increased to 65 ± 17 m-units/mg of cell protein ($n = 6$). Under these conditions, only $23.4 \pm 7.2\%$ of initial proteins were recovered in the supernatant. A poor, albeit

significant, stimulation (+13%) of the partially purified esterase activity was promoted by sodium taurocholate (10 mM).

However, when [^{14}C]oleoyl cholesterol was used, the activity of this roughly purified preparation from microsomes was not significantly lower (basal level, 0.67 ± 0.22 pmol/h per mg of protein) than that recovered on whole microsomes (1.30 ± 0.82 pmol/h per mg of protein). Only the addition of sodium taurocholate increased the activity level, up to 7.14 ± 0.82 pmol/h per mg of protein, with an optimal concentration of 10 mM. This activability differed from 3- to 10-fold between assays, whereas the ratio of proteins to detergent (w/w) was kept constant. The increase, higher than that obtained with *p*-nitrophenyl hexanoate as substrate, may have been due to the higher specificity of BSDL towards the radiolabelled [^{14}C]oleoyl cholesterol and corresponded with the bile-salt activation of the homogeneous pancreatic BSDL [27]. All measured BSDL or cholesterol esterase activities showed a neutral pH optimum at 7.4.

Under our measurement conditions, after incubation of cells in fresh medium for 18 h, the amounts of BSDL activity, released in the extracellular medium, were very low (close to the threshold of detection) and represented some $12.3 \pm 5.0\%$ ($n = 8$) of the total BSDL activity of the cell homogenate. This value was not significantly different than the LDH activity recorded routinely in the extracellular medium of HepG2 cells, which accounted for $8.1 \pm 3.3\%$ ($n = 8$) of the intracellular-medium LDH activity. The values of LDH activity released in extracellular medium were in good agreement with DNA values ($7.4 \pm 0.8\%$) recovered in the extracellular compartment, compared with the total DNA measured in the cell homogenate. As a consequence, one may suggest that the extracellular BSDL activity could have been due to cell lysis.

Anchorage of the BSDL immunoreactive form to microsome membranes

Although low, the BSDL activity recorded in microsomes isolated from HepG2 cells was not affected by Triton X-114. This allowed us to use the temperature-induced phase separation with Triton X-114 to determine the mode of BSDL association with the intracellular membranes. The localization of the enzyme in the different phases after treatment with Triton X-114 was only determined by Western blotting. After scanning of immunoblots (results not shown), it appeared that more than 97% of the initial material was recovered in the Triton X-114-rich phase, showing that the microsomal 62-kDa immunoreactive form of BSDL was tightly associated with membranes, as already found with the BSDL expressed by the human pancreatic tissue and pancreatic cell lines [28,29].

Sequence of the immunoreactive form of BSDL

The next issue was to better characterize the 62-kDa immunoreactive form of BSDL. For this purpose, RT-PCR, using RNA extracted from HepG2 cells as a template and a pair of primers covering the entire sequence of the mature pancreatic BSDL [30], was performed. This allowed us to isolate a transcript of about 1.7 kb (compared with 2.2 kb for the native human pancreatic BSDL). This transcript was cloned into the pCR2.1-TOPO vector, amplified and sequenced. The amino acid sequence was deduced from the nucleotide sequence of the cDNA (Figure 3). It was composed of 557 amino acids, and a molecular mass of about 62 kDa might be predicted, in agreement with the molecular mass observed after electrophoresis (see Figure 2). Compared with human pancreatic BSDL cDNA [31], a great homology (98.7%) was detected within the 5'-nucleotide sequence up to nucleotide 1426, where the sequence of exon 11

Figure 5 mRNA translation *in vitro*

About 10 μg of mRNA extracted and purified from HepG2 cells was used to programme a rabbit reticulocyte lysate system for translation *in vitro* in the presence of [^{35}S]methionine. After incubation, the translated products were immunoprecipitated with pAb-IP antibodies and separated by electrophoresis. Lane 1, control experiment where mRNA template was omitted; lane 2, translated product using mRNA from HepG2 cells.

