Phosphorylation of the rat pancreatic bile-salt-dependent lipase by casein kinase II is essential for secretion

Eric PASQUALINI, Nathalie CAILLOL, Anne VALETTE, Roland LLOUBES, Alain VERINE and Dominique LOMBARDO[1]
INSERM U.260, Faculté de Médecine/Timone, 27 bld Jean Moulin, 13385 Marseille cedex 05, FRANCE

Bile-salt-dependent lipase (BSDL, EC 3.1.1.–) is an enzyme expressed by the pancreatic acinar cells and secreted as a component of the pancreatic juice of all examined species. During its secretion route BSDL is associated with intracellular membranes. This association allows the complete glycosylation of the enzyme or participates in the inhibition of the enzyme activity, which can deleterious for the acinar pancreatic cell. Thereafter, the human BSDL is phosphorylated by a serine/threonine protein kinase and released from intracellular membranes. In the present study, we show that the rat pancreatic BSDL, expressed by AR4-2J cells used as a model, is phosphorylated by a protein kinase that is insensitive to inhibitors of protein kinases A, C or G and that the phosphorylation process is favoured by okadaic acid (an inhibitor of protein phosphatases 1 and 2A). However, 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB), which is a specific inhibitor of casein kinase II, abolishes the phosphorylation *in vitro* of BSDL within microsomes of AR4-2J pancreatic cells. We showed further that the α-subunit of casein kinase II co-locates with BSDL within the lumenal compartment of the Golgi. Genistein, which perturbs the *trans*-Golgi network, also inhibits the phosphorylation of BSDL, suggesting that this post-translational modification of BSDL probably occurred within this cell compartment. The inhibition of the phosphorylation of BSDL by DRB also decreases the rate at which the enzyme is secreted. Under the same conditions, the rate of α-amylase secretion was not modified. These data strongly suggest that phosphorylation is a post-translational event, which appears to be essential for the secretion of BSDL.

Key words: AR4-2J cell line, pancreas.

INTRODUCTION

Bile-salt-dependent lipase (BSDL, EC 3.1.1.–) is an enzyme involved in the duodenal hydrolysis of cholesteryl esters and lipid-soluble vitamin esters [1]. This enzyme, also referred to as carboxyl ester lipase or cholesterol ester hydrolase, is found in all examined species from fishes [2] to humans [3]. BSDL is synthesized within the endoplasmic reticulum of acinar cells, then follows the secretory pathway of these cells to be secreted as a component of the pancreatic juice [3]. However, contrary to other pancreatic enzymes such as α-amylase or colipase-dependent lipase, BSDL was shown as being associated with membranes of the endoplasmic reticulum and of the Golgi apparatus [4,5]. This association involved a membrane-folding complex including at least two proteins other than BSDL. The first one, with which BSDL interacts, is a 94-kDa protein immunologically related to glucose-regulated protein of 94 kDa (Grp94) [5]. The second one, of 46 kDa, is still uncharacterized. This association of BSDL with intracellular membranes could be essential for the complete O-glycosylation of the C-terminal domain of BSDL, which encompasses tandemly repeated identical sequences, rich in proline, threonine, serine and glutamic acid and referred to as PEST sequences [6,7]. It has been shown further that the glycosylation of these PEST sequences regulates the secretion of BSDL [7,8]. However, ATP, which is able to translocate into the endoplasmic reticulum [9], maintains BSDL in an unfolded or inactive conformation and triggers its association with membranes [10]. Consequently, the association of BSDL with membranes could be important to annihilate the enzyme activity which can perturb the lipid metabolism of the cell [11]. The question that now rises in mind is how and when is BSDL released from intracellular membranes and addressed towards secretion? We have already shown that the phosphorylation of human BSDL, probably on a serine residue, is essential for the dissociation of human BSDL with membranes [10]. This release should occur within a cell compartment once the glycosylation of the protein is completed [5,7,10]. This compartment, sensitive to monensin [5], could be the *trans*-Golgi network [10]. Furthermore, the stoichiometry of the phosphorylation, which is 1.2 ± 0.5 mol of phosphorus/mol of secreted BSDL [10], suggests that the phosphorylation could be an essential step for BSDL secretion.

The phosphorylation of human pancreatic BSDL seems to depend upon a protein-kinase cascade [10]. However, the protein kinase directly involved in the phosphorylation of BSDL is not yet characterized, although it could be calcium-sensitive and cyclic-nucleotide-independent [10].

The aim of this study was 3-fold: (i) to better characterize the protein kinase involved in BSDL phosphorylation, (ii) to locate the phosphorylation compartment and (iii) to demonstrate that the phosphorylation of the enzyme is an important step for its secretion. Most of the above-described data were obtained using human material. However, this material precluded in-depth study such as that designed to demonstrate the possible link between phosphorylation and secretion. Therefore, the present data were obtained using the rat pancreatic AR4-2J cell line, which has already been used as a model to study the secretion route of BSDL [5,12].

Abbreviations used: BSDL, bile-salt-dependent lipase; DRB, 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole; H-7, 1-(5′-isoquinolinylsulphonyl)-2-methylpiperazine; H-8, *N*-[2-(methylamino)ethyl]-5-isoquinoline sulphonamide; H-89, *N*-2-[(*p*-bromocinnamyl)amino ethyl]-5-isoquinoline sulphonamide; BAPTA-AM, bis-(*o*-aminophenoxy)ethane-*N,N,N′,N′*-tetra-acetic acid acetoxymethyl ester; RITC, rhodamine isothiocyanate; DMEM, Dulbecco's modified Eagle's medium; LDH, lactate dehydrogenase; Grp94, glucose-regulated protein of 94 kDa.

[1] To whom correspondence should be addressed (e-mail Dominique.Lombardo@medecine.univ-mrs.fr).

MATERIAL AND METHODS

Material

The protein-kinase inhibitors 1-(5′-isoquinolinylsulphonyl)-2-methylpiperazine (H-7), *N*-[2-(methylamino)ethyl]-5-isoquinoline sulphonamide (H-8), *N*-2-[(*p*-bromocinnamyl)amino ethyl]-5-isoquinoline sulphonamide (H-89), bis-(*o*-aminophenoxy)-ethane-*N*,*N*,*N*′,*N*′-tetra-acetic acid acetoxymethyl ester (BAPTA-AM), calphostin C, genistein and 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB) and the protein-phosphatase inhibitor okadaic acid were obtained from Sigma (St Louis, MO, U.S.A.) or from Calbiochem (La Jolla, CA, U.S.A). Recombinant α-subunit of casein kinase II and mouse monoclonal antibody specific for casein kinase II were from Transduction Laboratories (San Diego, CA, U.S.A.). Mouse monoclonal antibodies directed against the 58K Golgi protein (clone 58K-9), alkaline phosphatase-conjugated goat antibodies to mouse or rabbit immunoglobulins and rhodamine isothiocyanate (RITC)- and FITC-labelled goat antibodies to mouse or rabbit IgG were from Sigma. Polyclonal antibodies specific for rat BSDL were raised in our laboratory as described previously [13]. These antibodies specifically reacted with BSDL on Western blots and were able to immunoprecipitate this enzyme along with proteins involved in BSDL folding [5,7,12].

