Association of bile-salt-dependent lipase with membranes of human pancreatic microsomes is under the control of ATP and phosphorylation

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Bile-salt-dependent lipase (BSDL) is secreted by the pancreas into the duodenum, where it catalyses the hydrolysis of dietary lipid esters on activation by bile salts. The secretion pathway of BSDL is comparable with that of other digestive enzymes produced by pancreatic acinar cells. However, in contrast with these other enzymes, BSDL is partly associated with endoplasmic reticulum membranes as part of a folding complex, including a Grp94-related protein to which BSDL is transiently linked. The release of BSDL from membranes occurs once its glycosylation is completed [Bruneau and Lombardo (1995) *J. Biol. Chem.* **270**, 13524–13533]. In the present study, investigations concerning the mechanism of association/dissociation of BSDL with membranes of microsomes were performed. For this purpose the role of ATP and that of the possible phosphorylation of BSDL were examined. For the first time, it is shown that human pancreatic

BSDL is phosphorylated, probably at a serine residue, during its transport within the acinar cell. The phosphorylation of BSDL is provoked by calphostin C, an inhibitor of protein kinase C. In the presence of 1-(isoquinolinesulphonyl)-2-methylpiperazine, a non-specific inhibitor of serine/threonine protein kinase A, C or G, or of calcium chelator 1,2-bis(*O*-aminophenoxy)ethane-*N,N,N',N'*-tetra-acetic tetra(acetoxymethyl)ester, the phosphorylation of BSDL elicited by calphostin C is abolished. These data suggested that the phosphorylation of BSDL within human pancreatic microsomes is under the control of a cascade of protein kinases. We have also shown that the phosphorylation of BSDL appears to be involved in the release of the enzyme from microsome membranes. Nevertheless ATP, which modifies the conformation of BSDL, triggers this association, and an unhydrolysable ATP analogue was unable to promote it.

INTRODUCTION

Bile-salt-dependent lipase (formerly carboxyl ester hydrolase or cholesterol esterase; EC 3.1.1.–; BSDL) is secreted into the intestine where, once in the presence of bile salts, it catalyses the hydrolysis of lipid-soluble vitamin esters, cholesteryl esters, phospholipids and acylglycerols (for a review, see [1]). The physiological role of BSDL appears to be the facilitation of cholesteryl ester absorption, and probably lipid-soluble vitamin esters, by producing free alcohols that are easily absorbed [2,3]. The absorption of unesterified cholesterol is a saturable process [4], and the existence of proteins regulating this absorption has been hypothesized [5]. BSDL, in addition to its luminal hydrolytic role, has been proposed to be one of these regulatory proteins by acting as either a transport factor [6] or a cholesterol transfer protein [7]. It has also been postulated that BSDL could be involved in the esterification of cholesterol within enterocytes [8]. Immunological studies performed either on rat [9] or human [10] intestinal mucosa support this hypothesis.

All pancreatic digestive enzymes, including BSDL, are synthesized by pancreatic acinar cells. Intracellular aspects of the secretion process were established in the early 1970s by Palade [11] and co-workers and demonstrated to be correct for most pancreatic enzymes, such as α -amylase, trypsin and colipase-dependent lipase [12]. Immunolocalization studies indicated that, in contrast to the above-mentioned enzymes, BSDL was specifically associated with endoplasmic-reticulum (ER) membranes [13]. The size of the membrane-associated BSDL was compatible

with that of a fully processed enzyme [14]. It is known that BSDL carries out fucosylated O- and N-linked oligosaccharide structures. Consequently, the enzyme might be released from membranes between the *trans*-Golgi compartment, where fucose residues are added in the terminal position to oligosaccharide structures, and from the zymogen granules, where BSDL is mainly found in soluble material [10,13]. Further studies performed with human pancreatic microsomes and AR4-2J rat pancreatic cells [13,14] allowed us to characterize a membrane-protein ligand of 94 kDa (or p94), which was immunologically related to the glucose-regulated protein of 94 kDa (Grp94). It has been suggested that a multiprotein folding complex, including p94, and at least two other not-yet-characterized proteins of approx. 56 and 46 kDa (p56 and p46 respectively), are involved in the folding of BSDL [13,14]. Grp94 was originally identified as a protein that was co-ordinately regulated by agents that induced the glucose-regulated protein of 78 kDa (Grp78 or BiP) [15]. Recent studies suggested that Grp78 and Grp94 might act sequentially to fold ER proteins. Grp78 may bind to a less-oxidized form, whereas Grp94 would bind later to a more-oxidized form of proteins [16]. Consequently, and like Grp78, Grp94 appears to associate with unassembled or unfolded proteins [14,17]. Depletion of cellular ATP levels blocked the dissociation of complexes between Grp78 and unfolded peptide [18]. Grp78 was able to bind ATP, which translocated within the lumen of ER-derived vesicles [19]. ATP *in vitro* converted Grp78 oligomers to monomers and induced the dissociation of these complexes [20]. Grps autophosphorylate and display ATPase

Abbreviations used: BSDL, bile-salt-dependent lipase; ER, endoplasmic reticulum; TLCK, tosyl-lysylchloromethane; STI, soya bean trypsin inhibitor; H-7, 1-(isoquinolinesulphonyl)-2-methylpiperazine; H-8, *N*-[2(methylamino)-ethyl]-5-isoquinoline sulphonamide; H-89, *N*-(2{[3-(4-bromophenyl)-2-propenyl]-amino}-ethyl)-5-isoquinoline; BAPTA/AM, 1,2-bis(*O*-aminophenoxy)ethane-*N,N,N',N'*-tetra-acetic tetra(acetoxymethyl)ester; pAb, polyclonal antibody.

