Control of pancreatic bile-salt-dependent-lipase secretion by the glucose-regulated protein of 94 kDa (Grp94)

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Bile-salt-dependent lipase (BSDL; EC 3.1.1.13) is an enzyme expressed by the pancreatic acinar cell and secreted as a component of the pancreatic juice. During its route towards secretion, BSDL is associated with intracellular membranes by means of a multiprotein folding complex, which includes the glucose-regulated protein of 94 kDa (Grp94). We have postulated that the association of BSDL with membranes is required for the complete O-glycosylation of the protein, which diverts BSDL from a degradation route and consequently allows its secretion. To further characterize the role of Grp94 in BSDL secretion, we have studied the effect of a ribozyme specifically targeted to Grp94 mRNA. This ribozyme has been transfected into AR4-2J cells, and we have shown that a decrease in Grp94 expression leads to a concomitant decrease in BSDL secretion and ex-

pression. Geldanamycin (GA), which alters Grp94 functions, also affects the release of BSDL into the culture medium of AR4-2J cells. BSDL expressed in GA-treated AR4-2J cells is unstable. Furthermore, under conditions that decrease the level of BSDL secretion, no intracellular accumulation of the enzyme was observed, suggesting that BSDL that cannot associate with (or be structured by) Grp94 could be rapidly degraded. We have further shown that this degradation probably occurs via the ubiquitin-dependent pathway. Altogether, these results indicate that Grp94 has a pivotal role in BSDL folding and in the sorting of this pancreatic enzyme.

Key words: glucose-regulated protein, ribozyme, geldanamycin, butyrate.

INTRODUCTION

Bile-salt-dependent lipase (BSDL; EC 3.1.1.13) is an enzyme involved in the duodenal hydrolysis of cholesteryl esters [1]. The enzyme is synthesized within the endoplasmic reticulum (ER) of acinar cells, and then follows the secretory pathway of these cells to be secreted as a component of the pancreatic juice [2]. However, in contrast with other pancreatic enzymes, such as α -amylase, colipase-dependent lipase, chymotrypsinogen and pancreatic stone protein, BSDL has been shown to be associated with membranes of the ER and the Golgi apparatus [3,4]. This association involves a membrane multiprotein folding complex, and one of these proteins with which BSDL interacts is the chaperone known as the glucose-regulated protein of 94 kDa (Grp94) [4]. The association of BSDL with intracellular membranes could be essential for the complete O-glycosylation of a BSDL domain, rich in proline, threonine, serine and glutamic acid, and referred to as a PEST sequence, which acts as a signal for rapid degradation of proteins [5]. This domain is located within the C-terminal tandemly repeated identical mucin-like sequences of the protein [6]. We further demonstrated that O-glycosylation of mucin-like sequences masks the PEST domain and is required for BSDL secretion [7].

The quality control mechanism acting in the ER includes many molecular chaperones, such as calreticulin, calnexin, the protein disulphide isomerase, Grp78 and Grp94, which are involved in the secretion of proteins. At present the role of Grp94, which is the ER paralogue of the extensively studied heat shock protein (Hsp)90 is poorly understood. Nair et al. [8] recently gave a list of possible functions for the Hsp90/Grp94 chaperone family: (1) repression of the target's activity; (2) protection of the target from proteolysis; (3) dynamic docking of the target to regulate its oligomerization; and (4) provision of

phenotypic diversity for the target by stabilizing its alternative conformational states. Data suggest that Grp94 might act similarly to cytoplasmic Hsp90 by binding after a preceding pre-folding step by Grp78 [or immunoglobulin heavy-chain binding protein (BiP)] [9,10]. Thus Grp94 may recognize a protein segment with significant secondary structure, but with a fluctuating tertiary structure. Overexpression of Grp94 prolongs the folding of thyroglobulin [11], and treatment of cells with the Grp94-binding drug geldanamycin (GA) increases the transcription of ER chaperones via a stress pathway [12], with a concomitant increased association of unfolded proteins with Grp78, the gene of which is co-regulated with that of Grp94 [13]. We have recently shown that Grp94 also participates in the secretion of BSDL and in its internalization by the intestinal epithelium [14].

Based on these data, we reasoned that if Grp94 plays an essential role in BSDL folding and sorting along the entire secretory pathway of the enzyme, modulation of the expression of the chaperone should have effects on the secretion of the enzyme. We have used the cell line AR4-2J, which has been described as a model system for long term studies on pancreatic acinar cell function. In the present study we have designed a Grp94-mRNA-specific ribozyme, which upon cell transfection decreased the cellular level of the chaperone. Results suggested that Grp94 might be involved in the late folding steps of BSDL and that it might control the sorting of the active enzyme towards the secretion route.

MATERIALS AND METHODS

Reagents

Dulbecco's modified Eagle's medium (DMEM), methionine-free DMEM, glutamine, penicillin, streptomycin and trypsin/EDTA

Abbreviations used: BiP, immunoglobulin heavy-chain binding protein; BSDL, bile-salt-dependent lipase; CMV, cytomegalovirus; DMEM, Dulbecco's modified Eagle's medium; ER, endoplasmic reticulum; FCS, foetal calf serum; GA, geldanamycin; Grp94, glucose-regulated protein of 94 kDa; Hsp, heat shock protein; Ub-BSDL, ubiquitinated-BSDL.