Cell culture and microsome preparation

The rat pancreatic AR4-2J cells (European Collection of Animal Cell Cultures no. 93100618) were grown in Dulbecco's modified Eagle's medium (DMEM; Life Technologies, Grand Island, NY, U.S.A.) supplemented with 1 g/l glucose, 2 mM L-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin and 10% fetal calf serum, in 5% CO_2. Cells were washed with PBS buffer (10 mM sodium phosphate, pH 7.4/150 mM NaCl), then harvested in 5 ml of homogenization buffer (5 mM Tris/HCl, pH 7.4/250 mM sucrose/1 mM EDTA) and protease inhibitors (50 μg/ml soya bean trypsin inhibitor/2 mM benzamidine/2 mM PMSF). Cells were then pelleted by centrifugation (400 ***g***, 3 min, 4 °C) and homogenized with a Polytron (Kriens, Lucerne, Switzerland) in 0.5 ml of homogenization buffer.

Microsomes were isolated by serial centrifugation according to the method of Jamieson and Palade [14]. Cell homogenate was cleared by low-speed centrifugation (600 ***g***, 10 min, 4 °C). The supernatant was centrifuged successively at 3000 ***g*** (10 min), then at 8000 ***g*** (10 min) and at 15000 ***g*** (10 min) to obtain zymogen granules, mitochondria and lysosomes, respectively. Post-lysosomal supernatant was finally centrifuged for 1 h at 109000 ***g*** (4 °C) to isolate microsomes, which were washed with 10 ml of homogenization buffer and once again centrifuged under the latter conditions. Microsomes were then stored at −20 °C until use. This method allows us to obtain pancreatic microsomes with the correct membrane topology [10]. When required, freshly prepared microsomes were stripped by incubation in 0.25 M KBr as follows: microsomes (2 mg of proteins) were suspended in 1 ml of 20 mM Tris/HCl (pH 7.4) buffer (containing 2 mM benzamidine), and then 50 μl of 5 M KBr or vehicle only (water) was added and incubated for 20 min at 4 °C. Then microsome membranes were precipitated by centrifugation (109000 ***g***, 75 min) and the pellet was taken up in the Tris/HCl buffer before analysis.

Metabolic labelling of cell proteins

AR4-2J cells were cultured to reach 80% confluence and starved in methionine-free or phosphate-free DMEM (ICN, Costa Mesa, CA, U.S.A.) for 30 min. Then the cell-culture medium was supplemented with [^{35}S]methionine (20 μCi/ml, Trans ^{35}S-label; ICN) or [^{32}P]orthophosphate (0.5 mCi/ml; NEN, Boston, MA, U.S.A.). At the end of the metabolic labelling of cell proteins, cells were washed exhaustively with PBS and harvested. Cells were finally lysed in the appropriate buffer according to the experiment to be performed and the homogenate was cleared by low-speed centrifugation (600 ***g***, 10 min, 4 °C).

Phosphorylation *in vitro*

Microsomes (40 μg of proteins) suspended in 50 μl of 10 mM Tris/HCl buffer (pH 7.1; with 5 mM $MgCl_2$/5 mM $CaCl_2$/1 mM orthovanadate) were preincubated for 10 min at 30 °C in the presence of adequate inhibitor or in the presence of inhibitor vehicle only (< 10% DMSO by vol. or water). Then the phosphorylation was initiated by the addition of 50 μCi of [γ-^{32}P]ATP (10 μCi/μl; NEN); the final concentration of ATP was 0.2 μM. Samples were further incubated for 20 min at 30 °C. The reaction was stopped by the addition of an equal volume (50 μl) of a 100 mM Tris/HCl (pH 7.1)/2% SDS/5% glycerol buffer and heated for 2 min at 95 °C. Samples were analysed subsequently on SDS/PAGE or immunoprecipitated with polyclonal antibodies specific for BSDL.

DNA assay, protein and enzyme determinations, and BSDL purification

DNA was assayed by fluorimetry using Hoechst 33258 reagent [15] solubilized in 50 mM sodium phosphate buffer (pH 7.4; with 2 M NaCl/2 mM EDTA). Protein concentrations were determined using the bicinchoninic acid test from Pierce and BSA as standard. BSDL was assayed using 4-nitrophenyl hexanoate (0.1 mM) as substrate and sodium taurocholate (4 mM) as activator [2]. The α-amylase activity was determined at 410 nm on 4-nitrophenylmaltopentaoside in 0.1 M sodium phosphate/50 mM NaCl buffer (pH 7.4) in the presence of α-glucosidase [5,16]. The lactate dehydrogenase (LDH) activity was recorded as described by Goldberg [17].

BSDL was purified using affinity chromatography on a cholate-immobilized Sepharose column [18]. Sodium cholate from Sigma was first immobilized on CNBr-activated Sepharose according to the manufacturer's protocol (Pharmacia, Uppsala, Sweden). The gel was washed exhaustively with water, and 5 ml of swollen material was equilibrated in TBE buffer (25 mM Tris/HCl, pH 9.0/2 mM EDTA/1 mM benzamidine). Samples in TBE were loaded on the top of the gel and eluted with TBE containing 1% Triton X-100. The excess of detergent was eliminated with unabsorbed material by washing the column with TBE. Bound material was then eluted with 1% sodium cholate in TBE, dialysed against a large volume of water and concentrated by lyophilization.

Immunoprecipitation, polyacrylamide gels and Western blots

Samples (40 μg of proteins or more when required) to be analysed were firstly treated with 6 mg of protein A–Sepharose in TETN 250 buffer (25 mM Tris/HCl, pH 7.5/5 mM EDTA/250 mM NaCl/1% Triton X-100). After 4 h of incubation on a rotating wheel, the protein A–Sepharose pellet was eliminated by centrifugation (10000 ***g***, 3 min, 4 °C). The supernatant was diluted to 1 ml with TETN 250 buffer and 5 mg/ml BSA. Then, an appropriate amount of polyclonal antibody specific for rat BSDL was added. Antigen–antibody complexes were allowed to form by a further overnight incubation at 4 °C. Complexes were precipitated with 6 mg of protein A–Sepharose in TETN 250 buffer and centrifuged (10000 ***g***, 3 min, 4 °C). The pellet was

then washed twice with 1 ml of TETN 250 buffer and twice again with 1 ml of 10 mM Tris/HCl buffer (pH 7.5). Between each washing step, the material was recovered by centrifugation, and the final pellet was suspended in Laemmli buffer [19] and boiled for 3 min.

Proteins were separated on 10 % polyacrylamide gel in the presence of 0.1 % SDS (SDS/PAGE) according to Laemmli [19] using a Mini-PROTEAN® II apparatus (Bio-Rad, Rockford, IL, U.S.A.). Proteins were then fixed by Coomassie Brilliant Blue staining (0.25 % in ethanol/acetic acid, 91:18, v/v) and destaining in 5 % ethanol/7.5 % acetic acid solution. The gel was then dried and autoradiographed (BioMax MR, Eastman-Kodak, Rochester, NY, U.S.A.). Finally, autoradiograms were quantitated using the Image program (National Institutes of Health, Bethesda, MD, U.S.A.) [12].