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activity [21,22], which is believed to be involved in complex formation with denatured or newly synthesized proteins [23]. In the case of Grp94, autophosphorylation, which is stimulated by peptides [24], may lead to an overall active folding process, in contrast to a more passive chaperone function. Grp94 shares several features with a porcine protein kinase, such as activation by Mg^{2+} , ATPase activity [25] and stimulation by certain proteins such as histone. Because BSDL, which is a target protein for Grp94, displayed multiple sites for phosphorylation (analysis performed by ExPASy prosite program), the association/dissociation of BSDL with microsome membranes could be dependent on ATP and on phosphorylation/dephosphorylation processes. In the current study, we attempted to demonstrate that human pancreatic BSDL is a phosphorylated protein. In addition, we performed experiments to investigate the effect of phosphorylation/dephosphorylation on the enzyme association/dissociation with intracellular membranes, and to determine the role of ATP in this process. We showed for the first time that BSDL is a phosphorylated enzyme, and that its phosphorylation, which appears to be involved in its release from microsome membranes, is probably under the control of a cascade of protein kinases. However, the association of BSDL with microsome membranes was triggered by ATP but not by the unhydrolysable analogue β,γ -methylene ATP.

MATERIALS AND METHODS

Reagents

PMSF, benzamidine, β -phenyl propionate, poly(vinylpyrrolidone) P-40, Coomassie Blue R250, alkaline-phosphatase-conjugated anti-rabbit goat IgG, alkaline-phosphatase-conjugated anti-mouse goat IgG, monoclonal antibodies specific for *O*-phosphoserine (clone PSR-45), for *O*-phosphothreonine (clone PTR-8) and for *O*-phosphotyrosine (clone PT-66), fatty-acid-free BSA, bovine milk casein, tosyl-lysylchloromethane (TLCK)-trypsin, ATP-, ADP- and AMP-agarose, ATP ($MgCl_2$ and NaCl salts), β,γ -methylene ATP, Tween 20, Nitro Blue Tetrazolium, 5-bromo-4-chloro-3-indoyl phosphate, 4-nitrophenyl hexanoate, okadaic acid and soya bean trypsin inhibitor (STI) were from Sigma (St. Louis, MO, U.S.A.). Proteinase K was from Boehringer (Mannheim, Germany). Serine/threonine protein phosphatases 1 and 2A (catalytic subunits) were from Promega (Madison, WI, U.S.A.). Nitrocellulose (BA 83 type, 0.2 μm) membrane was from Schleicher and Schuell (Dassel, Germany). [γ - ^{32}P]ATP (> 6000 Ci/mmol) was from NEN (Les Ulis, France). Genistein and sodium orthovanadate were from ICN Biochemicals (Costa Mesa, CA, U.S.A.). Serine/threonine protein kinase inhibitors used in this study: 1-(isoquinoline-sulphonyl)-2-methylpiperazine (H-7), *N*-[2(methylamino)-ethyl]-5-isoquinoline sulphonamide (H-8), *N*-[2{3-(4-bromophenyl)-2-propenyl}-amino]-ethyl-5-isoquinoline (H-89), 1,2-bis(*O*-amino phenoxy)ethane-*N,N,N',N'*-tetra-acetic tetra(acetoxymethyl)-ester (BAPTA/AM) were from Calbiochem (La Jolla, CA, U.S.A.), and calphostin C was from Sigma. Stock solutions of these drugs were as follows: H-7, 10 mM in water; H-8, 1 mM in water; BAPTA/AM, 1 mM in DMSO; H-89, 10 mM in DMSO; calphostin C, 200 μM in DMSO; genistein, 37 mM in DMSO.

Biological materials

The secreted BSDL (100 kDa, mature form) was purified from human pancreatic juice as described previously [26]. Normal human, adult, pancreatic tissue was generously given by Professor J. R. Delpéro (Institut Paoli-Calmettes, Marseille, France). Immediately after resection, tissue samples were cut into small

cubes, frozen in liquid N_2 and subsequently stored at $-80^\circ C$ until use. Pancreatic microsomes were obtained after homogenization of pancreatic tissue (15 s) with a Polytron (Kriens, Luzern, Switzerland) in cold 5 mM Tris/HCl, pH 7.4, buffer (sucrose 0.25 M) with protease inhibitors (benzamidine, β -phenyl propionate and PMSF, 2 mM each, and STI, 100 $\mu g/ml$). After elimination of nucleus and cell debris by centrifugation (600 *g*, 10 min), microsomes were obtained by differential centrifugations [13] and stored frozen until use. Polyclonal antibodies (pAb L64), specific for human pancreatic BSDL, were raised as described previously [27] and purified on Protein A-Sepharose.

General methodology

BSDL was immunoprecipitated with pAb L64 using the specific protocol earlier described [28]. Controls were performed in the absence of antibodies. BSDL was quantified by ELISA [29], and protein concentrations by the bicinchoninic acid test from Pierce (Rockford, IL, U.S.A.). The esterolytic activity of BSDL on 4-nitrophenyl hexanoate was measured at 404 nm and pH 7.4, in a 0.1 M Tris/HCl buffer in a thermostatically controlled cell at $30^\circ C$, as described elsewhere [30].

