THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 273, No. 43, Issue of October 23, pp. 28208–28218, 1998
Printed in U.S.A.

Molecular Cloning of the Oncofetal Isoform of the Human Pancreatic Bile Salt-dependent Lipase*

(Received for publication, May 26, 1998, and in revised form, June 23, 1998)

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Specific transcripts for bile salt-dependent lipase (BSDL), a 100-kDa glycoprotein secreted by the human pancreas, were immunodetected in BxPC-3 and SOJ-6 pancreatic tumoral cell lines. Sequencing of fragments, obtained by mRNA reverse transcription and amplification, confirmed the presence of BSDL transcripts in these cancer cells. The protein was detected in lysates of pancreatic tumoral cells, where it was mainly associated with membranes. Only a minute amount of the enzyme was detected in the culture media. Immunofluorescence studies demonstrated that in SOJ-6 cells, BSDL colocates with the p58 Golgi protein and suggested that the protein may be sequestrated within the Golgi compartment. These results demonstrated that BSDL is expressed in human pancreatic tumoral cells and cannot be secreted (or for the least very poorly). Subsequently, a cDNA covering the entire sequence of BSDL was obtained by reverse transcription-polymerase chain reaction. The sequence of this cDNA indicated that the N-terminal domain encoded by exons 1–10 was identical to that of BSDL expressed by the human normal pancreas. However, the sequence corresponding to exon 11, which should code for the 16 tandem-repeated identical mucin-like sequences of BSDL, was deleted by 330 base pairs (bp) and encoded only 6 of these repeated sequences. We conclude that this truncated variant of BSDL would be its oncofetal form, referred to as feto-acinar pancreatic protein. We then investigated whether the deletion of 330 bp affected the secretion of the protein. For this purpose, the cDNA corresponding to the mature form of the BSDL variant expressed in SOJ-6 cells was cloned into an expression/secretion vector and transfected into CHO-K1 cells. Results indicated that the variant of BSDL isolated from SOJ-6 cells was expressed and secreted by transfected cells. However, the level of BSDL secreted by these transfected CHO-K1 cells was significantly higher than that observed for SOJ-6 cells. Consequently, the retention of the oncofetal variant of BSDL observed in human pancreatic tumoral cells might not result from inherent properties of the protein.

The bile salt-dependent lipase (BSDL,[1] EC 3.1.1) is a 100-kDa glycoprotein secreted by the pancreas into the duodenum, where it is thought to play an important role in cholesterol and lipid-soluble vitamin ester hydrolysis and absorption (1, 2). The cDNA for pancreatic BSDL has been isolated and sequenced (3). The amino acid sequence is rich in proline (12%), the majority of these residues (68%) being located within 16 C-terminal tandemly repeated sequences (repeats numbered 1 to 16) (3, 4). Further studies have shown that exon 11 encoded these tandem repeats (5), the size of which varied by species (5, 6). This accounts, in part, for the previously observed species variation in BSDL size and amino acid composition (7). In contrast to the other secretory pancreatic enzymes, BSDL is associated with membranes during its intracellular processing (8, 9). This association involves a multimeric folding complex including p94, a protein immunologically related to the glucose-regulated protein of 94 kDa (Grp94) and two other proteins of 56 and 46 kDa (9). It has been suggested that the interaction of BSDL with the Grp94-related p94 protein is essential for the *O*-glycosylation of C-terminal tandem-repeated sequences (9). These repeated sequences contain PEST regions, which are signals for rapid degradation (10). It is therefore possible that glycosylation of these PEST regions may contribute to the removal of BSDL from a possible degradation route (11). Once fully glycosylated, the enzyme is phosphorylated and released from membranes either in or after the *trans*-Golgi compartment (12). It is then aggregated in the *trans*-Golgi network (8, 9) with other digestive enzymes and enters the regulated secretion pathway. In a previous study, we have isolated an oncofetal variant of BSDL (13), later identified as the feto-acinar pancreatic protein (FAPP) (14).

FAPP was first characterized by the monoclonal antibody *J28* (*mAb J28*) (14). FAPP is expressed in human embryonic and fetal pancreas. The earliest expression of this protein was seen in undifferentiated mesenchymal cells and in nascent acini at the beginning of the morphological differentiation of the pancreas (15). Maximal synthesis of FAPP occurs at the time of intense proliferation of acinar cells and declines progressively thereafter (15) to reach a very low level of expression in normal adult pancreas (16). FAPP concentration is elevated in the blood of patients suffering from pancreatitis and pancreatic cancer, suggesting an enhanced synthesis in cases of pancreatic pathologies. This finding corroborates the increased level of FAPP in pathological pancreatic juices (17). FAPP was shown to be different from BSDL in many aspects: (*a*) FAPP is

* This work was supported by Association pour la Recherche sur le Cancer Grants Nb 6122 and 4050, the Conseil Général des Bouches-du-Rhône (Marseille, France), and the financial help from GEFLUC (Marseille, France). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF081673.

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[1] The abbreviations used are: BSDL, bile salt-dependent lipase; FAPP, feto-acinar pancreatic protein; CHO, Chinese hamster ovary; 4-NPH, 4-nitrophenyl hexanoate; bp, base pair(s); ER, endoplasmic reticulum; FITC, fluorescein isothiocyanate; TRITC, tetrarhodamine isothiocyanate; PBS, phosphate-buffered saline; LDH, lactate dehydrogenase; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; RT, reverse transcription; kb, kilobase(s).

much less active than BSDL; (*b*) the *N*-linked glycosylation of FAPP seems to be a high-mannose type, whereas that of BSDL is complex (18); (*c*) the amount of *O*-linked structures is largely decreased in FAPP (18); (*d*) carbohydrate accounts for 47% of the FAPP mass (14) instead of 20% for BSDL (19); and (*e*) the amino acid composition of FAPP differs from that of BSDL (13), although their N-terminal sequences are identical (20). Data suggest that the two proteins may have different C-terminal tails (18, 20). Finally, FAPP migration on SDS-PAGE is diffuse and lower than that of BSDL (13). Other studies have shown that FAPP is expressed in pancreatic tumor cells; however, the secretion of the protein was not detected (21). Miralles *et al*. (21) have postulated that the absence of secretion was due to the retention of the protein within the endoplasmic reticulum (ER) and suggested that FAPP would remain associated to ER resident protein(s) as a consequence of its improper folding. Recent findings suggested that glycosylation of the C-terminal region of BSDL regulates the secretion of the protein (11). The aim of this study was 2-fold: first, to investigate the molecular properties of BSDL expressed by human pancreatic tumoral cells; second, to determine whether the retention of the variant expressed by tumoral cells is due to inherent properties of the protein. We showed that part of C-terminal tandem repeated sequences were deleted in the BSDL variant expressed by pancreatic tumoral cells, suggesting that we are dealing with FAPP. Moreover, CHO-K1 cells transfected with the truncated cDNA secreted the protein. This result indicates that the retention of FAPP within tumor cells cannot be due to the truncation of the C-terminal region of the protein.

EXPERIMENTAL PROCEDURES

Materials—Glutamine, penicillin, trypsin-EDTA, and streptomycin were from Life Technologies, Inc. Fetal calf serum was from Dutscher (Brumath, France). Phenylmethylsulfonyl fluoride, benzamidine, and β-phenyl propionate were from Fluka (Buchs, Switzerland). Mouse antibodies specific for the p58 Golgi protein, FITC-conjugated anti-rabbit goat IgG, TRITC-conjugated anti-mouse goat IgG, saponin, 4-nitrophenyl hexanoate, and soybean trypsin inhibitor were from Sigma. [α-^{32}P]dCTP and ^{125}I-labeled protein A were from NEN Life Science Products. [^{35}S]Methionine (Tran^{35}S-label) was from ICN biochemicals (Costa Mesa, CA).

Cell Cultures and Tissues—BxPC-3 cells, obtained from the American Type Culture Collection (ATCC, Rockville, MD), were cultured in RPMI 1640 (Life Technologies, Inc.). SOJ-6 cells were kindly provided by Dr. M. J. Escribano (INSERM U 260, Marseille, France) and cultured in RPMI 1640. Media were supplemented with 10% fetal calf serum, glutamine (1%), penicillin (100 units/ml), and streptomycin (100 μg/ml). Cells were maintained at 37 °C in a 5% CO_2, 95% air atmosphere. Cell culture medium was removed after the required incubation time, collected, and stored frozen until use. CHO-K1 cells were also obtained from ATCC and cultured under the same conditions in Ham's F12 nutrient mixture (Life Technologies, Inc.) supplemented with 10% fetal calf serum. Normal human pancreatic tissues came from three donors (two females and one male, aged 60–65), and cancer tissue was from a 63-year-old female suffering with an adenocarcinoma of the pancreas. Diagnosis was confirmed by histology examination performed during the time course of surgery. All tissues were generous gifts from Prof. J. R. Delpéro (Institut Paoli-Calmettes, Marseilles, France). Tissue samples were immediately frozen in liquid nitrogen and stored at −80 °C until used.