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were obtained from Life Technologies (Gaithersburg, MD, U.S.A.). Foetal calf serum (FCS) was purchased from Dutscher (Brumath, France). Benzamidine, alkaline-phosphatase-conjugated goat antibodies specific for rabbit or rat immunoglobulins, sodium butyrate and 4-nitrophenyl hexanoate were obtained from Sigma (St. Louis, MO, U.S.A.). Nitrocellulose (BA 83 type; 0.2 μ m pore size) membranes were purchased from Schleicher and Schuell (Dassel, Germany). Taq DNA polymerase and dNTPs were obtained from Promega (Madison, WI, U.S.A.). Endoglycosidase H, avian myeloblastosis reverse transcriptase, human placenta RNase inhibitor and RNase A were purchased from Roche Diagnostics (Meylan, France). The pcDNA-3 vector was obtained from Invitrogen (Groningen, The Netherlands).

Biological materials

Polyclonal antibodies directed against rat BSDL were raised as previously described [15], and were purified on Protein A-Sepharose. Monoclonal antibodies specific for Grp94 (clone 9G10) and Grp78 (clone10C3) were obtained from StressGen Biotechnologies Corp. (Victoria, BC, Canada). Note that antibodies of the 9G10 clone do not recognize Hsp90, the cytosolic paralogue of Grp94. Rabbit polyclonal antibodies raised against bovine erythrocyte ubiquitin were purchased from StressGen Biotechnologies Corp.

Cell culture, cell extracts and metabolic labelling of cell proteins

The rat pancreatoma AR4-2J cell line was routinely cultured in DMEM containing 10% (v/v) FCS, 2 mM glutamine, 1 g/l glucose, 100 units/ml penicillin and 100 μ g/ml streptomycin. The cells were kept in a humidified incubator at 37 °C in an atmosphere of air/CO₂ (19:1). Cells grown to confluence were harvested with 0.25% trypsin/EDTA, after being rinsed twice with incomplete PBS buffer [10 mM phosphate buffer (pH 7.0) containing 0.15 M NaCl, but without Ca²⁺ and Mg²⁺]. The cells were suspended in DMEM supplemented with 10% (v/v) FCS, and were centrifuged at 1000 *g* for 2 min. The cells were then lysed in lysis buffer [10 mM Hepes (pH 7.4), 1.5% (v/v) Triton X-100, 200 mM NaCl, 2 mM CaCl₂ and 2 mM benzamidine] by sonication for 10 s (Biolock Vibra Cell, Strasbourg, France). The cell debris was pelleted by centrifugation (700 *g* for 15 min) and the supernatant, referred to as the intracellular fraction, was saved until use. When required, cell proteins were metabolically labelled with [³⁵S]methionine (20 μ Ci/ml; Tran ³⁵S-label; ICN Biomedicals, Costa Mesa, CA, U.S.A.) as previously described [15].

Transfection

Stable transfection of AR4-2J cells was performed using LIPO-FECTAMINE Plus™ reagent (Life Technologies) according to the manufacturer's protocol. Selection of transfected cells was performed essentially as previously described [7], using G418 (0.5 mg/ml; Life Technologies).

Protein determination, and DNA and enzyme assays

Protein content was measured using the Micro BCA kit from Pierce, using BSA as a standard. DNA was assayed by a fluorimetric method using the Hoechst 33258 reagent [16], solubilized in 50 mM sodium phosphate (pH 7.4), 2 M NaCl and 2 mM EDTA. The esterolytic activity of BSDL was measured using 4-nitrophenyl hexanoate as a substrate and monitoring the change in absorbance at 404 nm and pH 7.4 in a thermostated cell at 30 °C, as described elsewhere [17]. α -Amylase activity was determined using the Amylase kit from Sigma Diagnostic.

SDS/PAGE, immunoblotting and immunoprecipitation

SDS/PAGE was performed using slab gels containing 7.5% (w/v) polyacrylamide and 0.1% SDS, as described by Laemmli [18]. After migration, the proteins were electrophoretically transferred on to nitrocellulose membranes [19] in 50 mM Tris/HCl (pH 9.2), 40 mM glycine and 10% (v/v) methanol, at 18 mA overnight in a cold room. The completeness of the transfer was checked by staining the polyacrylamide gels with Coomassie Brilliant Blue R250 and destaining them in methanol/acetic acid/water (3:1:6, by vol.). After staining with 0.2% Ponceau S, to confirm the efficiency of the transfer, the free binding sites on the membranes were first blocked for 1 h in 50 mM Tris/HCl (pH 8.0) buffer containing 150 mM NaCl (TBS buffer) and 5% (w/v) skimmed dry milk (blocking buffer). Immunodetection was carried out using antibodies specific for BSDL, Grp78 or Grp94 as the primary antibodies. Incubation was carried out for 1 h in TBS buffer containing 2% (w/v) skimmed milk and 0.1% Tween 20. Replicas were then incubated in the same buffer containing alkaline-phosphatase-conjugated goat antibodies in order to visualize the bound antibodies. Double-immunoblots were performed using a mixture of rat antibodies specific for Grp94 and rabbit antibodies specific for BSDL, which were revealed with anti-rat and anti-rabbit antibodies conjugated to alkaline phosphatase. The membranes were developed with a mixture of 0.5 mM Nitro Blue Tetrazolium and 0.5 mM 5-bromo-4-chloroindol-3-yl phosphate in 10 mM Tris/HCl (pH 9.5), 10 mM NaCl and 1 mM MgCl₂. Between each incubation, membranes were extensively washed in TBS buffer containing 0.1% Tween 20. Alternatively, the Chemoluminescence Western Blotting Kit (Roche Diagnostics) was used to detect antigen-antibody complexes on nitrocellulose membranes.