Alternatively, proteins separated by SDS/PAGE were electro-transferred on to a nitrocellulose membrane [20]. Replicas were then saturated with 3 % BSA and developed with polyclonal antibodies specific for the rat BSDL [12] or for the α-subunit of casein kinase II as primary antibodies.

Immunofluorescence

Cells were grown on microscope coverslips until $\approx$ 50 % confluence. Coverslips were then washed in PBS buffer and cells fixed with 3 % paraformaldehyde. The aldehyde excess was eliminated by extensive washing of slides with 50 mM NH_4Cl. Cells were permeabilized with 0.05 % saponin in PBS and incubated with 10 % fetal calf serum. Cells were then treated successively with antibodies specific for rat BSDL, for the 58K Golgi protein or for casein kinase II α-subunit, washed with PBS and incubated with RITC- or FITC-labelled secondary antibodies. All incubation were performed in the presence of 0.05 % saponin. At the conclusion of the process, coverslips were put on microscope slides and mounted with a mounting solution (25 mM Tris/HCl, pH 8.0/75 % glycerol/0.1 % *p*-phenylenediamine). Slides were observed using a fluorescence microscope with the appropriate filter.

RESULTS

Phosphorylation of the rat BSDL

AR4-2J cells were cultured until 80 % confluence and starved in phosphate-free DMEM for 30 min. Once starved, cells were further cultured in fresh DMEM with 0.5 mCi/ml [^{32}P]orthophosphate for another 3 h. At the conclusion of the incubation, cell-free medium was withdrawn and stored at -20 °C until use. Cells were washed with PBS, harvested and lysed in 20 mM Tris/HCl buffer (pH 8.0; containing 100 mM EDTA, 100 mM NaCl, 0.5 % Nonidet P-40, and phosphatase inhibitors, 50 mM NaF, 30 mM sodium pyrophosphate and 0.2 mM sodium orthovanadate). After 30 min of incubation at 4 °C, the cell lysate was cleared by centrifugation. To isolate BSDL, the cleared supernatant of ^{32}P-labelled cell lysate (1 ml) was loaded on to the top of a cholate-immobilized Sepharose column, an affinity column that allows one-step purification of the enzyme [18]. The resin was suspended and incubated overnight at 4 °C on a rotating wheel. At the end of the incubation, the column was washed extensively with TBE containing 0.2 % Triton X-100 until the radioactivity reached a background level. The specifically adsorbed proteins were eluted with 1 % sodium cholate in TBE. This eluted material was dialysed against a large volume of cold water and concentrated by lyophilization. Proteins were then solubilized in 500 μl of water and an aliquot (25 μl) was analysed on SDS/PAGE and autoradiographed. Under these conditions, the phosphorylated material thus isolated migrated as a unique band at 74 kDa (Figure 1, lane 3). To ascertain that the 74-kDa protein was BSDL, the radiolabelled cell-free medium (1 ml) was immunoprecipitated using polyclonal antibodies specific for rat BSDL. As shown on Figure 1, only one ^{32}P-labelled protein migrating at 74 kDa was detected. Consequently, we concluded that BSDL expressed by AR4-2J cells was phosphorylated within the cells and subsequently secreted.

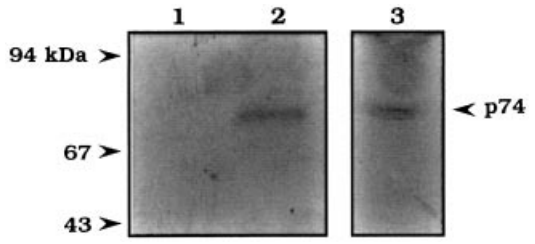

Figure 1 Phosphoylation of BSDL expressed by rat pancreatic AR4-2J cells

AR4-2J cells were starved in phosphate-free DMEM (30 min), and medium was supplemented with [^{32}P]orthophosphate (0.5 mCi/ml) for 3 h. The cell-free medium (1 ml) was then immunoprecipitated with antibodies specific for the rat BSDL and separated on SDS/PAGE (lane 2). Immunoprecipitation controls were performed by omitting antibodies specific for BSDL (lane 1). The radiolabelled cell homogenate was chromatographed on a cholate-immobilized Sepharose affinity column, and the material eluted with 1 % sodium cholate was dialysed and analysed as above (lane 3).

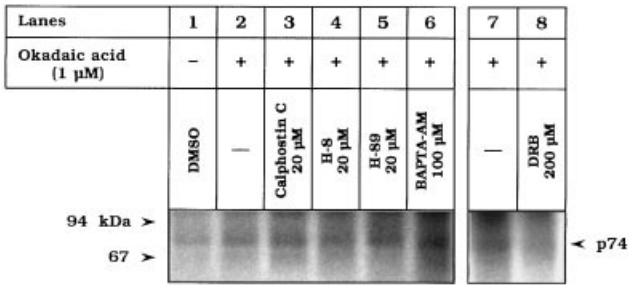

Figure 2 Phosphorylation *in vitro* of BSDL

Microsomes of AR4-2J rat pancreatic cells were isolated by serial centrifugations and 40 μg of proteins were pre-incubated (10 min, 30 °C) in the absence (vehicle alone, DMSO) or in the presence of protein-kinase inhibitors at the indicated concentrations with (+) or without (—) the protein-phosphatase inhibitor okadaic acid, and then incubated in the presence of [γ-^{32}P]ATP. The reaction was stopped and BSDL was immunoprecipitated using antibodies specific for rat BSDL. The immunoprecipitated material was analysed on SDS/PAGE and autoradiographed.

Phosphorylation *in vitro* of BSDL expressed by AR4-2J cells

Now, the question was to determine which protein kinase in the rat pancreatic AR4-2J cells was responsible for the phosphorylation of BSDL. To this end, microsomes were suspended in 10 mM Tris/HCl, pH 7.1/5 mM $MgCl_2$/5 mM $CaCl_2$/1 mM orthovanadate. A fraction of the suspended material (corresponding to 40 μg of proteins) was then treated under various conditions in the presence of [γ-^{32}P]ATP (0.2 μM). BSDL was then immunoprecipitated, analysed on SDS/PAGE and autoradiographed. The autoradiograms were finally quantitated by densitometric scanning. As shown in Figure 2, in the absence or presence of protein-kinase or protein-phosphatase inhibitors at

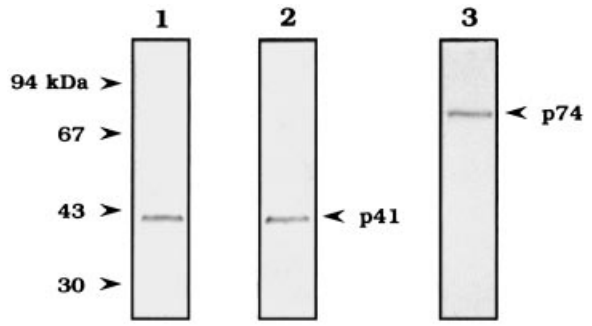

Figure 3 Detection of casein kinase II α-subunit in microsomes isolated from AR4-2J cells

Microsomes were isolated by serial centrifugations and 40 μg of proteins were separated on SDS/PAGE and electrotransferred on to nitrocellulose membranes. Replicas were then probed with mouse monoclonal antibody specific for the α-subunit of casein kinase II (lane 1) or with rabbit polyclonal antibodies specific for the rat BSDL (lane 3, p74). Lane 2 represents a control migration of recombinant α-subunit of casein kinase II (p41). The antigen–antibody complexes were then visualized using alkaline phosphatase-conjugated goat antibodies to mouse IgM or to rabbit IgG.