Cell Protein Extraction—Cells grown to confluence were rinsed twice with incomplete PBS buffer (10 mM sodium phosphate buffer, pH 7.4, with 0.15 M NaCl and no Ca^{2+} and no Mg^{2+} ions) and harvested with 0.25% trypsin-EDTA. Cells were suspended in culture medium and centrifuged at 400 × *g* for 3 min. Pellets were washed twice with PBS and suspended in PBS (500 μl) containing 100 μg/ml soybean trypsin inhibitor, 2 mM phenylmethylsulfonyl fluoride, 2 mM β-phenyl propionate, and 2 mM benzamidine and sonicated for 10 s. The cell membrane fraction was separated from the soluble fraction by centrifugation at 12,000 × *g* for 20 min at 4 °C (22). The pellet containing membrane proteins was solubilized in 0.125 mM Tris/HCl, pH 7.0, buffer (3% SDS) (21).

Protein Concentration and Enzyme Activity Determinations—Proteins were quantitated using the bicinchoninic acid test from Pierce using bovine serum albumin as standard. α-Amylase and lactate dehydrogenase (LDH) activities were determined using adequate methods (23, 24). BSDL was assayed on 4-nitrophenyl hexanoate (4-NPH) in the presence of 4 mM sodium taurocholate as specific activator of the enzyme (25). In BxPC-3 and SOJ-6 tumoral pancreatic cells, more than 90% of the activity on 4-NPH can be immunoprecipitated with antibodies specific for BSDL (22). The 4-NPH activity determined in CHO-K1 cells transfected with PCR products in pSecTag (see below) was corrected for the activity found in control cells (*i.e.* transfected with the empty vector). Note that the 4-NPH activity detected in control CHO-K1 cells represented less than 5% of that found in CHO-K1 cells transfected with PCR products.

Proteins and Antibodies—BSDL was purified from human pancreatic juice devoid of free proteolytic activity (a gift of Prof. R. Laugier, Hôpital de la Conception, Marseille, France) (13, 26) as judged from SDS-PAGE (13). α-Amylase was also purified to homogeneity (27). Antibodies against pure antigens were raised in rabbit as already described (28). Antibodies directed against α-amylase revealed, in human pancreatic microsomes, one band at 55 kDa (8), a migration compatible with that of α-amylase (27). Antibodies directed against BSDL (referred to as *pAbL64*) detected, in human pancreatic juice, one band associated with an M_r ≈100 kDa (13). *pAbL64* also reacted with the oncofetal variant of BSDL (*i.e.* FAPP) (13).

Polyacrylamide Gels and Western Blotting—Polyacrylamide gel electrophoreses (10% acrylamide, 0.1% sodium dodecyl sulfate, SDS-PAGE) were performed according to Laemmli (29). Proteins were electrophoretically transferred to nitrocellulose membranes (30) and probed using polyclonal antibodies specific for pancreatic proteins as primary antibody. The antigen-antibody complexes were then detected by ^{125}I-protein A (0.25 μCi/ml) overlay. After extensive washing, replicas were autoradiographed.

RNA Extraction, RT-PCR, Northern Dot-blots, and in Vitro Translation—Total RNA was extracted from pellets of human pancreatic cells or tissues using the method of Chirgwin *et al*. (31). RNA were reverse-transcribed as described by Roudani *et al*. (32). The cDNA(−) pool obtained was amplified by performing a polymerase chain reaction using a pair of primers designed to cover the entire sequence of secreted BSDL (3, 4) (BSDL-5′,5′-TTCGTaagcttGCGAAGCTGGGCGCCGTGT-ACAGAA; BSDL-3′,5′-TTTGTgaattcACGCTAAACCTAATGACTGCA-GGCATCTG) and using the GC-rich PCR kit from CLONTECH. These two primers include *Hin*dIII and *Eco*RI restriction sequences (lowercase letters), which were used for the subsequent cloning of transcripts. Bases were randomly added in 5′ of these primers to allow restrictive cleavage of PCR transcripts. The DNA was amplified using a 35 reaction cycles program as follows: denaturation (94 °C, 1 min), annealing (64 °C, 1 min), and extension (68 °C, 4 min). The reaction was terminated by an incubation at 68 °C for 10 min. PCR products were then analyzed on 1% agarose gel. After purification, transcripts were subcloned into pCR 2.1 TOPO vector (Invitrogen) and sequenced using M13 forward and reverse or T7 primers. Alternatively, RNA was quantitated by dot-blot dilution. For this purpose, RNA was blotted after cascade half-dilutions on nitrocellulose membrane. Prehybridization and hybridization using specific cDNA probes for BSDL (22), α-amylase (a gift of Prof. J-C Chaix, Univ. Aix-Marseille III), and actin (a gift of Dr. R. Planells, INSERM-U38, Marseille) were performed essentially as described (32). Before hybridization, probes were ^{32}P-labeled by random priming (Life Technologies, Inc.) using [α-^{32}P]dCTP at a specific radioactivity of $4{\cdot}10^8$ cpm/μg DNA probe. *In vitro* translations of RNA were performed using the rabbit reticulocyte lysate system from Promega in accordance with the manufacturer's instruction. 10 μg of RNA were incubated with the reticulocyte lysate and [^{35}S]methionine (0.4 mCi/ml) in a final volume of 50 μl. After 2 h of incubation at 30 °C, the translated products were immunoprecipitated with *pAbL64* and analyzed by SDS-PAGE followed by autoradiography.

Transfection—Transcripts obtained by RT-PCR were digested by *Hin*dIII and *Eco*RI and ligated into pSecTag expression vector (Invitrogen). Stable transfection of CHO-K1 cells was performed with the pSecTag vector comprising RT-PCR transcripts and using the LipofectAMINE-mediated transfection procedure according to the manufacturer (Life Technologies, Inc.). The selection of stable clones was performed for 6 weeks in Ham's F12 medium with zeocin (500 μg/ml, Invitrogen). Control cells, transfected with the empty pSecTag vector, were cloned under the same conditions.

Immunofluorescence—Cells grown to confluence on microscope slides were washed three times with incomplete PBS buffer and fixed with 3% (v/v) paraformaldehyde for 20 min. The excess paraformaldehyde was eliminated by washing slides in 50 mM NH_4Cl, and cells were perme-

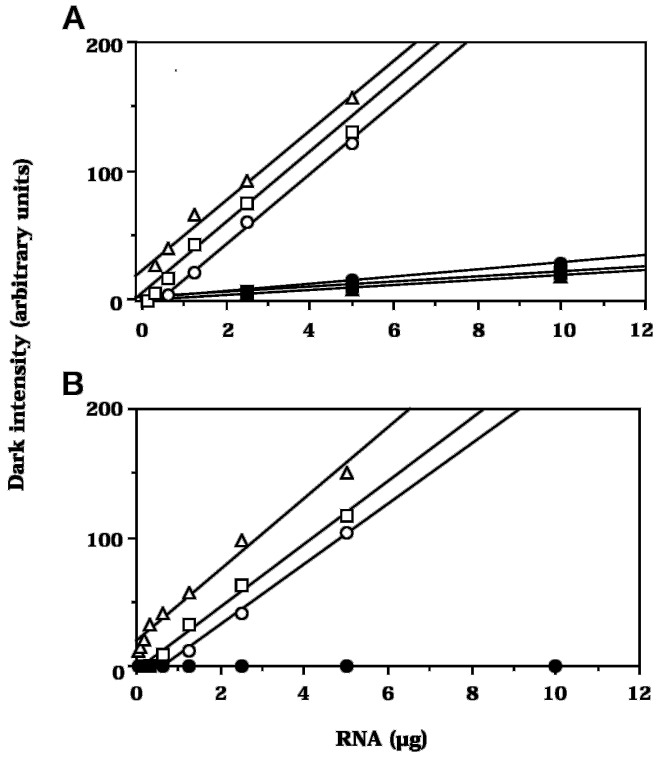

FIG. 1. **mRNA of pancreatic enzymes in pancreatic tumoral cell lines and tissue.** RNA was extracted from pancreatic cell lines and from human tumoral tissue (*filled symbols*) and normal pancreatic tissues from three different donors (*open symbols*). The RNA was dotted in decreasing rank order of half-dilution from 10 μg up to 0.015 μg. The presence of RNA encoding BSDL (*A*) and α-amylase (*B*) was detected using radiolabeled cDNA probes specific for each corresponding mRNA. Dots were then quantitated (dark intensity in arbitrary units) and plotted against the amount of RNA.