Immunoprecipitations were performed as follows. ³⁵S-labelled proteins (600 μ g) from clarified cell lysate or from identical volumes of cell-free medium were incubated overnight, under agitation at 4 °C, with 50 μ g of Protein A-purified antibodies specific for rat BSDL or with 10 μ g of monoclonal antibodies specific for Grp94. Protein A-agarose (10 mg; Sigma), prewashed three times with the lysis buffer, was added to the antibody-antigen complexes and was incubated for 4 h at 4 °C with agitation. The antibody-antigen-Protein A complexes were recovered by centrifugation (10000 *g* for 15 min, at 4 °C). The final pellet was then washed twice with washing buffer [10 mM Tris/HCl (pH 7.4), 5 mM EDTA and 0.5% Triton X-100], twice with the washing buffer supplemented with 1 M NaCl and 0.1% SDS, and finally twice with the washing buffer without any detergent. The pellet was then transferred into Laemmli's SDS/PAGE sample buffer, boiled for 5 min, centrifuged and resolved by SDS/PAGE. When required, a two-cycle immunoprecipitation procedure, slightly modified from Doolittle et al. [20], was performed. Proteins in the gels were fixed by staining with Coomassie Brilliant Blue R250, and were subsequently destained. Gels were then immersed (30–60 min) in Amplify (Amersham) and autoradiographed (BioMax films, Eastman-Kodak, Rochester, NY, U.S.A.). Finally autoradiograms were quantified using the Image program (National Institutes of Health, Bethesda, MD, U.S.A.).

Northern dot-blot

RNA was extracted from AR4-2J cells according to the method of Chirgwin et al. [21], and were quantified by Northern dot-blot dilution. Serial dilutions of RNA were blotted on to nitrocellulose membranes using a Bio-Dot microfiltration apparatus (Bio-Rad, Hercules, CA, U.S.A.). Prehybridization and hybridization using cDNA probes specific for BSDL [22], Grp94 [4] and β -actin were

performed essentially as described previously [23]. Before hybridization, probes were ^{32}P -labelled by the random-priming DNA-labelling kit (Life Technologies) using [α - ^{32}P]dCTP (NEN, Boston, MA, U.S.A.) at a specific radioactivity of 4×10^8 c.p.m./ μg DNA probe [22].

RESULTS

Synthetic ribozyme targeted against Grp94 mRNA

The aim of this study was to determine the effect of the modulation of Grp94 expression on BSDL secretion. For this purpose, and based on the 5'-terminal sequence of Grp94 [24], we have constructed a Grp94-mRNA-targeted hammerhead-type ribozyme [13,25]. To obtain the best efficiency, the mRNA cleavage by the hammerhead should occur close to the initiation codon of Grp94, in such a way that the truncated transcript, even if it remains stable, will not be able to be translated into Grp94 [12]. Additionally, antisense base-pairing between the ribozyme and the Grp94 mRNA, may also participate in inhibition of the translation, because steric hindrance occurs at the level of the AUG initiation codon. The ribozyme includes ten essential nucleotides that form three conserved stems (see the boxed residues in Figure 1B). The conserved stems constitute the catalytic core of the ribozyme [25]. The remaining part of the hammerhead catalytic centre was designed to specifically hybridize with Grp94 mRNA, and includes the GUC nucleotide sequence after which the catalytic cleavage is expected to occur. This cleavage site is 3' of the cytosine residue and is six nucleotides downstream from the initiation codon.

Therefore two complementary 63 bp oligonucleotides were synthesized (Genset Europe SA, Paris, France); one of them, which should be transcribed in the ribozyme, complies with all of the conditions described above. The duplex was formed by incubating these two oligonucleotides (15 μg of each, without prior purification) in water for 5 min at 95 °C, followed by further incubation at room temperature (20 min) and in ice (10 min). The duplex obtained, the sequence of which is given in Figure 1(A), was then purified on a 1.5 % (w/v) agarose gel and inserted into a pCDNA-3 vector at the *EcoRV* site. Under these

conditions, only two-end-blunt duplexes can be ligated into the vector.

The plasmid thus obtained was then amplified in *Escherichia coli* DH5- α strain and the clone producing the plasmid in the correct orientation [i.e. with the (+) strand facing the cytomegalovirus (CMV) promoter] was selected for by growing the bacteria on ampicillin-containing medium and was isolated after restrictive cleavages using *EcoRI* or *HindIII*. Cleavage of the plasmid by any of these enzymes should (Figure 2) give two fragments. Independently of the restriction enzyme used (*HindIII* or *EcoRI*) the correctly-orientated ribozyme should be cleaved into two fragments of approximately the same size (i.e. approx. 64 and 68 bp respectively), whereas the wrongly-orientated product should give two fragments of different size (i.e. 113 bp and 19 bp after cleavage by *HindIII* and *EcoRI* respectively). One clone was thus selected (Figure 2B) and this clone was sequenced using a T7 promoter universal probe. The sequence of the selected plasmid, termed pCD94 Rib⁺, was that expected. Therefore once transcribed this plasmid should give a product which will hybridize at the initiation codon level of the specific mRNA encoding Grp94 (see Figure 1B).