SDS/PAGE was performed in 10% (w/v) polyacrylamide gels and 0.1% (w/v) SDS, as described by Laemmli [31]. After migration, proteins were stained with Coomassie Blue R250 and destained in ethanol/acetic acid/water (2:3:35, by vol.).

Trypsin treatment of BSDL was performed as follows. Pure BSDL (0.4 mg/ml) was incubated for the required time at $30^\circ C$ in a 20 mM Tris/HCl, pH 7.4, buffer containing NaCl (0.15 M), $MgCl_2$ and EDTA (both 1 mM) in the presence or absence of ATP (2.5 mM) and 1% (w/w) TLCK-trypsin. At the end of incubation, the reaction was stopped by 1:10 dilution in a 5 mM Tris/HCl, pH 7.4, buffer containing 2 mM benzamidine and 50 $\mu g/ml$ STI. The remaining activity of BSDL was then determined as described above.

Proteinase K treatment of soluble BSDL (25 μg , ≈ 15 units) and microsome vesicles (250 μg , ≈ 15 BSDL units) was performed in a 10 mM Tris/HCl, pH 7.1, buffer, on ice, for various time periods in the absence or presence of a 1:10 amount of proteolytic enzyme. When required, ATP (2.5 mM) or calphostin C (20 μM) were added to the incubation mixture. At the end of the incubation, the remaining BSDL activity was recorded as described above.

Adenosine tri-, di- and mono-phosphate-agarose

ATP-, ADP- or AMP-agarose (1 ml) was used in a column binding procedure at $4^\circ C$. Each gel was equilibrated with sodium phosphate buffer (50 mM, pH 6.5) containing 10 mM $MgCl_2$. Gel was poured into an Econo-column (1 cm $\times$ 10 cm; Bio-Rad, Hercules, CA, U.S.A.), loaded with 10 μg of pure BSDL and incubated overnight at $4^\circ C$ on a roller. The column was then exhaustively washed with the sodium phosphate buffer and bound material was eluted with the same buffer containing 1 M NaCl and no $MgCl_2$. The presence of BSDL in washed-through and eluted fractions was determined by the enzyme activity on 4-nitrophenyl hexanoate.

Phosphate and O-phosphoamino acid determination

Pure BSDL was extensively dialysed against a large volume of double-distilled water and the required amount of protein was concentrated to dryness in an acid-washed glass tube. BSA (negative control) or bovine milk casein (positive control) was also dialysed. The same molar amount of each protein as that of BSDL was freeze-dried. Each sample was solubilized in 0.5 ml of

double-distilled water and mineralized for 24 h at 180 °C with 0.7 ml of 1 M H₂SO₄ [containing 10 % (v/v) perchloric acid]. Phosphorus was then determined according to Van Veldhoven and Mannaerts [32]. The presence of phosphoryl groups on the human pancreatic BSDL was also detected using a PhosphogelTM column from Pierce (Rockford, IL, U.S.A.) according to the supplier's protocol.

O-Phosphoamino acids were chromatographically detected on a Beckman System 6300 analyser after partial acid hydrolysis of a suitable amount of protein in 4 M HCl for periods of 3 h, 6 h and 10 h. Calculated values were extrapolated to zero time. Alternatively, O-phosphoamino acids were immunologically detected. For this purpose BSDL was dotted on to a nitrocellulose membrane, which was then blocked for 0.5 h in Tris/HCl, 50 mM, pH 8.0, buffer containing 150 mM NaCl and 3 % (w/v) poly(vinylpyrrolidone) P-40. Immunodetection was carried out using adequate anti-phosphoamino acid monoclonal antibodies as primary antibody. The antigen-antibody reaction was detected with alkaline-phosphatase-conjugated anti-rabbit goat IgG [33].

In vitro phosphorylation

Microsomes were suspended in 10 mM Tris/HCl, pH 7.1, buffer, containing 5 mM MgCl₂, 5 mM CaCl₂ and 1 mM sodium orthovanadate (*in vitro* phosphorylation buffer), to a final concentration of 1.4 mg/ml microsome proteins. Microsome proteins (40 µg) were preincubated with vector only (water or DMSO) or with the adequate inhibitor for 10 min at 30 °C. Then 50 µCi of [γ -³²P]ATP (0.2 µM, final concentration) was added (at this time the sample volume was 50 µl) and the mixture incubated for another 20 min at 30 °C. At the end of the incubation period, 50 µl of 100 mM Tris/HCl, pH 7.1, buffer with 2 % SDS and 5 % (w/v) glycerol were added and the reaction was stopped by boiling the sample in a water bath for 2 min. Endogenous BSDL was then immunoprecipitated with pAb L64. The immunoprecipitate was analysed by SDS/PAGE and phosphorylation was visualized by autoradiography of dried gels (Kodak X-OMAT AR film; Rochester, NY, U.S.A.).

CD

CD spectra of BSDL in the presence or absence of ATP were obtained using a Jobin-Yvon CD6 spectropolarimeter equipped with a thermostatically controlled cell holder under N₂ atmosphere. The temperature was maintained at 20 °C or 60 °C. The sample, as well as the buffer blank, spectra were obtained within the wavelength range of 260–190 nm. Cell path length was 50 µm; ten measurements per nanometre were recorded with an integration time of 10 s. Protein concentration ranged from 0.45 to 0.50 mg/ml in a 20 mM Tris/HCl, pH 7.4, buffer containing NaCl (0.15 M), MgCl₂ and EDTA (both 1 mM). No smoothing function was used for spectrum analysis.