abilized with 0.05% saponin in PBS buffer. Nonspecific sites were saturated by cell incubation for 30 min in 10% calf serum. The cells were then incubated for the same time with polyclonal antibodies specific for BSDL (*pAbL64*) and for the p58 Golgi protein. Slides were then exhaustively rinsed with 0.05% saponin in incomplete PBS and incubated for 20 min with FITC-conjugated antibodies directed against rabbit immunoglobulins (10 μg/ml) and with TRITC-conjugated antibodies directed against mouse immunoglobulins (10 μg/ml). Slides were washed with 0.05% saponin in incomplete PBS and mounted in 25 mM Tris/HCl, pH 8.0, buffer, 75% glycerol, and 0.1% *p*-phenylenediamine illuminated using adequate filters of a fluorescence microscope and photographed.

RESULTS

Presence of BSDL mRNA in Pancreatic Tumoral Cells and Tissue—The presence of a BSDL messenger has been detected by *in situ* hybridization performed on SOJ-6 cells (22). Therefore, the presence of mRNA encoding pancreatic enzymes was studied in BxPC-3 and SOJ-6 pancreatic tumoral cell lines. Radiolabeled probes specific for BSDL and α-amylase were used to detect transcripts of respective enzymes. A negative reaction was found when RNA was hybridized with the α-amylase probe, whereas that of BSDL detected a specific transcript in tumoral cell lines (Fig. 1). BSDL and α-amylase transcripts were detected in normal adult pancreas, whereas transcript encoding BSDL was only detected in tumoral pancreas; values were similar to those found with cancer cells. These data suggested that BSDL mRNA was expressed in tumoral tissue and cancer cells, whereas that of α-amylase was not. When normalized to actin mRNA transcript, it appeared that tumoral tissue and cells expressed 20-fold less BSDL than normal pancreatic tissue.

BSDL Is Poorly Secreted by Tumoral Pancreatic Cells—The

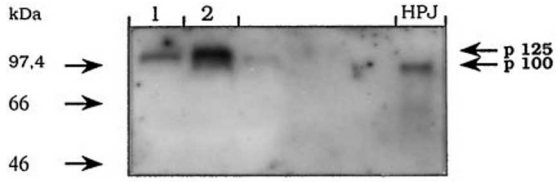

FIG. 2. **Bile salt-dependent lipase in culture medium of tumoral cell lines.** BxPC-3 and SOJ-6 cells were incubated in fresh medium for 6 h. For each cell line, 30 μl of culture medium were withdrawn, separated by SDS-PAGE, electrotransferred to a nitrocellulose membrane, and analyzed by Western blot using *pAbL64* as the primary antibody. The antigen-antibody complexes were then detected using ^{125}I-protein A and autoradiography. *HPJ*, human pancreatic juice.

secretion of BSDL was first investigated by Western blotting using polyclonal antibodies specific for BSDL and ^{125}I-protein A overlay. This very sensitive assay allowed us to detect BSDL in cell culture medium of BxPC-3 and SOJ-6 cell lines (Fig. 2). The migration of BSDL secreted by these tumoral cells, although diffuse, appeared lower (*arrow*, 110–125 kDa, p125) than that of the protein (*arrow*, 100 kDa, p100) present in normal human pancreatic juice (Fig. 2, *HPJ*). This could be related to high M_r glycoforms of BSDL also referred to as concanavalin A reactive forms (13, 18), which preferentially display the *J28* epitope. Two immunoreactive forms of BSDL were detected in cell culture of SOJ-6 cells, the lower M_r form having the same electrophoretic migration than that of the protein detected in normal pancreatic juice (100 kDa).

Second, BxPC-3 and SOJ-6 cells were incubated for 6 h in fresh medium, and BSDL activity was then recorded on 4-NPH. This activity was found in cell culture medium of SOJ-6 cells, whereas no activity could be detected in that of BxPC-3. BSDL activity represented approximately 12% of the total esterolytic activity detected in SOJ-6 cell line (Table I). The presence of BSDL in the extracellular medium did not correlate with that of the cytoplasmic marker LDH, the activity of which never exceeded 5% of its intracellular activity. This indicated that cell lysis may not be responsible for the presence of extracellular BSDL activity. Because BSDL activity represented at least 85% of the total esterolytic activity associated with tumoral cells (22), it seems that only a minute amount of BSDL activity was secreted by SOJ-6 cells. Attempts to detect α-amylase in the cell culture medium by Western blot or by recording activity were unsuccessful.

BSDL Is Associated with Membranes in Pancreatic Tumoral Cells—We next attempted to determine whether the BSDL expressed by tumoral cells was associated with membranes, as found in normal pancreatic tissue (8). For this purpose, cells were grown to 80% confluence and lysed, and the lysate was clarified as described previously (22). The pellet was then solubilized, in the same volume as the clarified lysate, with a 10 mM Tris/HCl, pH 7.4 (0.1% SDS), buffer. The presence of BSDL was then analyzed in clarified lysate and solubilized pellet. As shown in Fig. 3, BSDL could be detected in both fractions obtained from BxPC-3 and SOJ-6 cell lines. The detected protein migrated at the same position as BSDL present in microsomes isolated from a normal human pancreas (Fig. 3, *HPM*). From this figure, it is obvious that BSDL (*arrow*, p100) was mainly associated with the membrane pellet. Interestingly, the p46 immunoreactive form of BSDL (22) was detected in clarified lysates of BxPC-3 and SOJ-6 cells (*arrowhead*, p46). This pattern is similar to that obtained with the clarified lysate of normal pancreatic tissue (22). The release of BSDL from membranes can be obtained by treatment of the pellet with a 0.1 M sodium carbonate buffer (pH 10.0) or 0.25 M KBr (not shown). This suggests that the association of BSDL with

TABLE I
Secretion of BSDL by cell lines and transfected CHO cells

Cells were cultured in adequate fresh medium for 6h. Then, BSDL activity was recorded in cell-free medium (extracellular activity), and cells were harvested and lysed before determination of BSDL activity (intracellular activity).

Cells	Activity (10^{-3} units)		Extracellular activity (% Total activity)
	Intracellular	Extracellular	
SOJ-6	29.5 ± 1.3	4.1 ± 0.7	12.2 ± 2.4
BxPC-3	45.1 ± 4.1	0	0
CHO-pSecFAPP	2.8 ± 0.7	3.6 ± 0.4	56.3 ± 4.7
CHO-pSecBSDL	11.2 ± 2.0	130.7 ± 4.6	92.1 ± 1.4

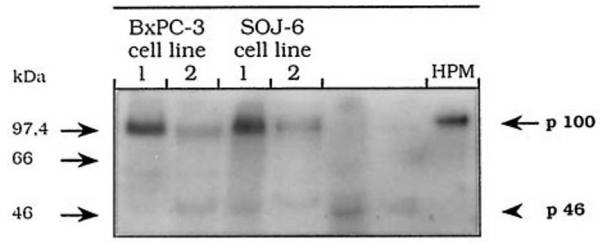

FIG. 3. **Presence of bile salt-dependent lipase in tumoral cell lines.** Proteins (50 μg) from membrane fraction (*lane 1*) and soluble fraction (*lane 2*) were separated on SDS-PAGE and electrotransferred to a nitrocellulose membrane. The analysis and detection were then performed as described in Fig. 2. *HPM*, human pancreatic microsomes.

membranes involves ionic interactions, as already described in normal human pancreatic tissue (8). Attempts to detect α-amylase using specific antibodies and specific enzymatic assays were unsuccessful.