AR4-2J cells which expressed Grp94 and secreted BSDL [4,15] were stably transfected with the empty pCDNA-3 vector (control) or with the pCD94 Rib⁺ plasmid. Cell clones were then selected for using G418 (0.5 mg/ml). By end-limiting dilution, ten control clones and ten pCD94 Rib⁺ clones were isolated. Two clones, representative of control clones and of pCD94 rib⁺ clones, and referred to as AR4-2J Rib⁻ and AR4-2J Rib⁺ respectively, were used in the present study. Immunoblotting using antibodies specific for Grp94 indicated that AR4-2J Rib⁺ clones expressed less Grp94 (Figure 3). Densitometric scanning of the bands showed that at steady state, AR4-2J Rib⁺ cells expressed approximately 30 % less (28.8 ± 13.0 %) Grp94 than AR4-2J Rib⁻ cells. This decrease is significant and comparable to that obtained by Little and Lee [13], who have constructed a similar Grp94-mRNA-targeted ribozyme using a more complex procedure. The basal expression of Grp94 may be due to a slow turnover of the chaperone in pancreatic cells or to cell viability, as a deficit in the expression of this chaperone can be lethal to the cell [13,26].

Figure 1 Sequence and structure of the ribozyme specifically targeted to Grp94 mRNA

(A) Sequences of the two paired oligonucleotides coding for the ribozyme. Restriction sites are underlined, and sequences complementary to the Grp94 mRNA are in italic. Nucleotides essential for the activity of the ribozyme are double underlined. (B) Hammerhead structure of the Grp94-mRNA-targeted ribozyme. The essential nucleotides are boxed, the cleavage site and the Grp94 mRNA initiation codon are indicated, and sequences complementary to the Grp94 mRNA are indicated by vertical lines. Unrelated sequences are in lower case letters.

Figure 2 Expression vector used to stably transfect rat pancreatic AR4-2J cells

(A) The parental pcDNA-3 vector was obtained from Invitrogen. The paired oligonucleotide encoding the ribozyme was inserted into the *EcoRV* site of the pcDNA-3 polylinker leading to an antisense- and sense-ribozyme-expressing vector mixture. (B) Vectors were amplified in *E. coli* DH5- α strain and the clone producing the plasmid in the correct orientation was selected by restrictive cleavages using *EcoRI* or *HindIII*. The correctly-orientated ribozyme should be cleaved into two fragments of approx. the same size (64–68 bp) whereas the wrongly-orientated one should give two fragments of 113 bp and 19 bp for *HindIII* and *EcoRI* respectively. After restrictive cleavage, fragments were separated on a 1.5% (w/v) agarose gel. As shown, the two clones examined (lanes b and c), gave fragment(s) of the expected size (i.e. 64–68 bp) that migrated at the level of the paired oligonucleotide (lane a), which was used as a migration control. Lane m displays a size ladder. Amp, ampicillin.

Also, an increase in the amount of misfolded proteins in the ER due to decreased constitutive expression of Grp94 could provoke an ER overload response and, in turn, the up-regulation of certain inducible chaperones including Grp94 [13,27]. It is worth noting that the expression of Grp78 was effectively increased in AR4-2J Rib⁺ clones compared with AR4-2J Rib⁻ clones (by 1.4-fold; results not shown). Contrary to Grp94, Grp78 has never been characterized as part of the BSDL folding complex [4], and might not be implicated in the folding of the enzyme. Consequently, if the Grp94-mRNA-targeted ribozyme affects the secretion of BSDL, it may not act by means of Grp78 up-regulation.

Grp94-mRNA-specific ribozyme decreases the intracellular level of BSDL and its rate of secretion

Accordingly, to further assess whether lowering the basal expression of Grp94 results in a modification of the secretion of BSDL, AR4-2J Rib⁻ and AR4-2J Rib⁺ cells were cultured under usual conditions [15] and the enzyme was allowed to concentrate

Figure 3 Expression of Grp94 in AR4-2J cells transfected with the Grp94-mRNA-targeted ribozyme

AR4-2J cells were transfected with the Grp94-mRNA-targeted ribozyme or with the empty vector to give AR4-2J Rib⁺ and AR4-2J Rib⁻ clones respectively. These clones were selected and allowed to grow until 80% confluence. Cells cultured for 4 h in three separate Petri dishes were harvested and lysed before analysis on SDS/PAGE (50 μ g of cellular protein/lane) and electrotransfer on to nitrocellulose membranes. Replicas were then probed using a monoclonal antibody specific for Grp94, and were quantified by densitometric scanning. This experiment was performed in triplicate (only one representative immunoblot is shown) and results are presented as means $\pm$ S.E.M.