RESULTS

Structural changes of secreted BSDL on ATP binding

To detect eventual structural changes of BSDL on ATP binding, the protein was incubated in the presence of 1 % (w/w) TLCK-trypsin at 30 °C. Under these conditions, secreted BSDL lost approx. 50 % of its activity in less than 3 h. This inhibition was abolished by 2.5 mM ATP (Figure 1). Consequently, ATP appeared to be capable of protecting BSDL against trypsin degradation, suggesting that ATP may be able to modify the conformation of BSDL. These possible conformational modifications of BSDL due to ATP were analysed by CD. The CD

Figure 1 Protection of secreted BSDL activity by ATP

BSDL (0.4 mg/ml) was incubated at 30 °C in 20 mM Tris/HCl, pH 7.4, buffer (0.15 M NaCl/1 mM MgCl₂/1 mM EDTA) in the presence (○) or absence (●) of ATP (2.5 mM) and 1 % (w/w) TLCK-trypsin. At the required time of incubation, aliquots were withdrawn and the reaction was stopped by dilution in 5 mM Tris/HCl, pH 7.4, buffer (2 mM benzamidine and 50 µg/ml STI) and the remaining activity of BSDL was recorded on 4-nitrophenyl hexanoate as substrate. Under these conditions ATP had no effect either on BSDL or on trypsin activities.

Figure 2 Effect of ATP on the structure of secreted BSDL, as analysed by CD

CD spectra of BSDL in the presence or absence of ATP were recorded on a Jobin-Yvon CD6 spectropolarimeter under N₂ atmosphere and at constant temperature as indicated. Further details are given in the Materials and methods section. (A) —, BSDL (0.5 mg/ml) at 20 °C; ···, BSDL (0.5 mg/ml) at 60 °C; ---, BSDL (0.47 mg/ml) at 20 °C and in the presence of 5 mM ATP. (B) —, BSDL (0.5 mg/ml) and ATP (0 mM); - - -, BSDL (0.49 mg/ml) and ATP (2.5 mM); ---, BSDL (0.47 mg/ml) and ATP (5 mM); ···, BSDL (0.45 mg/ml) and ATP (10 mM). Spectra are representative of at least two independent experiments.

spectrum of BSDL in the far ultraviolet region (Figure 2A) showed negative transition at 210 nm and a positive maximum at approx. 190 nm. The relatively low value of ΔA suggested a

Figure 3 Effect of ATP on the activity of secreted BSDL

(A) The activity of BSDL on 4-nitrophenyl hexanoate ($10^3 \times$ Remaining activity, units/ml) was recorded in the presence of ATP at the final concentrations indicated. (B) Dixon plot of $1/V_{sc}$ versus ATP concentration obtained at various concentrations of 4-nitrophenyl hexanoate: (○) 1 mM; (△) 2.5 mM; (▲) 5 mM; (□) 10 mM; (■) 15 mM. Values are means $\pm$ S.D. of three independent experiments, and error bars not shown are within the symbol size.

predominant contribution of β -sheets which, with β -turn, represent the major part of the ordered secondary structure of BSDL [34]. β -Structures contribute positive bands at 202 nm and 224 nm, which partially cancel the negative maximum of α -helix structures at 208–210 nm and 220–222 nm [35]. This CD spectrum is identical with that previously published, which indicated that the contribution assigned to α -helix structures was low (9%) and that human BSDL belongs to an 'all β ' class of proteins since 54–60% of its residues are in β -sheets and β -turns. At a concentration of 5 mM, ATP induced an increase in the negative ΔA at 205–210 nm and 215–220 nm, whereas the positive band at approx. 195 nm appeared to be unchanged. The negative bands at 205–210 nm and at 215–220 nm decreased with ATP concentration (Figure 2B), although the general shape of the spectral curve was not altered. These CD-spectrum modifications appeared to be rather specific, because a shift towards a random-coil conformation would lead to a decrease in the positive band at 195 nm to negative absorption, as shown by the CD spectrum of the BSDL recorded at 60 °C (Figure 2A) (at this temperature the enzyme was no longer active). These changes, which affected the absorption band of the spectrum rather than the overall shape of the curve, suggested that ATP modified the respective contribution of β -sheet and α -helix structures to the BSDL conformation.

Effects of ATP on secreted BSDL activity were also recorded. As shown in Figure 3(A), the enzyme activity decreased with increasing ATP concentration present in the assay cuvette and was totally abolished at a nucleotide concentration of 20 mM. Under these conditions, the ratio between the Michaelis–Menten

Figure 4 Binding of BSDL to ATP- and ADP-agarose

BSDL (10 μ g) was loaded on to a 1 ml (A) ATP-agarose, or (B) ADP-agarose, gel equilibrated in sodium phosphate buffer (50 mM, pH 6.5) containing 10 mM $MgCl_2$. After overnight agitation on a roller at 4 °C, the column was washed with the loading buffer and bound material was eluted with the sodium phosphate buffer containing 1 M NaCl and no $MgCl_2$.