The distribution of BSDL inside cells has been examined by immunofluorescence studies using *pAbL64* antibodies on permeabilized SOJ-6 cells. Although BSDL appeared dispersed throughout the ER, which in pancreatic cells largely occupied the cytoplasm (Fig. 4*C*), a typical Golgi region with a punctated staining around the nucleus was also revealed (Fig. 4*C*). Antibodies directed against the p58 Golgi protein detected identical structures around the nucleus (Fig. 4*D*). This pattern indicated that in SOJ-6 cells, BSDL colocates with Golgi probe and, as a consequence, would be sequestrated within this compartment.

Sequence of the PCR Transcript—The next objective was to obtain the entire BSDL transcript sequence expressed by tumoral cells. Because the *mAbJ28*, which characterizes FAPP (14), recognized a carbohydrate-dependent epitope (20), the screening of a cDNA library was precluded. Consequently, a pair of primers (BSDL-5′ and BSDL-3′) covering the entire sequence of the mature BSDL was used. As shown in Fig. 5, transcripts of approximately 1.8 kb were obtained using RNA extracted from BxPC-3 and SOJ-6 cells. Transcripts obtained from SOJ-6 cells were cloned into the pCR2.1 TOPO vector, amplified, and sequenced. Both strands of two different clones obtained from independent RT-PCR experiments were sequenced to confirm dissimilarities. The sequence matched 99.8% that of human BSDL including the sequence of exons 1–10, from nucleotide 1 to nucleotide 1424 (3). Two mutations, T→C and C→T at nucleotides 489 and 612, respectively, which did not change the amino acid sequence (residues Gly-163 and Thr-204, see below and Fig. 8) were detected. However, the sequence of the SOJ-6 transcript corresponding to that of exon 11, which encodes C-terminal tandem-repeated sequences of BSDL, showed a major difference (Fig. 6). Although the SOJ-6 transcript sequence from nucleotide 1425 to 1736 matched 99.7% that of exon 11 of BSDL up to the end of repeat number 4 (nucleotide 1736), it seemed that 330 bp were deleted. Two other repeated sequences, from nucleotide 1737 to 1803, were detected in the cancer cell transcript. The sequence of these two

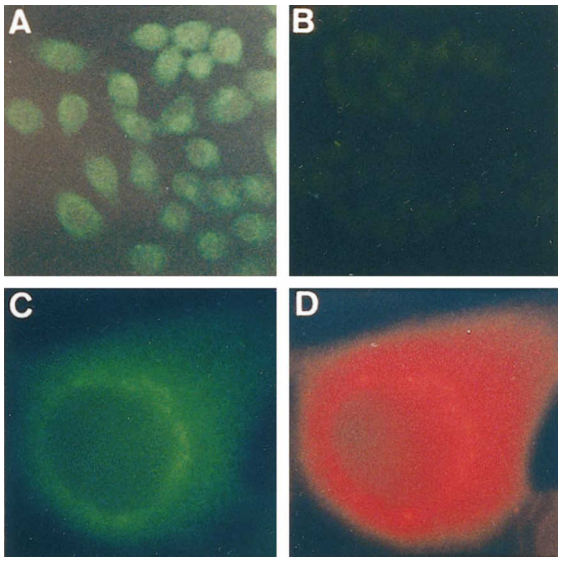

FIG. 4. **Detection of BSDL in SOJ-6 cells.** Detection of BSDL in SOJ-6 cells using *pAbL64*. *A*, SOJ-6 cells labeled with *pAbL64* and FITC-conjugated antibodies directed against rabbit immunoglobulins. *B*, SOJ-6 cells treated as in *A* but omitting *pAbL64* prior to immunofluorescence (control). *C*, magnification of SOJ-6 cells treated as in *A* prior to immunofluorescence. *D*, SOJ-6 cells labeled with mouse antibodies specific for the p58 Golgi protein and TRITC-conjugated antibodies directed against mouse immunoglobulins.

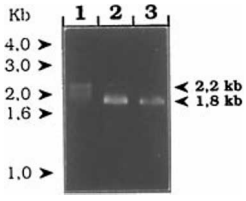

FIG. 5. **Amplification of RNA specific for bile salt-dependent lipase.** RNA was extracted from SOJ-6 (*lane 2*) and BxPC-3 (*lane 3*) cells. 1 μg of each RNA was reverse transcribed and amplified using sense and antisense primers designed to cover the entire sequence of the mature BSDL. Amplification products were separated on 1% agarose gel. *Lane 1* represents the migration of the normal transcript of BSDL (2.2 kb).

repeats was compared with that of repeats 1 to 16 of BSDL, successively. As shown in Table II, the sequence matched that of repeats 15 and 16 of BSDL. The remaining sequence of the SOJ-6 transcript, from nucleotide 1804 to the stop codon (nucleotide 1836), was identical to that of BSDL. Consequently, the sequence of BSDL between nucleotide 1736 and nucleotide 2066 (or between nucleotides 2476 and 2806 according to the numbering used in Ref. 3) was deleted in the SOJ-6 transcript. This nucleotide deletion leads to the excision of 10 repeated sequences, indicating that repeats 5 to 14 were missing in the SOJ-6 transcript. Therefore, this transcript possessed tandem repeats 1 to 4 and 15 to 16 of BSDL (Fig. 6). Only one mutation, A→G, which changes Asp (Asp-709 according to Ref. 3) into Gly (599), was detected in the SOJ-6 transcript. Except for the deletion of the sequence encoding repeats 5 to 14, the sequence of the SOJ-6 transcript matched more than 99% that of BSDL (3). The translation of the corresponding mRNA might lead to a protein of approximately 70 kDa, which is less than that of the BSDL protein core (nearly 80 kDa). To ascertain this point, RNA isolated from normal human pancreatic tissue and tumoral cells was used to program a rabbit reticulocyte lysate *in vitro* translation system in the presence of [^{35}S]methionine. At the conclusion of the translation program, translated products were isolated by immunoprecipitation with *pAbL64* and resolved on SDS-PAGE. The autoradiogram given in Fig. 7