Figure 4 Secretion of BSDL by AR4-2J Rib⁺ and AR4-2J Rib⁻ cell clones

AR4-2J Rib⁺ and AR4-2J Rib⁻ clones were cultured in three separate Petri dishes and were allowed to concentrate BSDL in the cell culture medium. At the indicated times BSDL activity was recorded in the culture medium of AR4-2J Rib⁺ (●) and AR4-2J Rib⁻ (○) clones. Data are presented as means $\pm$ S.E.M. for at least three independent experiments. Error bars not shown are within the symbols. mU, m-units.

in the culture medium. Samples were removed after 4, 10 and 18 h of culture and the cells were lysed to determine cellular DNA content. As shown in Figure 4 the rate of BSDL activity release into the culture medium by AR4-2J Rib⁺ cells was significantly decreased, by approximately 30% (as determined from the slope obtained after linear regression), when compared with the production of control cells (AR4-2J Rib⁻ clone). The decrease in secretion rate of BSDL corroborates the activity of BSDL detected within AR4-2J Rib⁺ cells after 4 h of culture, as this activity was lowered by the same proportion ($29.3 \pm 6.1\%$)

Figure 5 Expression of BSDL in AR4-2J Rib⁺ and AR4-2J Rib⁻ cell clones

Subconfluent AR4-2J Rib⁺ and AR4-2J Rib⁻ clones were cultured for 4 h, harvested and lysed. Cellular proteins were resolved by SDS/PAGE (50 μ g/lane) and were electrotransferred on to nitrocellulose membranes. Blots were then probed with antibodies directed against rat BSDL, and were quantified by densitometric scanning (only one representative immunoblot is shown). Data are from three separate experiments and are presented as means $\pm$ S.E.M.

when compared with AR4-2J Rib⁻ cells. This result may be interpreted in two ways: (1) unfolded or inactive BSDL, which cannot associate with Grp94, probably due to the lower expression of the chaperone, could accumulate within AR4-2J Rib⁺ cells; and (2) BSDL does not accumulate within these cells and is degraded. We attempted to discriminate between these two hypotheses by determining the levels of BSDL expressed in AR4-2J Rib⁻ and AR4-2J Rib⁺ cells after cell lysis. Data given in Figure 5, suggested that the level of intracellular BSDL was decreased by approx. 40% (38.4 ± 8.9) in AR4-2J Rib⁺ cells compared with AR4-2J Rib⁻ cells, a decrease similar to that observed for Grp94 (see Figure 3). Taken altogether, these results prompted us to favour the second hypothesis. Accordingly, if regulation of the cellular level of BSDL by Grp94 association took place, the amount of intracellular BSDL relative to the amount of Grp94 expressed in AR4-2J Rib⁺ and AR4-2J Rib⁻ cells should be constant. To ascertain this specific point, the ratio representing the expression level of Grp94 compared with that of BSDL was determined using double-immunoblots. This ratio was found to be identical in AR4-2J Rib⁺ (0.662 ± 0.097) and AR4-2J Rib⁻ (0.665 ± 0.150) cell clones. Furthermore, the amount of ³⁵S-labelled BSDL that was co-precipitated with antibodies specific for Grp94 was also identical in AR4-2J Rib⁺ and AR4-2J Rib⁻ clones, relative to the amount of precipitated ³⁵S-labelled Grp94 (0.58 ± 0.08). Altogether, these results suggest that Grp94, in part, controls the level of intracellular BSDL, probably by diverting the active enzyme from a degradation route towards secretion.

Figure 6 Northern dot-blot analysis of RNA in AR4-2J Rib⁺ and AR4-2J Rib⁻ cell clones

Total RNA was extracted from AR4-2J Rib⁺ (solid symbols) and AR4-2J Rib⁻ (open symbols) cell clones. Decreasing amounts were dotted on to nitrocellulose membranes and analysed using ³²P-labelled probes for (A) β -actin (■, □) or for (B) BSDL (▲, △) and Grp94 (●, ○). Graphs represent the regression analysis of dot-blot signal intensities versus μ g of dotted RNA and are representative of two independent experiments. (C) The synthesis rate of BSDL was determined by a pulse-chase experiment. AR4-2J Rib⁺ (●) and AR4-2J Rib⁻ (○) cells were cultured until subconfluence, were starved in methionine-free DMEM for 1 h and were pulsed with [³⁵S]methionine (20 μ Ci/ml) for 15 min. At the end of the pulse, cells were chased for the indicated time periods in fresh medium, and were subsequently harvested and lysed. Radiolabelled BSDL was immunoprecipitated from cell lysates and analysed by SDS/PAGE. Autoradiograms were quantified by densitometric scanning and the amount of precipitated radiolabelled BSDL (intensity in arbitrary units) was plotted according to the chase time period. Only a representative experiment is shown.

We wondered whether, during the selection process, we isolated AR4-2J cell clones expressing different levels of BSDL, and whether, fortuitously, the one expressing less BSDL was the AR4-2J Rib⁺ clone. To clarify this important point, we quantified mRNA specific for β -actin, BSDL and Grp94, in AR4-2J Rib⁻ and AR4-2J Rib⁺ cell clones. Using specific probes, the relative amounts of respective mRNA were determined using a Northern dot-blot as described in the Materials and methods section. As shown in Figures 6(A) and 6(B) densitometric quantification of Northern dot-blots indicated that at steady state, the abundance of mRNA specific for β -actin and BSDL was not

Figure 7 Effects of sodium butyrate

AR4-2J Rib⁺ (solid symbols) and AR4-2J Rib⁻ (open symbols) cells were grown until subconfluence, and were then cultured for 16 h in fresh medium in the presence of 0, 5 and 10 mM sodium butyrate. At the end of the incubation time, the culture medium was removed and the cells were lysed. **(A)** BSDL (▲, △) and **(B)** α-amylase (■, □) activities were recorded in the culture medium, and **(C)** the expression of Grp94 was determined in cell lysates by Western-blot analysis (50 µg of cellular protein/lane) using specific antibodies and densitometric scanning (●, ○). Data are presented as means ± S.E.M. for at least three independent experiments. Error bars not shown are within the symbols.