starts. The deduced amino acid sequence was consequently identical with that of the human pancreatic BSDL. In particular, the positions of amino acids involved in the catalytic sites, Ser¹⁹⁴ (in the consensus sequence Gly-Xaa-Ser-Xaa-Gly, recovered in the lipase superfamily), Asp³²⁰ and His⁴³⁵, as well as the putative site for of N-glycosylation at Asn¹⁸⁷ or that for heparin binding (Lys⁶¹-Lys-Arg-Cys-Leu-Gln⁶⁶), was conserved. However, the nucleotide sequence corresponding to exon 11, starting at nucleotide 1426, was different. In part, the number of repeated sequences was smaller than that of pancreatic cDNA [31]. In human pancreas, exon 11 of the BSDL gene encodes the C-terminal domain of the enzyme, which bears the 16 repeated identical sequences characterized by the consensus sequence Pro-Thr-Gly-Asp-Ser. If the HepG2 transcript matched well (98.6%) that of the pancreatic cDNA from nucleotides 1426 to 1630 and from 2125 to the stop codon (numbers correspond to human pancreatic BSDL sequence), it appeared that, between these two sequences, 495 bp were deleted. This nucleotide deletion led to the excision of 15 of the 16 repeated sequences (Figure 4). The remaining 'repeated' sequence located between nucleotides 1606 and 1638 matched well the first repeated sequence of the pancreatic BSDL. Nevertheless, one mutation, producing Asp⁵⁴⁴ instead of Gly, was detected in the nucleotide sequence (nucleotide 1631).

Translation *in vitro*

The translation of the 1.7-kb transcript amplified by RT-PCR from HepG2 mRNA might strengthen results described above. This transcript could result from a 1.7-kb mature mRNA that might encode a protein of approx. 62 kDa. RNA isolated from HepG2 cells was used to program a rabbit reticulocyte lysate-translation system *in vitro* in the presence of radiolabelled [^{35}S]methionine. After incubation, translated products were isolated by immunoprecipitation using pAb-IP. These products were then separated by SDS/PAGE and autoradiographed. The autoradiogram, compared with a control where the template mRNA was omitted, showed a protein migrating at approx. 63–65 kDa (Figure 5; arrow), which correlates well with the size of the protein detected in HepG2 cells (Figure 2) and with that of the protein, the sequence of which was deduced from the nucleotide sequence of the PCR transcript (Figure 3). Other bands, weakly stained, were detected around 100 and 46 kDa. It may be suggested that RNA extracted from the HepG2 cells encoded different forms of BSDL, as already found in pancreatic

tumour cells [32]. However, these bands could also represent proteins involved in the folding complex of BSDL [33].

DISCUSSION

In this study, Northern-blot experiments showed that mRNA specific to BSDL was present in HepG2 cells, a human tumoral hepatic cell line. We demonstrated further that an isoform of the human pancreatic BSDL was actually expressed by HepG2 cells. This BSDL isoform, on the one hand, has been characterized by immunodetection with an antibody specific for the human pancreatic BSDL and, on the other hand, was ascertained by RT-PCR methods and cDNA sequencing. A 1.7-kb transcript was thus obtained, the sequence of which should code for a 62-kDa protein. The sequence of this protein suggested that it is a truncated isoform of the pancreatic BSDL.

As already shown with other tumoral cells [34], the BSDL expressed by tumoral hepatic cells presented a poor esterase activity. This activity appeared weakly dependent on added bile salts. Nevertheless, when microsomal activity was partially purified, its ability to be activated by sodium taurocholate was increased and remained at the same magnitude as that obtained with purified human pancreatic BSDL, suggesting that proteins and/or lipids present in the microenvironment of the enzyme may greatly modify its activity. This confirmed especially the presence of a bile-salt-dependent esterase activity in HepG2 cells. The 62-kDa truncated BSDL isoform expressed by hepatoma cells was not secreted in the extracellular compartment; this observation was attested by several assays performed with different HepG2-cell-line lots and growth media. Under our experimental conditions, the BSDL isoform was sequestered within the tumoral cells and seemed to accumulate mainly within the cytosolic compartment, where its actual function remains to be clarified. As has been demonstrated previously by our group [35], one may not rule out the possibility that the cytosolic isoform of BSDL, expressed by HepG2 cells, acts as an acylase and is involved in the synthesis of cholesteryl esters used by tumoral cells to create new membrane structures, which are essential for cell proliferation. A similar role was also reported with human fetal cells, which also expressed BSDL [21]. From the rat liver, Camulli et al. [36] have isolated a neutral cholesterol esterase from cytosolic fractions. This activity, which was stimulated by sodium taurocholate, was immunodetected by antibodies raised against rat pancreatic cholesterol esterase but showed no significant modification of the molecular mass (about 67 kDa) compared with the rat pancreatic BSDL.