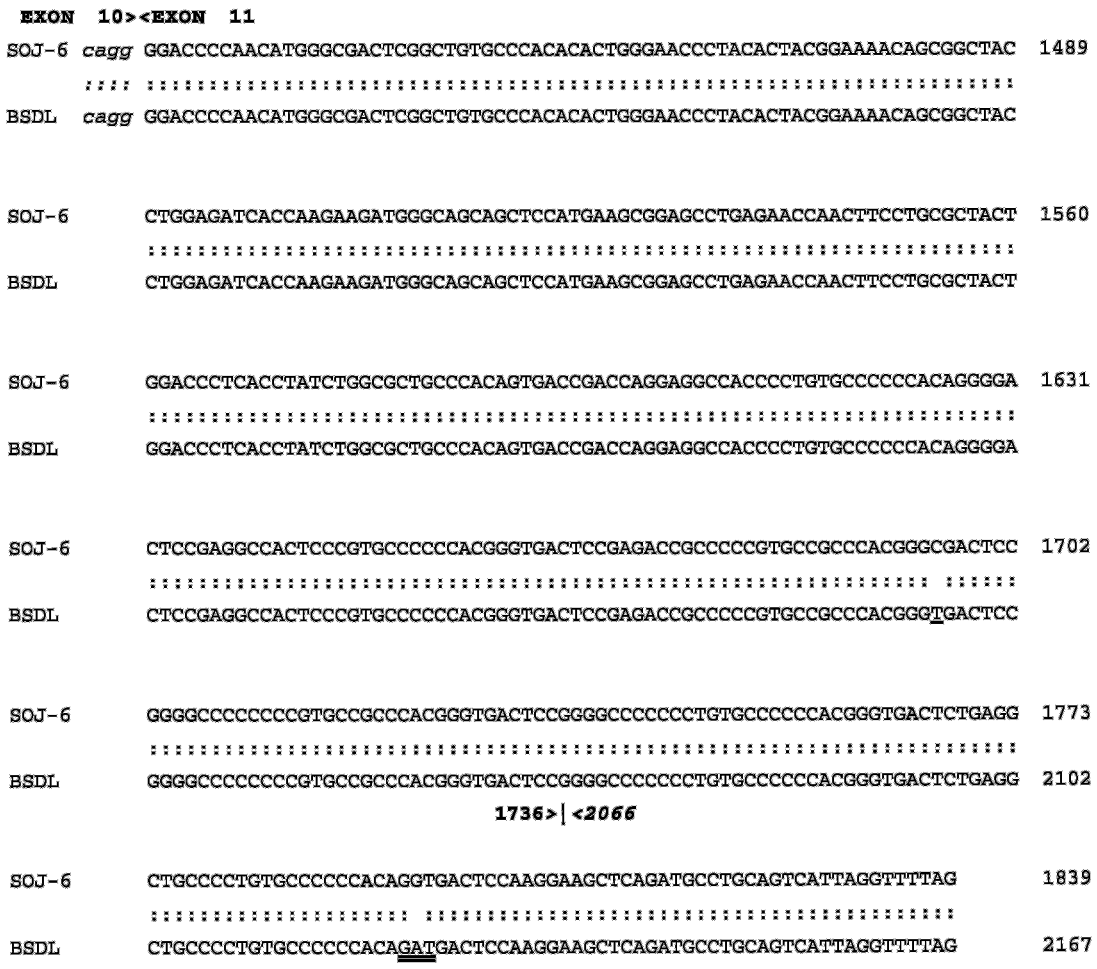

FIG. 6. **Nucleotide sequence of the RT-PCR transcript from SOJ-6 cells.** The RT-PCR transcript obtained from SOJ-6 was purified and cloned into pCR2.1 TOPO vector and sequenced. Only the sequence corresponding to the exon 11 of BSDL is given. The *single underlined* nucleotide locates a mutation at position 1696, which has no effect on the amino acid sequence, whereas *double underlined* nucleotide shows mutated codon GAT (Asp) to GGT (Gly). The sequence deleted in the transcript isolated from SOJ-6 cells between nucleotide 1736 and 2066 is indicated.

TABLE II

Comparison of repeated sequences 5 and 6 of the SOJ-6 transcript with the 16 repeated sequences of BSDL

The sequence of repeats from nucleotides 1737–1803 (GGGGCCCCCCCTGTGCCCCCCACGGGTGACTCTGAGGCTGCCCCTGTGCCCCCCACAGGTGACTCC) of the SOJ-6 transcript was successively compared with repeats 1–16 present in the cDNA of BSDL (3, 4).

BSDL repeat	Nucleotides		Match
	Start	End	
			%
1–2	1608	1675	85.9
2–3	1641	1704	85.9
3–4	1676	1737	83.9
4–5	1705	1765	86.4
5–6	1718	1803	86.4
6–7	1771	1836	86.4
7–8	1804	1869	84.6
8–9	1837	1902	84.6
9–10	1870	1935	83.3
10–11	1903	1968	81.5
11–12	1936	2001	83.3
12–13	1969	2034	84.8
13–14	2002	2067	87.9
14–15	2035	2097	86.9
15–16	2068	2133	98.5

showed that a reaction product can be immunoprecipitated as one band at approximately 80 kDa when the translation program used RNA extracted from normal human pancreatic tissue (p80, *lane 1*). The size of this product correlates with the translation of a 2.2-kb mRNA corresponding to that encoding BSDL (3). However, when RNA extracted from pancreatic tumoral cells were translated under identical conditions, a protein migrating at approximately 70 kDa was detected (Fig. 7, *lane 2*, *arrowhead*). The size of this protein suggests that it could be the translation product of the 1.8-kb transcript. Further translational modifications such as *N*- and *O*-glycosylation lead to the 100–125-kDa protein (Fig. 2). Another band, weakly stained and associated with an $M_r \approx 90$–100 kDa, can be immunoprecipitated from the translated material using RNA of tumoral cells. It is possible that RNA extracted from tumor cells encoded two different forms of BSDL. However, this material could also represent part of the membrane-folding complex of BSDL, which includes the chaperone Grp94-related 94-kDa protein (8, 9).

Examination of the BSDL sequence, deduced from the transcript obtained from SOJ-6 cells, indicated that the four Cys residues involved in disulfide bridges (Cys-64 linked to Cys-80 and Cys-246 linked to Cys-257), the amino acids involved in the catalytic site (Ser-194, Asp-320, and His-435) and the site for *N*-linked glycosylation (Asn-186) are well conserved. These data strongly suggested that the N-terminal domain of this protein should be correctly folded and, consequently, enzymatically active. The deletion of the repeats 5 to 14 leads to the presence of only six putative sites for *O*-linked glycosylation (4, 33) on Thr-538, -549, -559, -576, -587, and -577 (Fig. 8). When the amino acid sequence of the SOJ-6 transcript was compared

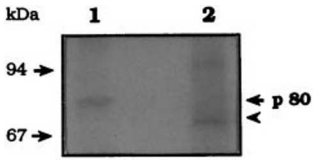

FIG. 7. ***In vitro* RNA translation.** RNA (10 μg) isolated from human pancreatic tissue and from tumor cells was used to program a rabbit reticulocyte lysate for *in vitro* translation in the presence of [^{35}S]methionine. After the required time of incubation, translated products were immunoprecipitated with *pAbL64* and resolved on SDS-PAGE. *Lane 1*, translated product using RNA from normal human pancreatic tissue. *Lane 2*, translated product using RNA from tumor cells. Control experiments from which no translated product was precipitated with *pAbL64* were carried out omitting template RNA (not shown).

with the sequence of the human pancreatic BSDL (3), similarities were obvious. The alignment (Clustal W program, Infobiogen, Paris) also suggests that repeats 5 and 6 of the SOJ-6 transcript matched repeats 15 and 16 of BSDL (Fig. 9).

The SOJ-6 Transcript Likely Encodes FAPP—As mentioned above, tumoral cells secreted, although poorly, a high M_r variant of BSDL. Two high M_r glycoisoforms of BSDL were previously isolated. The first one was a concanavalin A-reactive or ConA-reactive form (18), the second one was reactive with the *mAbJ28* and was referred to as FAPP (13). We have further shown that the ConA-reactive form of BSDL was related to FAPP (18). Both the ConA-reactive form of BSDL and FAPP were poorly active (13, 18). They also differed from the normal form of BSDL (ConA-unreactive and *mAbJ28*-unreactive) at the level of amino acids involved in tandem-repeated sequences, which are located on the C-terminal peptide (13, 18). The amount of sugar involved in the minimal *O*-linked structure, such as galactose and *N*-acetylgalactosamine residues, was largely decreased in FAPP (13) and in the ConA-reactive fraction as well (18), where it seems that no more than six *N*-acetylgalactosamine residues may initiate *O*-linked oligosaccharide structures (20). On BSDL, 14–16 sites for *O*-linked glycosylation, which are located on the C-terminal tail of BSDL, were described (18, 33). Only six of those sites were still present on the sequence of the SOJ-6 transcript (see Fig. 8). Therefore, the amino acid composition of the C-terminal peptide isolated from the ConA-reactive form of BSDL was compared with that deduced from the sequence of the SOJ-6 transcript from Met-510 to Met-606 (Fig. 8). As shown in Table III, the amino acid composition (in % of total amino acid) of the C-terminal peptide of the ConA-reactive form of BSDL was, within experimental error, very close to that deduced from the sequence of the SOJ-6 transcript. Of course, the experimental results obtained with the C-terminal tail of the ConA-reactive fraction could be partially erroneous due to contamination by an amino acid such as Gly or to some degradation of an amino acid such as Thr. However, the amino acid composition of the C-terminal peptide of BSDL expressed in SOJ-6 cells seemed far different than that of the C-terminal tail of the ConA-unreactive BSDL (18) or that predicted from the cDNA of BSDL (3). Consequently, one may suggest that the cDNA transcript isolated from SOJ-6 cells would encode FAPP.

Transfection of CHO Cells—To determine whether the impaired secretion of FAPP by tumoral cells was due to the shortened C-terminal tail compared with BSDL, the transcript obtained from SOJ-6 cells was subcloned, in frame, into the pSecTag vector; the plasmid thus obtained, referred to as pSecFAPP, carries the V-J2-C region of the mouse Ig*K* chains, which might drive expressed proteins toward secretion. The pSecFAPP plasmid was transfected into CHO-K1 cells. A clone expressing FAPP was selected in the presence of zeocin. As shown in Fig. 10*A*, immunofluorescence studies using *pAbL64* revealed that the enzyme was expressed by pSecFAPP-transfected CHO-K1 cells (CHO-pSecFAPP), whereas CHO-pSecTag transfected control cells displayed no reactivity (Fig. 10*B*). The labeling of CHO-pSecFAPP transfected cells with *pAbL64* (Fig. 10*C*) located in structures where the staining of the p58 Golgi protein was also detected (Fig. 10*D*). Therefore, these structures likely represent Golgi stacks. The full-length cDNA of the human BSDL, including the sequence coding for the 16 C-terminal repeats, in pS429 (34) was amplified by PCR using primers BSDL-5′ and BSDL-3′. The transcript of 2.2 kb was subcloned directly into pSecTag under the same conditions as the 1.8-kb transcript amplified from SOJ-6 cells. The plasmid (pSecBSDL) was also transfected into CHO-K1 cells, and a homogeneous clone (CHO-pSecBSDL) was selected.