different within AR4-2J Rib⁻ and AR4-2J Rib⁺ cells. However, the relative abundance of mRNA specific for Grp94 was decreased by some 25% in AR4-2J cells expressing the ribozyme targeted against the Grp94 mRNA (Figure 6B), a result which correlates with the decreased expression of Grp94 in AR4-2J Rib⁺ compared with AR4-2J Rib⁻ cells (see Figure 3). To assess whether the ribozyme affected the biosynthesis of BSDL, pulse-chase experiments were performed. For this purpose AR4-2J Rib⁻ and AR4-2J Rib⁺ cells were pulse-labelled for 15 min with [³⁵S]methionine and chased for the time periods indicated in Figure 6(C). At the end of the chase, cells were lysed and BSDL was immunoprecipitated with specific antibodies. The immunoprecipitated material was then resolved by SDS/PAGE, autoradiographed and quantified by densitometric scanning. Data shown in Figure 6(C) indicated that the rate of BSDL synthesis at time 0 of the chase was identical in AR4-2J Rib⁺ and AR4-2J Rib⁻ cells. However, some degradation should occur in AR4-2J Rib⁺ cells, as suggested by the decrease in labelling with time, which is a little faster in AR4-2J Rib⁺ than in AR4-2J Rib⁻ cells.

Figure 8 Effect of GA on the rate of BSDL secretion by AR4-2J cells

(A) Subconfluent AR4-2J cells were incubated in fresh DMEM in the absence (control; □) or presence (■) of 1 µg/ml GA. Aliquots of the culture medium were removed at the indicated times and BSDL activity was determined, using 4-nitrophenyl hexanoate as a substrate. The insert displays an immunoblot performed in cell culture medium after a 6 h incubation in the presence (+) or absence (-) of GA and using antibodies specific for rat BSDL. **(B)** AR4-2J cells were incubated in the absence (-) or in the presence (+) of 1 µg/ml GA for 6 h. At the end of the incubation period, the cell culture medium was removed and the cells were harvested and lysed. The α-amylase activity was then determined in the cell culture medium (secreted activity) and in the cell lysate (intracellular activity). Data are presented as means ± S.E.M. for at least three independent experiments. Error bars not shown are within the symbols.

Sodium butyrate decreases the secretion rate of BSDL

It is known that sodium butyrate increases the expression of recombinant plasmids [28]. Upon sodium butyrate treatment, relaxation of some chromosomal regions occurs, and plasmids that are integrated into these regions acquire responsiveness to sodium butyrate. Therefore AR4-2J Rib⁺ and AR4-2J Rib⁻ cells were treated for 16 h in the presence of 0, 5 and 10 mM sodium butyrate. At the end of the treatment, BSDL and α-amylase activities were recorded in the cell-free medium, and Grp94 expression was determined by Western-blot analysis performed on cell lysates using anti-Grp94 antibodies, and was quantified by densitometric scanning. As shown in Figure 7(A) BSDL activity was increased by sodium butyrate treatment of AR4-2J Rib⁻ cells, whereas the opposite effect was observed following treatment of AR4-2J Rib⁺ cells. This decrease in BSDL secretion rate seemed to be specific for AR4-2J Rib⁺ cells, since sodium butyrate increased α-amylase secretion in both AR4-2J Rib⁺ and AR4-2J Rib⁻ cells (Figure 7B). It is worth noting that the

Figure 9 Effect of GA on intracellular levels of BSDL

Subconfluent AR4-2J cells were incubated in the absence (control; empty column) or presence (dashed column) of 1 μ g/ml GA for the period of time indicated. At the end of the incubation period, cells were harvested and lysed. **(A)** After clearing by centrifugation, the BSDL activity expressed as m-units/mg cellular protein, and reported relative to control values, was recorded (average of two separate experiments). **(B)** BSDL was analysed by SDS/PAGE and immunoblotting (50 μ g of cellular protein/lane) using the polyclonal antibodies specific for rat BSDL. Immunoblots shown are representative of two separate experiments. Immunoblots were then quantified, and the data presented in the histograms are averages of two independent experiments. **(C)** AR4-2J cells were incubated in the absence (control) or presence of 1 μ g/ml GA for 6 h. At the end of the incubation period the cells were lysed at 4 °C by sonication (for 15 s) in 20 mM Tris/HCl (pH 7.4). Lysates were incubated for either 30 min at 37 °C (right panel) or were immediately (left panel) treated with Laemmli's sample buffer and analysed by SDS/PAGE and Western blotting using antibodies specific for rat BSDL. Immunoblots are representative of two separate experiments (40 μ g of cellular protein was loaded per lane) and the data presented in the histograms are averages of two separate quantifications. Empty columns, BSDL; single-dashed columns, Ub-BSDL; double-dashed columns, 60 kDa peptide. **(D)** Aliquots of cell lysate were immunoprecipitated (+) or not (–) with antibodies specific for rat BSDL. The immunoprecipitated material was subsequently resolved by SDS/PAGE and subjected to an immunoblot using either anti-BSDL antibodies (lane 2) or anti-ubiquitin antibodies (lane 4). Lanes 1 and 3 show Western blots of cell lysates (80 μ g of cell proteins/lane) before immunoprecipitation and probed with anti-BSDL antibodies and anti-ubiquitin antibodies respectively.

ribozyme did not affect the secretion of α -amylase. We subsequently examined the expression of Grp94 by these two clones upon sodium butyrate treatment. Interestingly, sodium butyrate increased Grp94 expression by AR4-2J Rib[–] cells, but that expressed by AR4-2J Rib⁺ cells remained constant (Figure 7C). The relationship between the increase in expression of Grp94 and that in BSDL secretion cannot be stated due to the effect of sodium butyrate, which also increased α -amylase secretion. However, in AR4-2J Rib⁺ cells the increase in Grp94 expression is obviously balanced by a higher level of expression of the Grp94-specific ribozyme. In turn, this leads to a decrease in BSDL secretion by AR4-2J Rib⁺ cells upon sodium butyrate treatment. Therefore all of the results supported the proposal that the less Grp94 that is expressed the less BSDL that is secreted by AR4-2J cells.