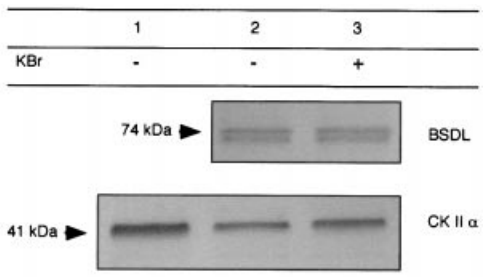

Figure 4 Localization of casein kinase II α-subunit and BSDL at the lumenal side of microsomes isolated from AR4-2J cells

Microsomes were isolated by serial centrifugations, stripped in 20 mM Tris/HCl buffer, pH 7.4 (with 2 mM benzamidine), and treated with 0.25 M KBr (+) or mock-treated (−) for 20 min at 4 °C. Then microsomes were pelleted by centrifugation at 109000 ***g*** for 75 min. Pellets were resuspended in buffer and analysed on SDS/PAGE and Western blot as described in Figure 3. Lane 1, recombinant α-subunit of casein kinase II detected with specific antibodies. Lane 2, BSDL (upper panel) and the α-subunit of casein kinase II (CKIIα; lower panel) were detected in mock-treated microsomes using specific antibodies. Lane 3, as lane 2 but performed on KBr-stripped microsomes.

the required final concentration, a 2–3-fold increase in phosphorylation of BSDL over control in the presence of the vehicle only (DMSO) was detected (Figure 2, lanes 1 and 2, p74 arrowhead). Calphostin C, H-8 and H-89, which are susceptible to inhibiting calcium-dependent protein kinase C, G and A activities, after correction for background level in each lane, had no effect on BSDL phosphorylation promoted by okadaic acid (Figure 2, lanes 3–5). The lack of pronounced effect of a calcium-chelating drug such as BAPTA-AM (Figure 2, lane 6, less than 30% inhibition under conditions used) and of H-7 (results not shown) corroborates this result. The stimulating effect due to okadaic acid was significantly decreased by DRB (Figure 2, lanes 7 and 8), which has been described as a specific inhibitor of casein kinase II [21]. Consequently, the phosphorylation state of BSDL in AR4-2J cells could be under the control of protein phosphatase(s) and casein kinase II.

Localization of BSDL and casein kinase II

Casein kinase II is a ubiquitous protein serine/threonine kinase localized predominantly within the cytoplasm and nucleus of cells [22,23]. A casein kinase II activity was also detected in the endoplasmic reticulum of osteoblast-like ROS 17/2.8 cells [24]. In rat liver, casein kinase II activity is predominantly cytosolic, with the remaining activity divided among the nuclear, mitochondrial and microsomal fractions [25]. We therefore determined whether casein kinase II was associated with microsomes of AR4-2J cells. Immunofluorescence studies using monoclonal antibody specific for the α-subunit of casein kinase II and polyclonal antibodies directed against rat BSDL suggested that these proteins locate within a reticulated compartment (results not shown). To further this study, microsome proteins (40 μg) were separated on SDS/PAGE and electrotransferred on to a nitrocellulose membrane. Replicas were then probed with the monoclonal antibody specific for the catalytic α-subunit of casein kinase II. As shown in Figure 3 (lane 1), the α-subunit of casein kinase II, migrating at the same position as the recombinant protein used as control (lane 2, p41), can be detected in microsomes of AR4-2J cells, where BSDL can also be detected (lane 3, p74) [5]. These results strongly suggested that BSDL and casein kinase II co-locate within the same cell compartment,

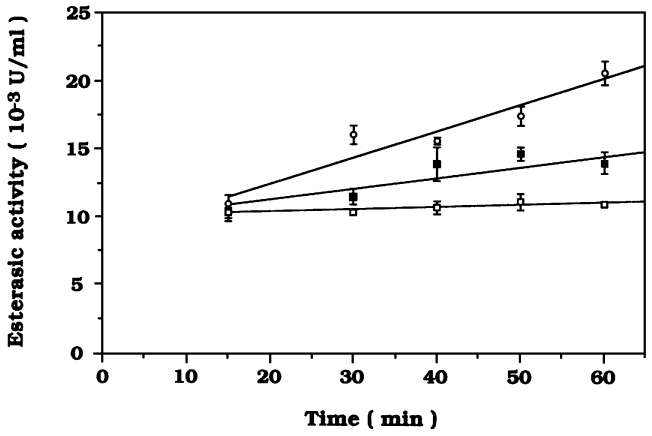

Figure 5 Effect of genistein on the rate of secretion of BSDL by AR4-2J cells

At the beginning of the experiment, culture medium of AR4-2J cells was replaced with 1 ml of fresh DMEM supplemented with genistein at 0 (○), 30 (■) and 60 (□) μg/ml, respectively. At the required time period of incubation, aliquots of the culture medium were withdrawn and the BSDL activity recorded. Results are means ± S.D. of at least three independent experiments.

where they are likely to be associated with membranes [10,23]. To confirm this point, freshly prepared microsomes (2 mg of proteins) were stripped with 0.25 M KBr to eliminate trapped cytosolic proteins. The membrane pellet containing lumenal proteins was obtained after centrifugation of the incubation mixture. The pellet was then suspended in buffer and analysed by SDS/PAGE and Western blotting using antibodies specific for BSDL and the α-subunit of casein kinase II. Under these conditions the amount of BSDL detected (Figure 4) in stripped microsomes did not differ from mock-treated microsomes (i.e. microsomes treated with the KBr vehicle only). The same result was obtained with the α-subunit of casein kinase II (Figure 4). These data indicated that BSDL and casein kinase II locate together in the lumens of microsomes.

Table 1 Effect of genistein on α-amylase and BSDL activities

AR4-2J cells were cultured in DMEM supplemented with genistein at the indicated final concentration. After 1 h of incubation, cells were harvested and lysed. Cell homogenates were cleared by centrifugation and parameters, as indicated (DNA, protein, LDH, α-amylase and BSDL activities), were recorded. Values are means ± S.D. of at least three independent experiments. All intra- and extracellular activities are given as 10^{-3} units/mg of cell protein. N.D., not determined.