constant (K_m) and the saturation velocity (V_{sc}) was unaltered [$(16.1 \pm 1.4) \times 10^3$ min] by ATP, as shown by the parallelism of the reverse function $1/V_{sc}$ versus ATP concentration (Figure 3B). This pattern is typical for uncompetitive inhibition and suggested that ATP does not interact with the catalytic site. The inhibition observed in the presence of ATP appeared to be reversible because 80% of the enzyme activity was recovered after a preincubation of BSDL with 20 mM ATP (30 °C for 10 min) and a 1:500 dilution of the preincubation medium before assay. These data indicated that BSDL may be able to bind ATP. To further substantiate this possibility, BSDL was loaded on to a column filled with ATP-agarose. As expected, BSDL bound to ATP-agarose (Figure 4A), and more than 90% of the BSDL activity loaded on to the column was eluted with sodium phosphate buffer containing 1 M NaCl. BSDL was also retained on the ADP-agarose column (Figure 4B). However, BSDL activity was recovered in wash-through fractions of the AMP-agarose column and no esterolytic activity was eluted with buffer containing 1 M NaCl (result not shown). Taken together, these data support the conclusion that BSDL was able to bind adenine nucleotide [36]. This binding affects the protein conformation, in a reversible concentration-dependent manner.

ATP triggers the association of endogenous BSDL of pancreatic microsomes with membranes

Previous work showed that BSDL in pancreatic microsomes is partitioned between the aqueous phase and the membrane phase [13]. The BSDL associated with the membrane was compatible in size with that of a fully processed enzyme that displayed

Table 1 Protection of microsomal BSDL from proteinase K proteolysis

Endogenous BSDL of microsomes vesicles (250 μ g of protein) and soluble BSDL (25 μ g) were treated with proteinase K (1:10) on ice for the time period indicated. When required, Nonidet P40, ATP or calphostin C was added at the final concentration shown. BSDL activity was then recorded on 4-nitrophenyl hexanoate as substrate. Controls (100% remaining activity) were performed under each set of experimental conditions in the presence of the compounds as indicated except proteinase K. Results are means $\pm$ S.D. determined from three independent experiments.

Fraction	Treatment	Time (h)	Remaining activity (%)
Soluble BSDL	Proteinase K (1:10)	4	23.2 $\pm$ 0.7
	Proteinase K (1:10) + ATP (2.5 mM)	4	25.8 $\pm$ 0.6
	Proteinase K (1:10)	24	0.5 $\pm$ 0.1
	Proteinase K (1:10) + Nonidet P40 (1%)	24	22.8 $\pm$ 3.9
	Proteinase K (1:10) + ATP (2.5 mM) + calphostin C (20 μ M)	24	67.2 $\pm$ 2.1
	Proteinase K (1:10) + ATP (2.5 mM) + calphostin C (20 μ M)	72	39.3 $\pm$ 3.7
Microsomes	Proteinase K (1:10)	4	89.3 $\pm$ 4.7
	Proteinase K (1:10) + ATP (2.5 mM)	4	97.9 $\pm$ 2.1
	Proteinase K (1:10) + Nonidet P40 (1%)	24	16.6 $\pm$ 1.0
	Proteinase K (1:10) + ATP (2.5 mM) + calphostin C (20 μ M)	24	87.6 $\pm$ 6.2
	Proteinase K (1:10) + ATP (2.5 mM) + calphostin C (20 μ M)	72	94.9 $\pm$ 3.8

Figure 5 Effect of phosphorylation inhibitors on the phosphorylation of endogenous BSDL of pancreatic microsomes

Microsomes were isolated from human pancreatic tissue and incubated with [γ - 32 P]ATP (1 mCi/ml, 20 min, 30 $^{\circ}$ C) in the presence of protein kinase or protein phosphatase inhibitors, at the indicated concentrations (final volume 25 μ l). The reaction was stopped by addition of 50 μ l of buffer containing 2% SDS and 5% glycerol and by boiling for 2 min in a water bath. Endogenous BSDL was then immunoprecipitated with pAb L64 before analysis on SDS/PAGE and autoradiography. Each box represents a typical experiment performed with pancreatic microsome preparations obtained from different donors.

fucosylated O- and N-linked glycan structures [14]. We next wanted to determine the topographical orientation of BSDL associated with microsomes. For this purpose, soluble (i.e. secreted) BSDL and microsome vesicles were treated with proteinase K. Under our experimental conditions, soluble BSDL lost a large fraction of its activity on 4-nitrophenyl hexanoate, whereas endogenous BSDL of microsomes was insensitive to proteolysis (Table 1). However, when microsomes were lysed by Nonidet P40, endogenous BSDL became sensitive to proteinase K (Table 1). Therefore one may conclude that BSDL was within the lumen of microsome vesicles. Addition of ATP or calphostin C did not modify the intravesicular orientation of BSDL, although the action of proteinase K in the presence of calphostin C was reduced (Table 1). Consequently, ATP, which translocates within the lumen of microsomes [19] and promotes changes in BSDL conformation, may be involved in the membrane association/dissociation process of this protein. Therefore human pancreatic microsomes were treated or mock-treated with 2.5 mM MgATP (10 mM Tris/HCl, pH 7.1, buffer) for 15 min at 30 $^{\circ}$ C and centrifuged for 1 h at 150 000 *g*. Endogenous BSDL was then quantified in the supernatant containing soluble proteins

and in the pellet containing membranes and associated proteins (resuspended in the same volume of starting buffer) by specific ELISA and by enzyme activity. Under these conditions, the fraction of membrane-associated BSDL represents the amount of enzyme present in the membrane fraction and normalized with the total amount of BSDL. This value, which represents 56.3 $\pm$ 3.7% in the absence of MgATP, significantly increased ($P < 0.01$, impaired Student's *t* test, determination performed from four independent experiments), up to 94.2 $\pm$ 1.5%, in the presence of the nucleotide. This result suggests that MgATP triggered the association of BSDL with membranes. This association appeared to be energy-dependent because, in the presence of 2.5 mM unhydrolysable ATP analogue, β , γ -methylene-ATP, the fraction of membrane-associated BSDL was no different from that obtained in the control experiment.