To determine whether the translated products can be secreted, CHO-pSecFAPP and CHO-pSecBSDL cells were allowed to stand in fresh RPMI medium for 6 h. At the conclusion of the incubation, the cell-free medium was withdrawn and stored. Cells were washed, harvested, pelleted, and lysed. The lysate was cleared, and BSDL and LDH activities were recorded in cell-free medium and lysate. The LDH activity recorded in the extracellular medium of the two clones was very low (<5% of total activity) and indicated that no lysis of transfected cells occurred during the incubation time. As shown in Table I, secreted BSDL activity (corrected for the endogenous esterolytic activity of control cells transfected with the empty pSecTag vector), which represents 56% of the total esterolytic activity expressed in CHO-pSecFAPP cells, was detected in the cell-free medium. This value was higher than those determined with tumoral cells but lower than those recorded in extracellular medium of CHO-pSecBSDL. These data strongly suggest that the FAPP, which contains six repeated sequences, can be secreted once transfected into CHO-K1 cells.

DISCUSSION

Most of the human pancreatic cell lines were established from tumors displaying a ductal morphology. This is not surprising, as 90% of human pancreatic tumors are adenocarcinoma, with most of these presenting a ductal phenotype (35). A significant variability in the expression of different biochemical markers has been observed. Ductal phenotype marker such as carbonic anhydrase II was detected in some cell lines such as BxPC-3 but not in PANC-1 (36). Trypsinogens 1 and 2, which are markers for the acinar phenotype, were detected in CFPAC-1 and CAPAN-1 cells but not in PANC-1 cells (37). In a previous study, we showed that human pancreatic tumoral cell lines BxPC-3 and SOJ-6 and human pancreatic adenocarcinoma tissue expressed a 46-kDa (p46) immunoform of BSDL, whereas the 100-kDa (p100) immunoform could not be detected in clarified cell extracts (22). In this study, we demonstrated that the p100 immunoform of BSDL is expressed by BxPC-3 and SOJ-6 cell lines. This protein is also expressed by human pancreatic adenocarcinoma, and BSDL was mainly found associated with intracellular membranes of tumoral cells as also described in normal human pancreatic tissue (8). Providing an explanation to previous results (22), the p100 immunoform of BSDL cannot be detected in the soluble fraction of tumor cells, whereas the p46 immunoreactive form partitioned between soluble and membrane fractions. The expression of BSDL by SOJ-6 cells was ascertained by RT-PCR and DNA sequencing. PCR amplification, using RNA extracted from BxPC-3 cells, also confirmed the presence of a BSDL mRNA in these cells. Attempts to detect α-amylase were all negative. Consequently, it is suggested that BSDL is expressed in tumoral pancreatic cell lines and in tumoral pancreatic tissue, whereas α-amylase cannot be detected. The still unanswered question concerns the nature of the p46 immunoreactive form of BSDL, which was

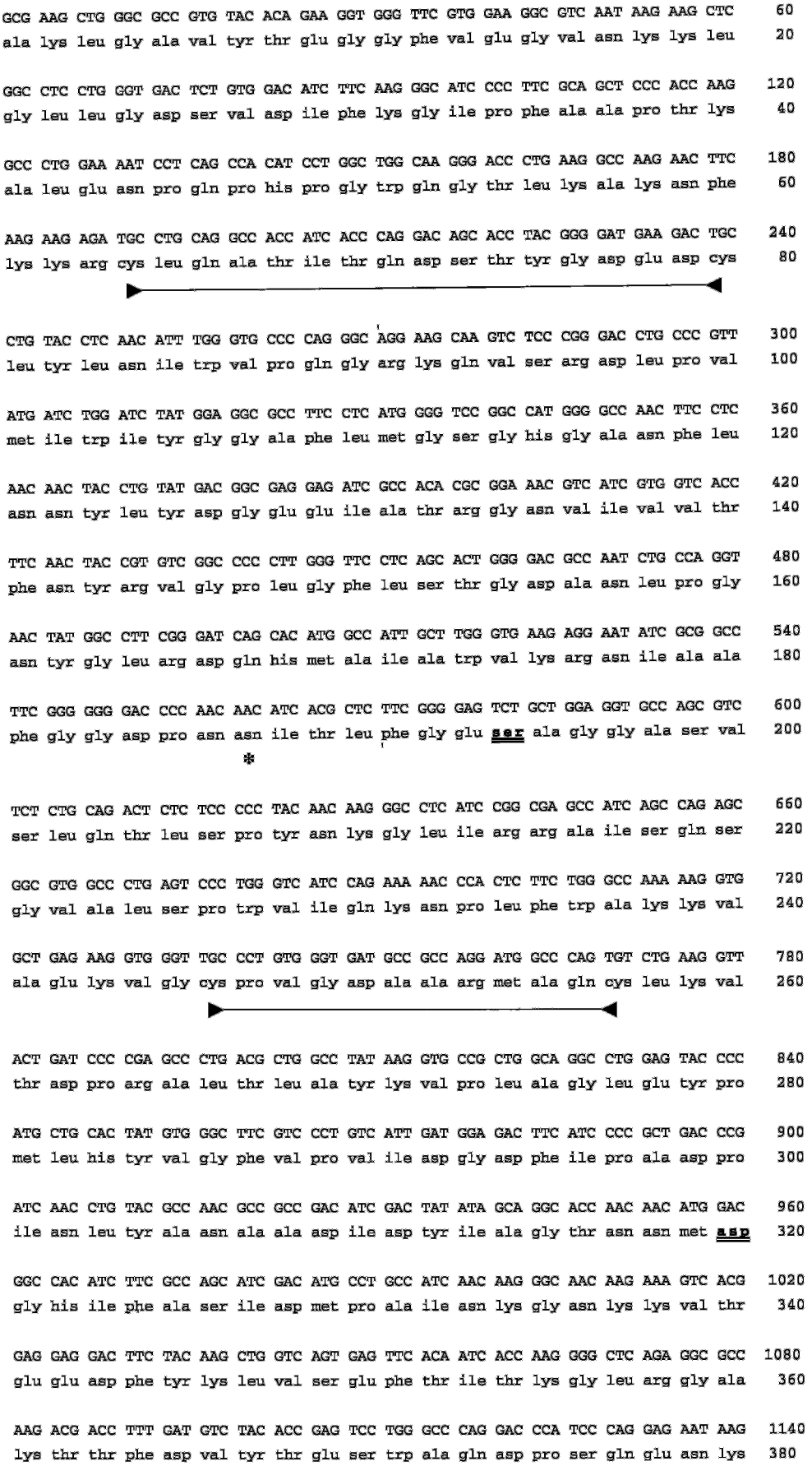

FIG. 8. **Amino acid sequence of the BSDL expressed by SOJ-6 cells.** The amino acid sequence of the BSDL expressed in SOJ-6 cells was deduced from the cDNA sequence of the RT-PCR transcript. *Bold double underlined* amino acids are involved in the catalytic site; *arrowheads* indicate disulfide bridges. The *asterisk* and *dots* indicate putative sites for *N*- and *O*-linked glycosylation, respectively. *Numbers* locate tandem-repeated sequences. Residue Gly-599 in *bold* represents the mutated residue Asp → Gly.