			Intracellular activities		Extracellular activities		
Genistein (μg/ml)	DNA (μg/ml)	Protein (mg/ml)	LDH	BSDL	LDH	α-Amylase	BSDL
0	290.5 ± 18.1	1.42 ± 0.04	1520 ± 400	206.0 ± 21.2	43.8 ± 5.4	6.24 ± 2.14	18.5 ± 4.8
30	297.1 ± 9.3	1.56 ± 0.09	1230 ± 70	210.3 ± 11.0	36.6 ± 10.5	N. D.	12.5 ± 1.0
60	312.1 ± 18.5	1.51 ± 0.09	1080 ± 110	207.7 ± 1.6	45.0 ± 3.0	2.10 ± 0.42	7.5 ± 0.9

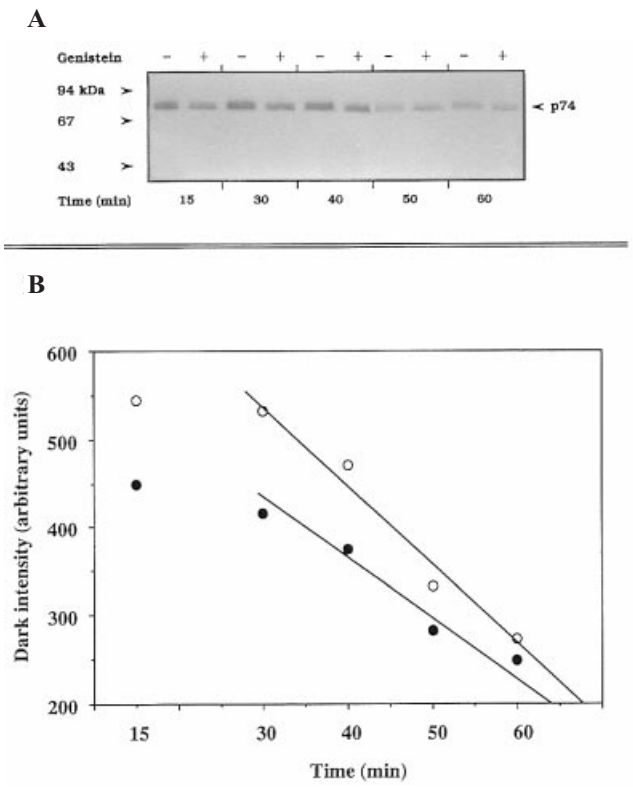

Figure 6 Effect of genistein on the rate of BSDL synthesis by AR4-2J cells

(**A**) AR4-2J cells grown to 80% confluence were pulsed for 5 min with [^{35}S]methionine (20 μCi/ml) then chased for the times as indicated, in the absence (control, −) or in the presence (+) of 60 μg/ml genistein. At the required time, cells were washed, harvested and lysed. The clear lysate was immunoprecipitated with antibodies specific for rat BSDL, and analysed by SDS/PAGE and autoradiography. Results are representative of at least two independent experiments performed under identical conditions. (**B**) Autoradiograms obtained as in (**A**) were quantitated by densitometric scanning and the dark intensity, in arbitrary units, was plotted as a function of the duration of the chase (○, no genistein; ●, genistein, 60 μg/ml). Results are the average of two independent experiments performed under identical conditions.

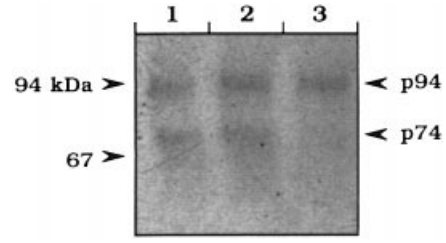

Figure 7 Effect of genistein on the phosphorylation of BSDL by AR4-2J cells

AR4-2J cells were grown until 80% confluence, starved for 30 min in phosphate-free DMEM, and incubated with [^{32}P]orthophosphate in the absence or presence of genistein for 3 h. Cells were then harvested, lysed and cleared by centrifugation. The material corresponding to 250 μg of cell proteins was immunoprecipitated with antibodies specific for rat BSDL. The material thus immunoprecipitated was separated on SDS/PAGE and autoradiographed. Concentrations of 0, 30 and 60 μg/ml genistein were used (lanes 1, 2 and 3, respectively).

The phosphorylation of BSDL occurs within a genistein-sensitive compartment of the cell

The isoflavone genistein has been described as a specific inhibitor of protein tyrosine kinases. The inhibition of intracellular protein tyrosine kinases by this drug leads to the concomitant inhibition of the formation and the release of cargo vesicles in the *trans*-Golgi network [26]. As a consequence, the secretory mechanism is interrupted. Therefore, the effect of genistein on the secretion of proteins by AR4-2J cells was determined and, as expected, 60 μg/ml genistein inhibited the secretion of BSDL (Figure 5) and α-amylase to the same extent (i.e. ≈ 60%; Table 1). The intra- and extracellular LDH activities were not significantly modified by genistein, meaning that this drug probably did not interfere with the cell viability (Table 1). The effect of genistein was dose-dependent, and 30 μg/ml genistein did not totally inhibit BSDL secretion (Figure 5). We have also studied the effect of genistein on the rate of BSDL biosynthesis. For this purpose, AR4-2J cells were pulsed with [^{35}S]methionine for 5 min, then chased in fresh medium without (control cells) or with genistein (60 μg/ml) for time periods between 15 and 60 min. At the end of each chase period, cells were harvested and lysed. Cell homogenates were cleared and immunoprecipitated using specific antibodies for the rat BSDL. The immunoprecipitated material was separated on SDS/PAGE and analysed by autoradiography (Figure 6A). Densitometric scanning of autoradiograms and linear-regression analysis (Figure 6B) indicated that the rate of BSDL new synthesis was poorly modified by genistein (less than 20–25%).

Surprisingly, and although genistein inhibited the BSDL secretion and had a weak effect on its synthesis, no accumulation of the BSDL activity was detected within AR4-2J cells treated with this drug (Table 1). This result suggested that a rapid degradation of BSDL, which cannot be detected under our experimental conditions [5,7], occurred or that inactive BSDL accumulated. In fact we have shown that the membrane-associated BSDL was poorly active [4] and that phosphorylation of the enzyme led to the release of the protein from intracellular membranes [10]. Therefore, the inhibition of BSDL phosphorylation by genistein could explain the inhibition of enzyme

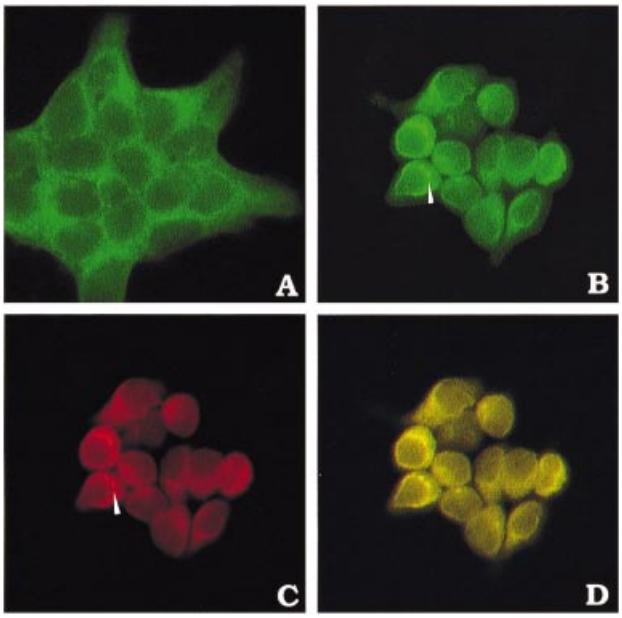

Figure 8 Localization of BSDL and the 58K Golgi protein in AR4-2J rat pancreatic cells upon genistein treatment