Disruption of the association between Grp94 and BSDL, by GA, leads to degradation of BSDL

The next step was to determine the behaviour of BSDL that cannot be accommodated by Grp94. For this purpose we determined the influence of GA (1 μ g/ml), known to disrupt the association between Grp94 and unfolded proteins [29], on BSDL fates. Figure 8(A) illustrates experiments in which GA treatment decreased BSDL secretion by drug-treated AR4-2J cells, both in terms of activity and protein, by approx. 60%. In addition, after incubation for 6 h, GA slightly decreased (approx. 15–20%) intracellular and extracellular α -amylase activity (Figure 8B). This decrease was probably due to the systemic effect of GA on protein synthesis, as this drug, under these conditions, decreased the amount of ³⁵S-labelled trichloroacetic acid-precipitable cellular proteins by the same percentage (i.e. 15–20%) relative to cellular DNA (results not shown). Therefore the effect of GA on BSDL secretion appeared specific. Furthermore, GA did not provoke any accumulation of BSDL within AR4-2J cells (Figures 9A and 9B). To determine the behaviour of BSDL upon dissociation from Grp94, cells were treated or mock-treated with GA (1 μ g/ml) for 6 h, and were subsequently lysed by sonication (15 s) in 20 mM Tris/HCl (pH 7.4). Cell lysates (4 mg of cellular protein/ml) obtained at 4 °C were immediately resolved by SDS/PAGE and were analysed by Western-blot analysis using antibodies specific for rat BSDL and ubiquitin. Alternatively, cell lysates were incubated at 37 °C for 30 min, incubations were then stopped with Laemmli's sample buffer [18] and analysed as above. As shown in Figure 9(C), antibodies specific for BSDL detected two extra bands on Western blots performed with GA-treated AR4-2J cells and control cell lysates incubated at 37 °C. The first one has a lower electrophoretic migration (96 kDa) than BSDL (74 kDa), whereas the second one migrates as a 60 kDa polypeptide. Attempts were performed to characterize the 96 kDa immunoreactive form of BSDL. Proteins of AR4-2J cell lysates were immunoprecipitated with antibodies raised against rat BSDL. The immunoprecipitated material was then resolved by SDS/PAGE and analysed by Western-blot analysis using antibodies specific for either BSDL or ubiquitin. As shown in Figure 9(D), antibodies specific for rat BSDL that recognized the 96 kDa and 74 kDa forms of BSDL present in the cell lysate (lane 1) were still reactive with these two proteins after immunoprecipitation with antibodies raised against rat BSDL (lane 2). Antibodies specific for ubiquitin, which recognized many proteins in AR4-2J cell lysates (lane 3), only detected the 96 kDa protein after immunoprecipitation with antibodies raised against rat BSDL (lane 4). This latter result strongly suggested that the 96 kDa protein corresponded to ubiquitinated-BSDL

Figure 10 Stability of BSDL in GA-treated AR4-2J cells

AR4-2J cells were untreated (control) or treated with 1 μ g/ml GA for 6 h. Cells were then lysed, and the lysate was cleared by centrifugation. The thermal stability of BSDL expressed under control conditions or expressed in the presence of GA was examined by incubation of the cell lysate [diluted 10-fold in 100 mM sodium phosphate buffer (pH 7.4) containing 2 mM benzamidine] at 40 °C (○) or at 50 °C (●) and monitoring the BSDL activity in aliquots withdrawn at the indicated times. Results are reported relative to values obtained under control conditions, and are expressed as percentages of the control data. Data are presented as means $\pm$ S.E.M. for three independent experiments.

(Ub-BSDL). The 60 kDa polypeptide could be a degradation product of Ub-BSDL. When compared with the control, the amount of Ub-BSDL and that of the 60 kDa degradation peptide increased in GA-treated cells (see Figure 9C, lower right-hand panel). It is worth noting that the amount of Ub-BSDL was also higher in GA-treated cells than in mock-treated cells analysed just after lysis of cells at 4 °C (Figure 9C, lower left-hand panel). These results indicated that BSDL that is dissociated from (or that may not interact with) the Grp94 chaperone was probably degraded by the ubiquitin–proteasome-dependent pathway [30]. Furthermore, we attempted to determine whether BSDL expressed by GA-treated AR4-2J cells was less stable than that expressed under control conditions. For this purpose, AR4-2J cells were either treated or not treated (control) with 1 μ g/ml GA for 6 h. At the end of the incubation period cells were washed and lysed. Cell lysates were cleared by centrifugation (700 g for 15 min, at 4 °C) and diluted 10-fold in 100 mM sodium phosphate (pH 7.4) and 2 mM benzamidine. Diluted cell lysates obtained from GA-treated cells were incubated at either 40 °C or 50 °C, and the remaining BSDL activity was recorded over time and expressed relative to control cell activity determined under identical conditions. As expected, BSDL synthesized in the presence of GA appeared to be less heat stable than the enzyme expressed under control conditions. At 40 °C the activity of BSDL expressed in the presence of GA decreased a little with time when compared with control values, and this process was emphasized when the temperature was increased to 50 °C (Figure 10). These results support the view that the fraction of BSDL that cannot be accommodated by Grp94 is less stable.