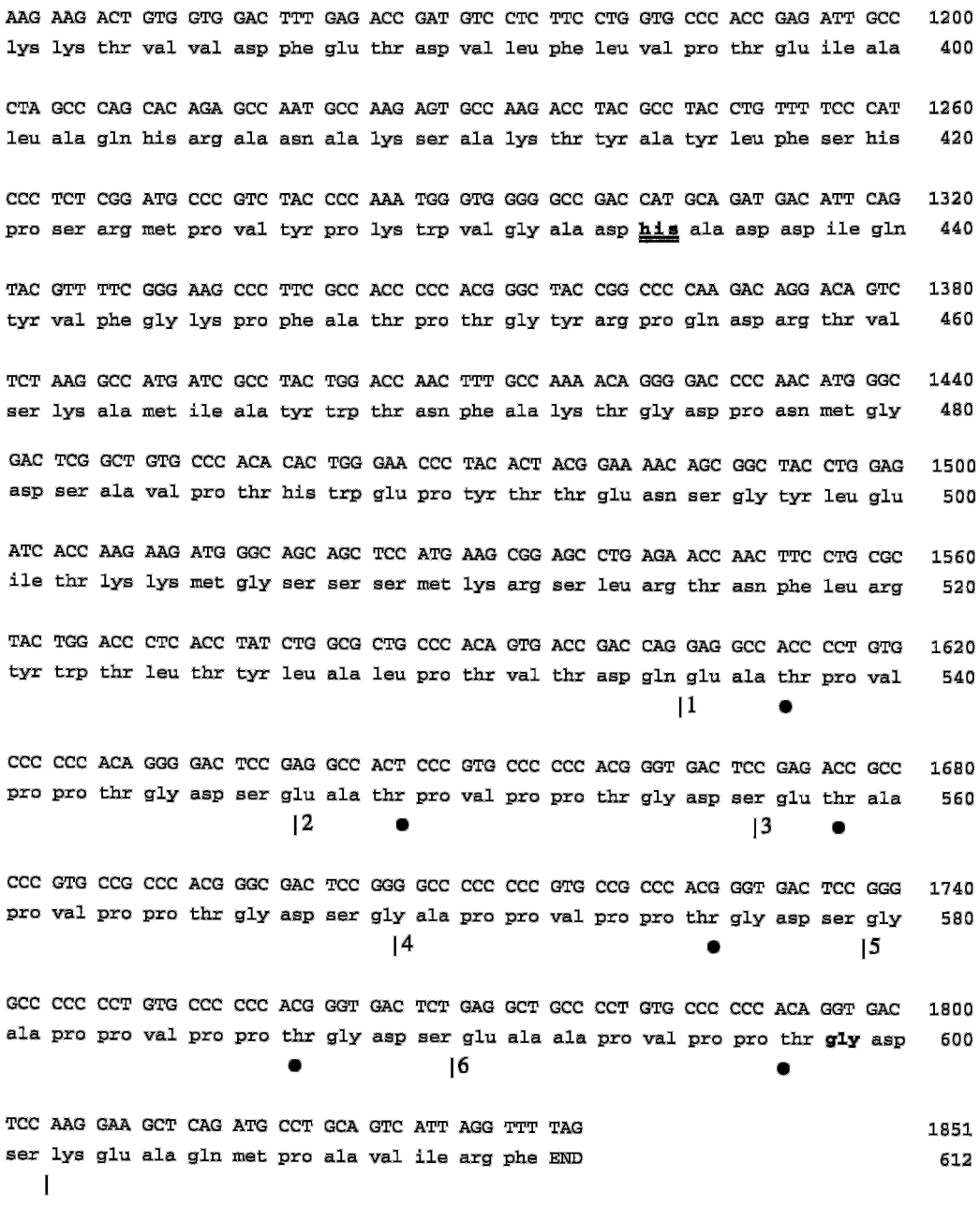

FIG. 8—*continued*

detected in tumoral pancreatic cells (Ref. 22 and this study). In light of recent data showing that BSDL secretion involves an association with a membrane-folding complex, including proteins of 94 kDa (Grp94-related p94 protein) and 46 kDa (9), one may wonder if p46 is complexed with BSDL within cancer cells and therefore coprecipitates with the enzyme. Accordingly, p46 may not be related to BSDL, and studies are in progress to clear this specific point. We went further in this investigation and examined the secretion of the p100 immunoreactive form of BSDL. For this purpose, BSDL activity was recorded in the culture medium of SOJ-6 cells. Extracellular LDH activity was extremely weak, and consequently cell lysis may not be responsible for the presence of BSDL in SOJ-6 cell culture medium. Thereby, immunoreactive forms of BSDL associated with $M_r \sim$ 100,000 and 125,000 were detected in culture medium of SOJ-6 cells. Although no BSDL activity was recorded in BxPC-3 cell culture medium, Western blots allowed us to detect the protein. An explanation for that could be the different sensitivity of methods used (colorimetric enzyme assay and ^{125}I-protein A overlay) or a poor activity of BSDL expressed by tumoral cells. The high M_r form of BSDL (125 kDa) was the unique form detected in BxPC-3 cell culture medium.

Therefore, tumoral cells expressed a high M_r variant of BSDL, the nature of which was further investigated. The cDNA covering the entire sequence of BSDL expressed in SOJ-6 cells was obtained by RT-PCR, and data indicated that the N-terminal domain encoded by exons 1–10 was strictly identical to that of BSDL expressed by normal human pancreas (3). However, the size of the C-terminal region encoded by the SOJ-6 transcript was shorter than that of BSDL (3), and a deletion of 330 bp was observed. The 1.8-kb cDNA fragment, amplified from the SOJ-6 cell RNA, could be produced by eliminating a large loop occurring in the 2.2-kb mRNA structure in RT or in PCR reactions. Prediction of the putative secondary structures of mRNA sequence coding for the C-terminal repeats of BSDL was performed according to the stems-and-loops program (Stemlo program, Infobiogen) and the free energy minimization model for RNA folding (38). Both programs predict that stems

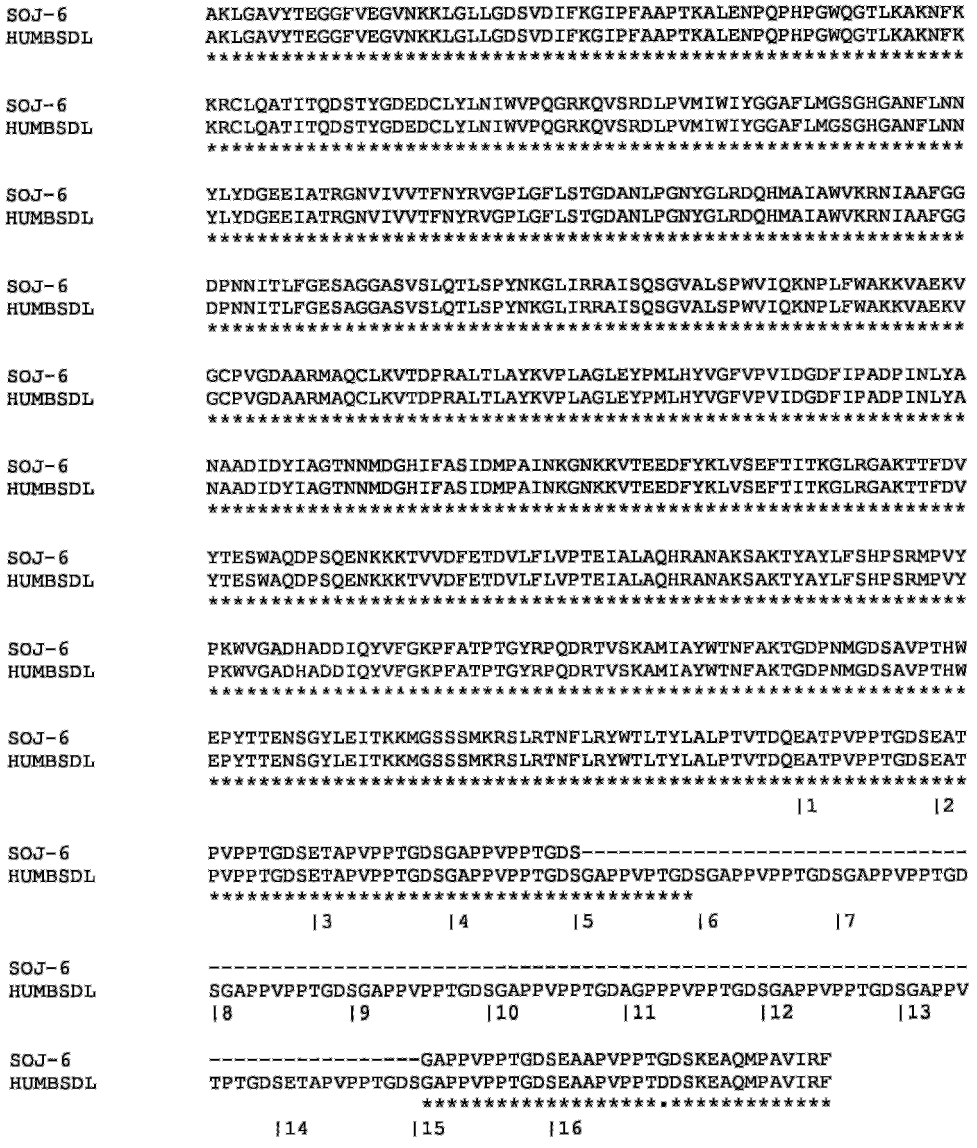

FIG. 9. **Comparison of the amino acid sequence of the SOJ-6 transcript and BSDL.** The amino acid sequence deduced from the SOJ-6 transcript (SOJ-6) was compared with that of the human pancreatic BSDL (BSDL) (3). Matches are indicated by an *asterisk*, mismatching amino acids are indicated with a *dot*, and the deleted sequence is denoted by the *dashed line*. *Numbers* locate tandem-repeated sequences.