AR4-2J cells were grown on coverslips and incubated for 1 h in the absence (**A**) or in the presence of 60 μg/ml genistein (**B**, **C** and **D**). At the end of the incubation cells were permeabilized with 0.05% saponin in PBS buffer. Cells were treated with rabbit polyclonal antibodies to rat BSDL and with mouse monoclonal antibodies to 58K Golgi protein. The antigen–antibody complexes were then detected with secondary antibodies to rabbit or mouse IgG, conjugated with FITC and RITC, respectively. Coverslips were mounted on microscope slides and observed with a fluorescence microscope. (**A**) Control experiment, detection of BSDL with rabbit anti-BSDL antibodies and FITC-conjugated anti-rabbit IgG. (**B**, **C**) AR4-2J cells were treated with genistein and incubated with antibodies specific for BSDL (**B**) or for the 58K Golgi protein (**C**). Antibody–antigen complexes were detected using FITC-conjugated anti-rabbit IgG (**B**) or RITC-conjugated anti-rabbit IgG (**C**). (**D**) Represents the merged photograph (**B** + **C**).

secretion and its constant intracellular activity upon genistein treatment by a retention of inactive BSDL within a genistein-sensitive compartment. We firstly determined whether the genistein inhibited BSDL phosphorylation. For this purpose, cells were starved (30 min) in phosphate-free DMEM and radiolabelled with [^{32}P]orthophosphate (0.5 mCi/ml) for 3 h in the absence or presence of genistein. Then, cells were harvested and homogenized; BSDL was immunoprecipitated from the cleared homogenate (250 μg of proteins) and analysed by SDS/PAGE followed by autoradiography. As shown in Figure 7, the phosphorylation of BSDL (p74) was only partially inhibited by 30 μg/ml genistein (less than 20 % inhibition), but was almost totally abolished (more than 90 % inhibition) when this drug was used at the final concentration of 60 μg/ml. These results indicated that this drug, which perturbs cargo-vesicle traffic in the *trans*-Golgi network, also acted as an inhibitor of both phosphorylation and secretion of BSDL. As a consequence, the phosphorylation of BSDL should occur within this cell compartment. Then casein kinase II, which phosphorylates BSDL, might also be associated with this compartment. A 94-kDa ^{32}P-labelled protein (p94) co-precipitates with BSDL independently of the phosphorylation state of the enzyme. This 94-kDa phosphoprotein is likely the (auto)phosphorylatable Grp94 [27]. This chaperone, which co-precipitates with the enzyme, is involved in the folding of human [4] and rat [5] BSDL. Secondly, we attempted to demonstrate that BSDL protein accumulated within the Golgi compartment consecutively to genistein treatment. For this purpose, AR4-2J cells were grown on coverslips and treated with either genistein vehicle only (DMSO, control) or genistein. Coverslips were then treated for immunofluorescence studies. As shown on Figure 8(A), in AR4-2J control cells, BSDL located within a reticulated compartment and did not display retention. However, once treated with genistein at a concentration that inhibited BSDL secretion and phosphorylation (60 μg/ml), we observed an accumulation of the enzyme (Figure 8B, arrowhead) in a cell compartment where it had co-located with the 58K Golgi protein (Figure 8C, arrowhead), as demonstrated by the merged picture (Figure 8D).

BSDL phosphorylation and secretion

In a previous study [10] we have shown that the phosphorylation of human BSDL is important for the release of the protein from microsome membranes. Taken together with data presented here, this result supports strongly that the phosphorylation of BSDL might be essential for its secretion. To ascertain this point, AR4-2J cells were incubated in the presence of the casein kinase II inhibitor DRB [21] or in the presence of the drug vehicle only (controls). At the conclusion of the incubation, the cell-free medium was withdrawn and used to record BSDL and α-amylase activities. Alternatively, cells were harvested, lysed and the homogenate was cleared by centrifugation. BSDL and LDH activities of the cleared homogenate were determined. Under these conditions, the amount of BSDL activity present in cell-free medium of DRB-treated cells was significantly decreased by some 30 % ($P < 0.05$, Student's t test) compared with controls (Table 2), whereas no modification was observed when α-amylase activity was recorded in cell-culture medium. No LDH activity was detected in this medium, and that determined in the cell homogenate was not modified. These results strongly suggest that the phosphorylation of BSDL is an essential step for its secretion process. As already found with genistein, the inhibition of BSDL secretion by DRB did not induce a concomitant increase in intracellular BSDL activity.

Table 2 Effect of DRB on α-amylase and BSDL activities

AR4-2J cells were cultured in DMEM supplemented with DRB. After 15 min of incubation, cells were harvested and lysed. Cell homogenates were cleared by centrifugation and parameters, as indicated (DNA, protein, LDH, α-amylase and BSDL activities), were recorded. Values are means ± S.D. of at least three independent experiments. All intra- and extracellular activities are given as 10^{-3} units/mg of cell protein. $^*P < 0.05$ (Student's t test).

			Intracellular activities		Extracellular activities	
	DNA (μg/ml)	Protein (mg/ml)	LDH	BSDL	α-Amylase	BSDL
Control	20.4 ± 2.2	0.41 ± 0.06	5360 ± 240	223.6 ± 14.1	35.5 ± 1.8	7.2 ± 0.6
DRB (60 μM)	23.4 ± 2.7	0.45 ± 0.08	5100 ± 840	211.4 ± 20.2	33.5 ± 0.4	5.2 ± 0.8*

DISCUSSION

In its fundamental attributes, the secretion pathway of pancreatic BSDL follows that described for all enzymes involved in regulated secretion. However, BSDL is subjected to translational modifications. The first one, N-glycosylation of the enzyme at Asn-187, seems essential to establish the correct conformation of the enzyme [12]. This correct folding state is compatible with the full expression of BSDL activity and its secretion [12]. Once transferred *en bloc* to the nascent BSDL, the oligosaccharide precursor can be matured further without any effect on the enzyme activity or on its rate of secretion [12]. This result corroborates the fact that the N-glycosylation of BSDL may vary with pancreatic pathologies without affecting the presence of the enzyme in the pancreatic juice [28,29]. Amino acid sequences rich in Pro, Glu, Ser and Thr, referred to as PEST sequences, are common to rapidly degraded proteins [8]. Such sequences are present on pancreatic BSDL, where they are associated with tandemly repeated mucin-like sequences located in the C-terminal domain of the enzyme [6]. The second translational modification consists in the O-glycosylation of Thr residues [30] of these PEST sequences. The O-glycosylation is suspected to mask PEST sequences and to drive the protein towards the secretion pathway instead of a degradation route [7].