The cDNA covering the mature sequence of the BSDL isoform of HepG2 cells was obtained by RT-PCR using specific primers. The transcript thus obtained (1.7 kb) was shorter than that isolated from the pancreatic tissue [31]. The deduced amino acid sequence of this isoform revealed a number of interesting features. Our results indicated that the N-terminal domain coded by exons 1–10 was strictly identical with that of BSDL expressed by the normal human pancreas. Similar observations have been described for other different tumoral cells [34]. Like all carboxyl esterases, it contains the consensus sequence GXSXG around the serine residue of the active site, which is shared by the lipase family [20,37]. On the contrary, the C-terminal domain coded by the HepG2-cell transcript was shorter than that of pancreatic BSDL and the deletion of a 165-amino acid segment was observed. In contrast with the pancreatic BSDL C-terminal domain, which displays 16 tandem-repeated sequences, a single repeat sequence was present on the C-terminal domain of BSDL isoform described here. This number of repeated sequences appeared closely related to the expression of catalytic properties

and to the intracellular traffic of the protein. It is known that repeated sequences of native BSDL offer *O*-glycosylation sites and are composed of 11 amino acids, among which proline, threonine, serine and glutamic acid are well represented. These four amino acids constitute the well-known PEST (Pro-Glu-Ser-Thr) sequences, which are constitutive or conditional signals for the rapid degradation of domestic proteins. PEST sequences are found in key metabolic enzymes, transcription factors, protein kinases or protein phosphatases [38]. Nevertheless, it was postulated that, in the pancreas, PEST sequences present on the BSDL may be partly masked by numerous and large glycosylation motifs located at the surface of the enzyme structure and, thus, may contribute to the regulation of the secretion of the BSDL [39]. If this number of glycosylation sites was decreased (and/or if the structure of the glycosylation motif was modified) as a consequence of a reduced number of repeated sequences, it may lead to a routing of the BSDL isoform to the cytosol, then probably to a degradation cell compartment, with a concomitant decrease in secreted material [40]. The reduced glycosylation level may also be correlated with a weak esterase-activity efficiency [29]. For the HepG2 BSDL isoform, it may be postulated that the glycosylation level could be limited, as indicated by the weak difference observed between the molecular masses determined from SDS/PAGE, from the predicted amino acid sequence and from translation *in vitro*.

Studies *in vitro* have suggested that recombinant BSDL expressed in eukaryotic cells and bearing various amounts of repeated sequences have similar esterase activities [41]. However, results recorded under constant conditions from our group have indicated that the enzyme activity varied strongly according to the C-terminal structure, independent of the substrate used (*p*-nitrophenyl acetate, *p*-nitrophenyl hexanoate or cholesteryl esters). The oncofetal variant of the human BSDL, which possesses six repeated sequences [34], is about 10-fold less active than the human BSDL, which has 16 of these sequences [31]. Also, the cod enzyme (no repeated sequence on the enzyme) is about 6-fold less active than the human enzyme [42]. Finally, the ferret enzyme, which presents seven repeated sequences, is much more active (approx. 10 times) than human BSDL [15,20].

Besides the well-known hydrolysing function towards cholesteryl esters of BSDL, it was shown that this enzyme, under appropriate conditions, may esterify free cholesterol with free fatty acids [36]. Nevertheless, conditions regulating this dual function remain unknown, although bile-salt concentration and pH may be important. The actual function of the BSDL isoform found in the tumoral liver cells remains to be elucidated. However, one cannot exclude that the structure of the C-terminal domain may influence the routing and running modes of the enzyme: highly glycosylated structures, rich in repeated sequences, would orient the enzyme towards secretion and acting as an esterase, whereas poorly glycosylated shorter forms would route the enzyme to the cytosol and towards acting as an acyltransferase involved in the synthesis of cholesterol esters and/or in cholesterol homeostasis [5].

Complementary experiments will be essential to test this hypothesis and, in particular, the transfection of HepG2 cells with an antisense message of this BSDL isoform will be able to explain the actual function of this isoform in the metabolism of cholesterol. The studies of the intracellular traffic of the BSDL isoform may help us understand why it is sequestered completely in the cytosol.

This study was in part financed by grant no. 9912 to A. V. awarded by the Association pour la Recherche sur le Cancer (ARC, Villejuif, France). We thank Mr. C. Tognotti for his expert assistance and his fruitful discussions.

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Received 2 February 1999/27 April 1999; accepted 4 June 1999