Phosphorylation of endogenous BSDL in pancreatic microsomes

These findings raised the possibility that the phosphorylation process of BSDL may be involved in the association of the

protein with pancreatic microsome membranes. Therefore human pancreatic microsomes isolated from different donors were suspended in the *in vitro* phosphorylation buffer and incubated with [γ - 32 P]ATP in the presence of various protein kinase inhibitors. Endogenous BSDL was then immunoprecipitated with pAb L64, analysed by SDS/PAGE and autoradiographed. In the presence of vector only (i.e. water or DMSO), no phosphorylation of BSDL (Figure 5, lanes 1 and 2) could be detected in the precipitated material (Figure 5, arrow). This suggested that no autophosphorylation of BSDL occurred. Instead, a phosphoprotein associated with an M_r of approx. 48000 (Figure 5, arrowhead) was precipitated by pAb L64. It is noteworthy that this material was detected independently of microsome treatment, meaning that the protein of M_r approx. 48000 may (auto)phosphorylate independently. Contrary to all expectations, 32 P-labelled material corresponding to BSDL was precipitated by pAb L64 in the presence of calphostin C, a protein kinase C inhibitor (Figure 5, lane 5). The same result, although the signal for BSDL was less intense, was obtained following incubation of human pancreatic microsomes with H-8 (Figure 5, lane 3) and H-89 (Figure 5, lane 4), which are inhibitors for protein kinases A and G and for protein kinase A respectively. At the concentration used, protein kinase C is poorly inhibited by H-8 and H-89.

In the presence of H-7 (Figure 5, lane 8), which inhibits indistinctly protein kinases A, G and C, the phosphorylated signal of BSDL cannot be detected. Other potential phosphorylation inhibitors, such as genistein (Figure 5, lane 6) or BAPTA/AM (Figure 5, lane 7), also did not allow us to detect BSDL phosphorylation. Nevertheless, BAPTA/AM (Figure 5, lane 12) did abolish phosphorylation induced by calphostin C, whereas genistein did not (Figure 5, lane 13). The combined action of calphostin C with inhibitors such as H-7 (Figure 5, lane 9), H-8 (Figure 5, lane 10) and H-89 (Figure 5, lanes 11) indicated that the BSDL phosphorylation signal decreased with H-7 and H-8, whereas it was not modified by H-89 at concentrations up to 40 μ M (compare with lane 5 of the same box). Cyclic nucleotides such as cAMP or cGMP, at concentrations up to 1 μ M, neither induced phosphorylation of BSDL nor modified the level of phosphorylation promoted by calphostin C (results not shown). Taken together, these data suggested that the phosphorylation of BSDL in pancreatic microsomes depended on a cascade of protein kinases, one of them, sensitive to Ca^{2+} , being directly involved in the BSDL phosphorylation. The involvement of a protein phosphatase can be ruled out as okadaic acid did not increase the phosphorylation promoted by calphostin C (Figure 5, lane 14, compare with lane 5 in the same box). Interestingly the phosphorylation of BSDL was sensitive to protein phosphatase 1 but not to protein phosphatase 2A (results not shown). This last result indicates that the site for BSDL phosphorylation may be a serine or a threonine residue.

Evidence for the presence of phosphoryl group(s) on secreted BSDL

As shown above, BSDL was phosphorylated during its transit within the acinar cell. Consequently the enzyme present in human pancreatic juice might be a phosphoprotein. Chemical determination of phosphorus was therefore performed on BSDL isolated from the pancreatic juice of six normal donors. Taking into account the controls (background due to contamination of dialysis tube, correction for protein interferences as determined with albumin), 1.2 ± 0.5 mol of phosphorus/mol of BSDL (range 0.3–1.8) was detected. We then attempted to determine whether

Figure 6 Phosphoryl groups on secreted BSDL

(A) Human pancreatic BSDL purified from pancreatic juice was analysed on a PhosphogelTM column according to the manufacturer's protocol. Absorbed material was eluted with 0.1 M sodium phosphate solution. The BSDL activity of each fraction was recorded with 4-nitrophenyl hexanoate as substrate (O), and the BSDL concentration was determined by ELISA ($\bullet$). (B) Human pancreatic BSDL was dotted (from 5 to 20 μ g) on to nitrocellulose membrane. Phosphoamino acids were then detected using monoclonal antibodies specific for O-phosphoserine, O-phosphothreonine and O-phosphotyrosine. Control was performed with the secondary antibody and omitting antibodies specific for O-phosphoamino acids.