In human as well as in rat pancreas, BSDL was shown to be associated with intracellular membranes by means of a multiprotein complex including a 94-kDa Grp94-related chaperone [4,5]. This complex plays an essential role in the folding and transport of BSDL. Because the membrane-associated BSDL was N- and O-glycosylated [5], we have suggested that it might be released from intracellular membranes in the *trans*-Golgi compartment where glycans of BSDL experience terminal fucosylation. This terminal fucosylation is implicated in the establishment of the fucosylated oncofetal structure termed J28 epitope [31]. This oncofetal epitope, located on the C-terminal mucin-like structure of the oncofetal isoform of human BSDL [31–33] and which co-locates with PEST sequences, is generated by the core 2-β(1-6) *N*-acetylglucosaminyltransferase and the α(1-3/4) fucosyltransferase (FUT 3) [34], two glycosyltransferases associated with *trans*-Golgi membranes [35].

Another interesting point was to show that ATP, which is able to translocate within the endoplasmic reticulum [9], triggers the association of human BSDL to intracellular membranes [10]. In fact, ATP energy may be used to alter the structural properties of BSDL and allows the enzyme to associate with the membrane-folding complex [10]. In light of data showing that ATP helps BSDL to adopt an enzymically inactive conformation, we have postulated that the association of BSDL with the folding complex may be important for protecting the pancreatic cell from any alteration of lipid turnover [11]. Another possibility could be that the association of BSDL with endoplasmic reticulum and Golgi membranes is important in keeping the enzyme in close interaction with membrane glycosyltransferases.

The last-known post-translational modification is the phosphorylation of human BSDL at one site, probably a serine residue [10], and work is currently in progress to identify this residue. In human pancreas, a cascade of protein kinases controls the phosphorylation of BSDL [10]. The simplest model that can be deduced from published data could be that a first protein kinase sensitive to calphostin C, probably a protein kinase C, would inactivate by phosphorylation a second Ca^{2+}-sensitive and cyclic-nucleotide-independent kinase [10]. The latter kinase, when up-regulated by inactivation of the protein kinase C, phosphorylates BSDL [10]. After its phosphorylation, in a late Golgi compartment, BSDL is released from intracellular membranes and, consequently, could enter into the normal secretion pathway of pancreatic enzymes.

Although we strongly suspect that the release of human BSDL from intracellular membranes, consecutive to its phosphorylation, is an essential event in the enzyme-secretion process, no direct link was established between phosphorylation and secretion of the protein. In the present study, we have demonstrated that BSDL expressed by rat pancreatic AR4-2J cells is phosphorylated. This phosphorylation is most likely to be promoted by casein kinase II, which was further co-localized with BSDL within the lumens of microsomes of these cells. The casein kinase II is sensitive to cations [25] and could possibly be inhibited by cation chelators such as BAPTA-AM, which abolishes the phosphorylation *in vitro* of human BSDL [10] and decreases that of the AR4-2J cells. Therefore, the Ca^{2+}-sensitive and cyclic-nucleotide-independent protein kinase involved in the phosphorylation of the human BSDL could be casein kinase II. Interestingly, a protein kinase sensitive to EDTA and EGTA, and cAMP-independent, has been located within the Golgi apparatus (but not in the endoplasmic reticulum) of rat liver cells [36]. This kinase, which phosphorylates the apolipoproteins B100 and B48 [36], may be important in the assembly and secretion of apolipoprotein B-containing lipoproteins. Although casein kinase II autophosphorylates on the β-subunit [37], it seems that its activity, expressed by the α-subunit, could not be regulated by phosphorylation, and consequently could not depend upon another protein-kinase activity. A way to conciliate data obtained on human microsomes with these present results on rat microsomes is to postulate that the inhibition of the calphostin C-sensitive protein kinase, which promotes the phosphorylation of BSDL by casein kinase II, might be a contaminant of our human-microsome preparation. This contaminant protein kinase may act indirectly on the activity of casein kinase II by, for example, affecting the synthesis of phospholipids that modulate the casein kinase II activity. The lack of effect of inhibitors of protein kinases A, C and G such as H-7, H-8, H-89 and calphostin C argues in favour of the absence, in our rat-microsome preparation, of any other kinases which may alter the casein kinase II activity. However, we can also argue for a species difference (rat compared with human) and for a different pathophysiological state of the cell model, normal human tissue versus tumoral rat cell line, used. More studies will help us to elucidate this point.

Furthermore, the phosphorylation of BSDL occurred in a genistein-sensitive compartment of AR4-2J cells. Because genistein inhibits the formation and the release of cargo vesicles in the *trans*-Golgi network [25], and the phosphorylation of BSDL as well, one may postulate that BSDL is phosphorylated within this subcellular compartment. We further show that, upon treatment of cells by genistein, BSDL accumulates in a compartment where it co-locates with the 58K Golgi protein. Therefore, BSDL appears to be phosphorylated in a late compartment of the Golgi apparatus. This result agrees with data indicating that the release of BSDL from membranes, which occurs after its phosphorylation, is sensitive to monensin [5]. It also agrees with the full glycosylation of the membrane-associated BSDL [4,5]. Another important result was to show that DRB, a casein kinase II-specific inhibitor [21], inhibits the phosphorylation of BSDL and alters its secretion. However, DRB did not affect the α-amylase secretion and the LDH intracellular activity, meaning that neither the secretory mechanisms nor the transcription processes were affected by this drug under our experimental conditions. Taken altogether, these data suggest strongly that the phosphorylation of BSDL by casein kinase II takes place within the *trans*-Golgi compartment, where the

enzyme is released from membranes and secreted consecutively. Genistein and DRB, which alter BSDL secretion, did not induce an increase of BSDL intracellular activity; however, the former drug induces the accumulation of the protein within the Golgi. Monensin also inhibits the enzyme secretion by impairing the Golgi transport of proteins [5]. This results in a higher association of inactive BSDL with intracellular membranes and its routing to a degradation compartment [5]. Thus genistein, which alters the Golgi functions, and DRB, which inhibits BSDL phosphorylation, could increase the association of BSDL with membranes (probably in an inactive conformation) and/or target the protein to degradation.

Another important item is that Grp94, which normally resides within the endoplasmic-reticulum vesicles tends, under stressful conditions, to redistribute to the Golgi apparatus [38]. Grp 94 was found in the secretion of exocrine pancreatic cells [39]. This chaperone can be phosphorylated by numerous kinases, including a Ca^{2+}- and Mg^{2+}-dependent autophosphorylation activity [27] and a labile kinase activity, which shares some similarity with casein kinase II [40]. Grp94, the role of which is still uncertain [41], is also known to be associated with casein kinase II [42] and seems to be phosphorylated in another compartment than the endoplasmic reticulum [43]. Grp94 has been reported to exist in a C-terminally truncated form [44], which may help its escape from the endoplasmic reticulum by losing the C-terminal KDEL retention signal, suggesting that the localization of Grp94 may be versatile. Then it seems that, as BSDL, Grp94 could reach the *trans*-Golgi, where its phosphorylation, coupled to that of BSDL, could help to dissociate the folding complex from intracellular membranes [5]. However, and as suggested by results presented in Figure 7, a 94-kDa phosphoprotein, which could be Grp94, can be co-precipitated with specific antibodies to the rat BSDL, even when the phosphorylation of the enzyme was abolished by genistein. Although the phosphorylation of BSDL leads to its release from intracellular membranes [10] or from its folding complex [5], the dissociation of the BSDL–Grp94 complex [5] could not depend upon the phosphorylation state of BSDL. This allows the secretion of BSDL, a fraction of which can be still complexed to Grp94, in pancreatic juice [45].