and loops can be formed within this GC-rich sequence. However, neither of them lead to the excision of a 330-bp loop giving rise to the exact deletion of ten repeats. Moreover, a reading frameshift with the occurrence of new stop codons would occur. However, the amplification of a 2.2-kb transcript of BSDL has been obtained from the complete cDNA (*i.e.* including the 16 repeated sequences) using the same protocol. This ruled out any PCR artifacts. Northern blot experiments, using a probe that hybridizes with the 5′ region of the BSDL mRNA, suggested that an mRNA strand of approximately 1.8–2.0 kb was often associated with the 2.2-kb mRNA encoding for human BSDL (3, 32, 39). In this study using pancreatic tumoral cells, the shorter mRNA could have been amplified as the 1.8-kb cDNA. The translated product corresponding to this mRNA should have a protein core of approximately 70 kDa as indeed was found after *in vitro* translation of RNA from tumoral cells. However, taking into account possible post-translational modifications of the protein (9, 40), it is conceivable that variants of BSDL expressed by tumoral cells may be the product of this short mRNA, which possibly lacks the nucleotide fragment encoding repeats 5 to 14. Obviously, glycan structures present on this protein should be larger than those present on BSDL and may correlate with previous data showing that the carbohydrate moiety of FAPP accounted for some 47% of its apparent mass (14). All observations and results (13, 18, 20, 33) suggested that the cDNA transcript isolated from SOJ-6 cells would encode for the feto-acinar glycoisoform of BSDL, referred to as the feto-acinar pancreatic protein or FAPP (13, 14).

Previous studies have shown that FAPP (characterized by its reactivity with the *mAbJ28*) cannot be detected in conditioned medium of pancreatic tumoral cells (14, 21). It has been postulated that the absence of secretion was due to the retention of the protein within the ER and suggested that FAPP would remain associated with ER resident protein(s) as a consequence of its improper folding (21). We have further shown that a multiprotein membrane complex including the chaperone Grp94-related p94 protein may play an essential role in the folding and transport of BSDL (9). Moreover, the glycosylation of the C-terminal tandem-repeated sequences regulates the secretion of BSDL (11). The distribution of FAPP in tumoral cells indicated that the protein, which is mainly membrane associated, distributes within the ER and the Golgi where FAPP colocates with the p58 Golgi protein. These data suggested that FAPP would be sequestrated within either of these

TABLE III
Amino acid composition of C-terminal fragment of BSDL

Residue	C-terminal fragments			
	Predicted[a]	Reported[b]		Deduced[c] SOJ transcript
		ConA-unreactive	ConA-reactive	
Ala	9.2	9.1	8.3	9
Asx	9.2	9.3	8.8	8
Arg	1.5	1.6	2.7	3
Gly	12.6	12.4	16.5	8
Glx	3.9	3.6	7.7	7
His	0	0	0.7	0
Ile	0	0	1.5	0
Leu	2.4	2.8	4.2	5
Lys	1.0	0.7	2.8	2
Met	0.5	0	0.4	1
Phe	0.5	0.9	1.9	1
Pro	29.1	30.1	18.6	21
Ser	7.8	7.0	9.0	7
Thr	12.6	12.5	8.6	14
Tyr	1.0	1.1	1.6	2
Trp	0.5	ND	ND	1
Val	8.3	8.7	6.7	7
Cys	0	ND	ND	0
Total	100.1	99.8	100	96

[a] The predicted amino acid composition indicates the amino acid composition corresponding to that of the C-terminal peptide of BSDL from Met-510 to Met-716 (3).

[b] Reported amino acid composition of the C-terminal peptide of BSDL either unreactive (ConA-unreactive) or reactive (ConA-reactive) to concanavalin A (18).

[c] The amino acid composition of the C-terminal fragment of SOJ-6 cells was deduced from the sequence of the cDNA transcript from Met-510 to Met-606 (see Fig. 8). ND, not determined.

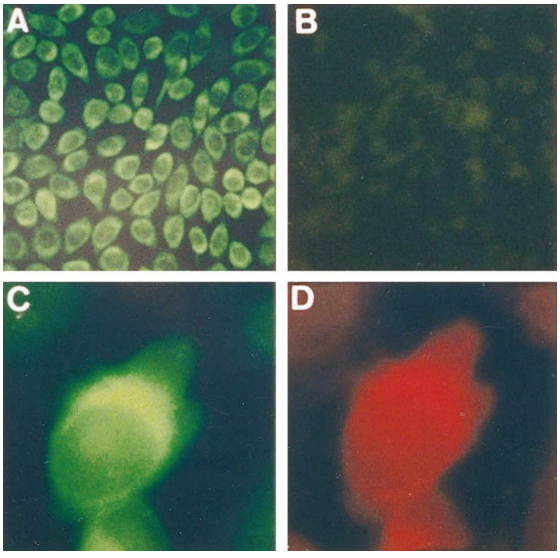

FIG. 10. **Transfection of CHO-K1 cells.** The RT-PCR transcript obtained with SOJ-6 cells was cloned into pSecTag vector and used to transfect CHO-K1 cells. *A*, immunofluorescence using *pAbL64* and FITC-conjugated antibodies directed against rabbit immunoglobulins was performed on an homogeneous transfected clone. *B*, control CHO cells transfected with the empty pSecTag vector. *C*, magnification of transfected CHO-K1 cells treated as in *A* prior to immunofluorescence. *D*, transfected CHO-K1 cells labeled with mouse antibodies specific for the p58 Golgi protein and TRITC-conjugated antibodies directed against mouse immunoglobulins.

compartments and corroborated the fucosylated nature of the oncofetal epitope recognized by *mAbJ28*, which is present on this protein (14, 20). Accordingly, the retention of FAPP in tumoral cells could be inherent to properties of the protein, in part to the shortened C-terminal tail. Indeed, the transfection of CHO-K1 cells with the cDNA encoding FAPP into an expression/secretion vector (CHO-pSecFAPP) led to the detection of the protein within the Golgi compartment, where the protein, as already found in SOJ-6 cells, was associated with membranes. As a consequence, the level of secretion of FAPP (encompassing 6 C-terminal repeated sequences) by transfected cells was lower than that of BSDL (including 16 repeated sequences). This confirms that the C-terminal tail of BSDL may regulate the secretion of the protein (11). However, the amount of FAPP secreted by CHO-pSecFAPP was relatively high when compared with that secreted by tumoral cells (see Table I). These results indicate that the retention of FAPP in tumoral pancreatic cells could not be inherent to the protein properties or to its improper folding. More likely, other function(s) essential for secretion processes could be altered in tumoral pancreatic cells.

The significance of the expression of FAPP (which is active on BSDL substrates (13)) in tumoral pancreatic tissue and cell lines remains to be elucidated. With regard to this point, BSDL transcripts were ubiquitously found in various human fetal cells and in human embryo as early as the 6th week of gestation (32). Therefore, one may suggest that tumoral pancreatic cells may have regressed back to a fetal-like differentiated state. As postulated for fetal cells (32), BSDL lipolytic activity could be essential for the metabolism of lipids during the intense growing phase of cancer cells.

Acknowledgments—We gratefully acknowledge Prof. J. R. Delpéro (Institut Paoli-Calmettes, Marseille) and Prof. J. C. Chaix (University Aix-Marseille III) for the kind gift of human pancreatic tissue and cDNA probe specific for α-amylase. We are also indebted to Dr. D. Gauteret (CNRS, Marseille) for help in computer modeling of the BSDL mRNA structure, to V. Sbarra for technical assistance, to Drs. M-J. Escribano and L. Bläckberg for the release of SOJ-6 cell line and of the cDNA encoding the full-length BSDL in pS429 vector, to Dr. F. Haigler for reading the manuscript, and to V. Mariottini for typing the manuscript.

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