all BSDL molecules were equally phosphorylated. For this purpose, pure human BSDL containing approximately one phosphoryl group per enzyme molecule was chromatographed on a PhosphogelTM column, which specifically binds phosphoproteins. After elution of unbound material, representing less than 5% of loaded material (determined by protein assay after dialysis) and devoid of BSDL activity, bound material was eluted with 0.1 M sodium phosphate solution (Figure 6A). This bound material represented 88% of loaded proteins and was associated with the enzymic activity of BSDL. ELISA indicated that only fractions eluted with phosphate contained BSDL. Overall these data suggested that phosphoryl group(s) was (were) present on BSDL. To determine which amino acid residue carries out the phosphoryl groupment on the BSDL molecule, the protein was subjected to acid hydrolysis and analysed for O-phosphoamino acid content. A unique peak eluted in the O-phosphoserine and O-phosphothreonine position was observed on amino acid analyses. Results agreed with the presence of approximately one mole of phosphorylated amino acid per mole of protein. Dot blots performed with specific monoclonal antibody to O-phosphorylated amino acid indicated that the site for phosphorylation was probably a serine residue, although a very slight reactivity was also detected with the antibody specific for O-phosphothreonine. No reactivity with the monoclonal antibody to O-phosphotyrosine was detected (Figure 6B).

Phosphorylation of endogenous BSDL promotes its release from pancreatic microsome membranes

Previous work showed that BSDL was associated with membrane of pancreatic microsomes [13,14]. Because BSDL in pancreatic

juice is phosphorylated, the release of BSDL from membranes may depend on its phosphorylation state. Consequently, we next investigated whether phosphorylation regulated the interaction of the enzyme with membranes. For this purpose, human pancreatic microsomes were suspended in 10 mM Tris/HCl, pH 7.1, buffer and treated with 20 μ M calphostin C and 2.5 mM MgATP for 20 min at 30 °C to provoke the phosphorylation of BSDL (see Figure 5), or with MgATP alone. Then membranes were separated from soluble proteins by centrifugation (150 000 g, 4 °C, 1 h). Endogenous BSDL was quantified by the ELISA technique in supernatant and pellet (after resuspension in the starting buffer volume). Under conditions inducing the phosphorylation of the enzyme, the fraction of BSDL present in the soluble fraction and normalized with the total amount of BSDL was significantly higher ($33.0 \pm 2.4\%$, $n = 4$, $P < 0.05$) than in the control with MgATP only ($24.1 \pm 3.0\%$). This suggests that the phosphorylation of BSDL appeared to be essential for its release from microsome membranes.

DISCUSSION

The mechanism by which a correctly folded polypeptide is released from chaperones is not yet fully understood. Results are consistent with the idea that chaperones such as Grp78 bind to extended hydrophobic stretches that are exposed to the hydrophilic medium on nascent or unassembled polypeptides [23]. These stretches would become inaccessible after complete folding of the protein. Although questionable for Grp94 [37] and for its cytosolic counterpart Hsp90 [36], chaperones bind ATP and have a weak ATPase activity [22] that promotes the release of bound proteins [38]. Depletion of cellular ATP inhibits protein folding and results in a longer association of some proteins with chaperones [18]. These results, along with the translocation of ATP into the ER, support a role for ATP in chaperone function. We have shown that pancreatic BSDL is associated with membranes from the ER to a *trans*-Golgi compartment where the enzyme is released once its glycosylation is fully completed [13,14]. Furthermore, we have speculated that the association of BSDL with membranes and the subsequent glycosylation of PEST sequences may contribute to protect the enzyme from degradation and to target BSDL for secretion [14]. In the present study, we attempted to gain information on the mechanism of release of BSDL from intracellular membranes. For this purpose, we incubated human pancreatic microsomes, in which BSDL is partitioned between the lumen soluble phase and the membrane phase [13], with ATP. Against all expectations, we found that ATP triggered the association of BSDL with microsome membranes. We also showed that BSDL binds ATP, and that its conformation changes on nucleotide binding. These structural changes appeared to be rather specific because the CD spectrum showed that ATP modified the respective contribution of β -sheet and α -helix structures to the BSDL conformation. We conclude, from the effect of ATP on enzyme activity and conformation and from the total recovery of BSDL activity on ATP dilution, that conformational modification of the protein is reversible and concentration dependent. Therefore it is conceivable that ATP stabilizes an unfolded state of BSDL and may somehow contribute to the efficient folding of BSDL. Whether the ATP-mediated folding of BSDL proceeds via a series of ATP-dependent binding and release reactions has to be elucidated. Another intriguing aspect of the present work is the requirement for the energy derived from ATP, as shown by the inefficiency of the unhydrolysable ATP analogue to trigger the association of BSDL with microsome membranes. This indicates that ATP energy may be used to alter the structural properties of BSDL

and allow it to associate with membrane. In addition, the phosphorylation of either BSDL or of another molecule within the complex that folds BSDL [14] could be important. It is worth noting that this folding complex comprises the Grp94-related p94 protein and two additional proteins of 56 and 46 kDa [14]. The size of the latter correlates quite well with the phosphorylated protein, which always co-precipitates with BSDL (see Figure 5), and the nature of which must be further characterized. Nevertheless, these data raise the possibility that BSDL (which presents multiple sites for phosphorylation) may be phosphorylated and that its phosphorylation is required for its interaction with membranes.