This work was supported by Grants in Aid nos. 9912, 6122 and 4050 from the Association pour la Recherche contre le Cancer (ARC, Villejuif, France). E.P. received a fellowship from the Fondation pour la Recherche Médicale (FRM, Paris, France), and N.C. was a recipient of a fellowship from the ARC.

REFERENCES

1 Lombardo, D. and Guy, O. (1980) Biochim. Biophys. Acta **611**, 147–155
2 Gjellesvik, D. R., Lombardo, D. and Walther, B. T. (1992) Biochim. Biophys. Acta **1124**, 123–134
3 Lombardo, D., Guy, O. and Figarella, C. (1978) Biochim. Biophys. Acta **527**, 142–149
4 Bruneau, N., Lechene de la Porte, P., Sbarra, V. and Lombardo, D. (1995) Eur. J. Biochem. **233**, 209–218
5 Bruneau, N. and Lombardo, D. (1995) J. Biol. Chem. **270**, 13524–13533
6 Reue, K., Zambaux, J., Wong, H., Lee, G., Leete, T. H., Ronk, M., Shively, J. E., Sternby, B., Borgstrom, B., Ameis, D. and Schotz, M. G. (1991) J. Lipid Res. **32**, 267–276
7 Bruneau, N., Nganga, A., Fisher, E. A. and Lombardo, D. (1997) J. Biol. Chem. **272**, 27353–27361
8 Rogers, S., Wells, R. and Rechsteiner, M. (1986) Science **234**, 364–368
9 Clairmont, C. A., De Maio, A. and Hirschberg, C. B. (1992) J. Biol. Chem. **267**, 3983–3990
10 Pasqualini, E., Caillol, N., Bruneau, N., Lexa, D. and Lombardo, D. (1997) Biochem. J. **327**, 527–537
11 Le Petit-Thevenin, J., Bruneau, N., Nobili, O., Lombardo, D. and Vérine, A. (1998) Biochim. Biophys. Acta **1393**, 307–316
12 Abouakil, N., Mas, E., Bruneau, N., Benajiba, A. and Lombardo, D. (1993) J. Biol. Chem. **268**, 25755–25763
13 Abouakil, N., Rogalska, E., Bonicel, J. and Lombardo, D. (1988) Biochim. Biophys. Acta **961**, 299–308
14 Jamieson, J. D. and Palade, G. E. (1967) J. Cell Biol. **34**, 577–596
15 Labarca, C. and Paigen, K. (1980) Anal. Biochem. **102**, 344–352
16 Wallenfels, K., Földi, P., Niermann, H., Bender, H. and Linder, D. (1978) Carbohydrate Res. **61**, 359–368
17 Goldberg, E. (1972) J. Biol. Chem. **247**, 2044–2048
18 Wang, C. S. and Johnson, K. (1983) Anal. Biochem. **133**, 457–461
19 Laemmli, U. K. (1970) Nature (London) **227**, 680–685
20 Burnette, W. N. (1981) Anal. Biochem. **112**, 195–203
21 Zandomeni, R. and Weinmann, R. (1984) J. Biol. Chem. **259**, 14804–14811
22 Penner, C. G., Wang, Z. and Lichtfield, D. W. (1997) J. Cell Biochem. **64**, 525–537
23 Sarrouilhe, D., Filhol, O., Leroy, D., Bonello, G., Baudry, M., Chambaz, E. M. and Cochet, C. (1998) Biochim. Biophys. Acta **1403**, 199–210
24 Sfeir, C. and Veis, A. (1995) J. Bone Mineral Res. **10**, 607–615
25 Edelman, A. M., Blumenthal, D. K. and Krebs, E. G. (1987) Annu. Rev. Biochem. **56**, 567–613
26 Austin, C. D. and Shields, D. (1996) J. Cell Biol. **135**, 1471–1483
27 Csermely, P., Miyata, Y., Schnaider, T. and Yahara, I. (1995) J. Biol. Chem. **270**, 6381–6388
28 Sugo, T., Mas, E., Abouakil, N., Endo, T., Escribano, M. J., Kobata, A. and Lombardo, D. (1993) Eur. J. Biochem. **216**, 799–805
29 Mas, E., Abouakil, N., Roudani, S., Franc, J. L., Montreuil, J. and Lombardo, D. (1993) Eur. J. Biochem. **216**, 807–812
30 Wang, C. S., Dashti, A., Jackson, K. W., Yeh, J. C., Cumming, R. D. and Tang, J. (1995) Biochemistry **34**, 10639–10644
31 Mas, E., Crotte, C., Lecestre, D., Michalski, J. C., Escribano, M. J., Lombardo, D. and Sadoulet, M. O. (1997) Glycobiology **7**, 745–752
32 Pasqualini, E., Caillol, N., Panicot, L., Mas, E., Lloubes, R. and Lombardo, D. (1998) J. Biol. Chem. **273**, 28208–28218
33 Mas, E., Abouakil, N., Roudani, S., Miralles, F., Guy-Crotte, O., Figarella, C., Escribano, M. J. and Lombardo, D. (1993) Biochem. J. **289**, 609–615
34 Panicot, L., Mas, E., Pasqualini, E., Zerfaoui, M., Lombardo, D., Sadoulet, M.-O. and El Battari, A. (1999) Glycobiology **9**, 935–946
35 Colley, K. J. (1997) Glycobiology **7**, 1–13
36 Swift, L. L. (1996) J. Biol. Chem. **271**, 31491–31495
37 Hathaway, G. M. and Traugh, J. A. (1982) Curr. Top. Cell Regul. **21**, 101–127
38 Booth, C. and Kock, G. L. E. (1989) Cell **59**, 729–737
39 Takemoto, H., Yoshimori, T., Yamamoto, A., Miyata, Y., Yahara, I., Inove, K. and Tashiro, Y. (1992) Arch. Biochem. Biophys. **296**, 129–136
40 Ramakrishnam, M., Schonthal, A. H. and Lee, A. S. (1997) J. Cell. Physiol. **170**, 115–129
41 Csermely, P., Schnaider, T., Söti, C., Prohàszha, Z. and Nardai, G. (1998) Pharmacol. Ther. **79**, 129–168
42 Shi, Y., Brown, E. D. and Walsh, C. T. (1994) Proc. Natl. Acad. Sci. U.S.A. **29**, 2767–2771
43 Cala, S. E. and Jones, L. R. (1994) J. Biol. Chem. **269**, 5926–5931
44 Poola, I. and Kiang, J. G. (1994) J. Biol. Chem. **269**, 21762–21769
45 Bruneau, N., Lombardo, D. and Bendayan, M. (1998) J. Cell Sci. **111**, 2665–2679

Received 9 April 1999/6 September 1999; accepted 21 October 1999