In the present study, we have demonstrated that BSDL was most probably phosphorylated on (a) serine residue(s). Results also showed that this protein did not autophosphorylate, nor phosphorylate on a tyrosine residue. Paradoxically, BSDL was phosphorylated when the inhibitor of protein kinase C, calphostin C, was added to human pancreatic microsomes. This result indicates that the potential protein kinase C activity did not directly phosphorylate BSDL, but may have acted upstream of a cascade leading to the phosphorylation of the enzyme. In the presence of H-7, which was described as a non-selective inhibitor of protein kinases A, C or G, no BSDL phosphorylation could be detected. These data suggested that a second kinase sensitive to H-7 inhibitor, and different from the first one, which was inhibited by calphostin C, was also involved in the phosphorylation process of endogenous BSDL in human pancreatic microsomes. The simplest model that is compatible with these data could be that the first protein kinase, sensitive to calphostin C and possibly to H-7, would inactivate another protein kinase sensitive to H-7 by phosphorylation. The inhibition of the first kinase by calphostin C, probably a protein kinase C, would positively regulate the second kinase, which in turn may be responsible for BSDL phosphorylation. In the presence of inhibitors such as H-8 and H-89, BSDL phosphorylation, although less intense, was still detectable, implying that a second protein kinase was not inhibited by these compounds. When BSDL phosphorylation was induced by calphostin C, H-89 did not modify the band intensity, whereas H-7 and H-8 inhibitors decreased the phosphorylation of BSDL. The last result supports the protein kinase cascade as described above. The second protein kinase, which is directly involved in BSDL phosphorylation, appeared to be depending on the presence of calcium because BAPTA/AM inhibited the phosphorylation of the protein promoted by calphostin C. Because the phosphorylation of BSDL appeared to be insensitive to cyclic nucleotides such as cGMP or cAMP, one may propose that the kinase involved in BSDL phosphorylation did not behave as a cAMP-dependent kinase nor as a cGMP-dependent kinase. This agrees with the ineffectiveness of H-8 and H-89 inhibitors at abolishing BSDL phosphorylation promoted by calphostin C. The sensitivity of the protein kinase involved in BSDL phosphorylation to H-7, suggests that it is not a calmodulin-dependent protein kinase. The fact that okadaic acid did not inhibit BSDL phosphorylation induced by calphostin C allowed us to rule out the possible involvement of such protein phosphatases 1 and 2A. However, protein phosphatases other than 1 and 2A may be implicated. Protein kinases have been localized along the secretory pathway [39], and their role in the final event of exocytosis, fusion of the pancreatic secretory vesicles with the plasma membrane, has long been postulated [40–44]. Based on the cytoplasmic orientation of these kinase activities, one may argue that they cannot be involved in BSDL phosphorylation. Lastly, BSDL could be a substrate for Grp94, which displays protein kinase activity, but this would occur in the absence of protein kinase inhibitors [25].

Consequently, BSDL is probably not phosphorylated by the Grp94-related p94 protein, although the 48 kDa protein that co-precipitates with BSDL (see Figure 5) may be a substrate for the chaperone [25]. Obviously, the mechanism by which BSDL is phosphorylated remains obscure. The localization and characterization of protein kinases present in pancreatic microsomes and susceptible to phosphorylating BSDL is currently in progress and should allow us to determine the role of the phosphorylation of BSDL.

Data collected until now ([13,14,28,45] and this study) allow us to propose a model for specific transport of BSDL along the secretory pathway of pancreatic cells. The translocation of ATP within the lumen of the ER helps BSDL to adopt an enzymically inactive conformation and triggers the association of the protein with intracellular membranes. This agrees with recent data that suggest that the membrane-associated BSDL was poorly active or inactive [13,14], in contrast to the soluble form. Preliminary results indicate that the esterolytic activity of BSDL does not depend on the phosphorylation of the enzyme. In the light of early data obtained on BSDL specificity, showing that this enzyme was able, in the absence of bile salts, to hydrolyse phospholipids to generate lysogenic lysophospholipids [46], it is conceivable that ATP may contribute to inhibit the BSDL expressed by pancreatic cells. The inhibition should be effective immediately after the release of the signal peptide of BSDL [11]. The soluble BSDL thus produced should be otherwise active and potentially deleterious for the cell-membrane integrity. Accordingly, the association of BSDL with the so-called membrane folding complex, including the Grp94-related p94 protein [13,14], may be essential to protect the cell from any alteration of membrane-lipid turnover. This may represent another way, with the pancreatic secretion of inactive zymogens, to protect the cell from digestion. Another possibility, which is not exclusive of the first one, could be that the association of BSDL with ER and Golgi membranes should also be important to keep the enzyme, which is highly glycosylated [34], in close interaction with membrane glycosyltransferases [47]. As postulated, the glycosylation of C-terminal PEST sequences [13,14] may target BSDL for secretion instead of degradation [48]. When glycosylation is fulfilled in the *trans*-Golgi area [13,14], BSDL should be released from membranes and co-precipitated with other digestive enzymes under the acidic conditions of the *trans*-Golgi network [49] and finally enter the pancreatic secretion. As shown herein, it is possible that the release of BSDL from microsome membranes may depend on phosphorylation. Based on these results, one may postulate that the phosphorylation of BSDL should occur in a late Golgi compartment. This is likely in the light of accumulated studies suggesting that the Golgi apparatus may be an intracellular site for the Ca^{2+} -dependent cAMP-independent phosphorylation of secretory proteins (see [50] and refs. therein). From this point, one may conceive that a minor part of BSDL could elude the phosphorylation process and reach the secretory granules while still associated with membranes. In this context, and in the absence of ATP, BSDL may exhibit measurable phospholipase activity [46] and could play the role ascribed to the zymogen granule-membrane-associated BSDL by Withiam-Leitch et al. [51] in the fusion of membranes occurring during exocytosis.

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