DISCUSSION

Co- and post-translational modifications of human pancreatic BSDL, include N-glycosylation of Asn¹⁸⁴ and O-glycosylation of the C-terminal mucin-like region [7,15,31–34]. The N-glycosylation seems to be essential for the correct folding of the enzyme [15]. Secretion of BSDL occurs once O-glycosylation masks PEST sequences, which are signals for protein degradation [5,7].

To achieve correct and complete glycosylation, BSDL associates with Grp94 [4]. Once phosphorylated by casein kinase II, BSDL is released from intracellular membranes in the *trans*-Golgi network [35,36]. After this post-translational modification, soluble BSDL precipitates with other pancreatic enzymes in condensing vacuoles and finally follows the pancreatic secretion pathway until it reaches the duodenal lumen where it accomplishes its physiological role [1]. In view of this we wondered whether Grp94 helps BSDL to enter into the secretion pathway instead of a degradation route, as we have previously proposed [4,7].

In the present study we have attempted to answer this question and have constructed a Grp94-mRNA-targeted ribozyme. AR4-2J cells, which secrete BSDL [15], were used as a model for BSDL secretion, and were transfected with the ribozyme. Under the conditions used, the steady-state level of Grp94 mRNA was specifically decreased, leading to a concomitant decrease in Grp94 expression by some 30%. A parallel reduction in BSDL – activity and protein – was recorded within ribozyme-transfected cells. Also, the secretion rate of BSDL was lowered in a comparable proportion, whereas that of α -amylase was not affected. Both BSDL and α -amylase secretion, and Grp94 expression were enhanced upon treatment of AR4-2J Rib[–] cells with sodium butyrate. Sodium butyrate had a comparable effect on the secretion profile of epithelial cells [37–39]. However, sodium butyrate also stimulated the ribozyme expression from the CMV promoter of the pCDNA-3 plasmid [28,39] including the ribozyme construction (pCD94 Rib⁺). This increase in ribozyme expression led to a decrease in both Grp94 expression and BSDL secretion levels in AR4-2J Rib⁺ cells compared with AR4-2J Rib[–] cells. No difference in α -amylase secretion was observed when the ribozyme expression was modulated by sodium butyrate treatment of either AR4-2J Rib[–] cells or AR4-2J Rib⁺ cells. Altogether these results suggested that the intracellular level and secretion rate of BSDL could be directly linked to the amount of BSDL that can interact with Grp94.

We have hypothesized that the association of BSDL with a membrane multiprotein complex is important for enzyme secretion [4]. In this complex BSDL is in part interacting with Grp94 [4]. The complex, including Grp94, could have a pivotal role in BSDL secretion, because it maintains the enzyme in close proximity to reticular- and Golgi-membrane glycosyltransferases [4]. The activity of these transferases masks PEST degradation sequences present within the O-glycosylated mucin domain of BSDL [7]. Data presented in the current study using GA, strengthened this hypothesis. BSDL molecules that cannot associate with Grp94 are unstable and cannot be secreted. Therefore Grp94 [3,4] may assist the sorting of BSDL towards the secretion pathway. Under these circumstances, the fraction of enzyme that does not associate with Grp94 (or with the folding complex) could be stored within the ER before entering another folding cycle, as described for folding mechanisms involving calnexin [40], and being targeted towards secretion. Alternatively, BSDL molecules that are not accommodated by Grp94 are less stable and may be degraded. Conditions that lower the basal expression of the Grp94 chaperone result in a decrease in the rate of BSDL secretion with a parallel decrease in the intracellular enzyme activity, suggesting that the fraction of BSDL that cannot associate with the chaperone would be effectively degraded. Such degradation of BSDL, probably following the ubiquitin-conjugation of the protein, which is a hallmark for proteasomal degradation [41], has been observed in the presence of GA, which promotes the dissociation of Grp94 from its substrate [11]. Interestingly, after GA treatment, the Grp94–p185-erbB2 complex dissociates and the released

p185-erbB2 protein is degraded by the proteasome [42]. Also, perturbing Hsp90, the cytosolic paralogue of Grp94, interaction with the cystic fibrosis transmembrane conductance regulator accelerates its degradation by the proteasome [43]. The compartment where sorting and/or degradation of BSDL occur(s) needs further characterization.

The precise role of Grp94 is still unclear [26]. This protein was originally identified as a protein that was co-ordinately regulated by agents that induced Grp78 (or BiP) synthesis [44]. Evidence for its involvement in ER-protein folding has been difficult to obtain and is limited to only a few proteins. Like Grp78, Grp94 appears to preferentially associate with unassembled proteins [9], and binds ATP [45] and calcium [46]. The involvement of Grp94 in the late stages of protein folding [10] and its presence in late secretory compartments [14] is more compatible with a role in the sorting of active BSDL molecules towards secretion and the targeting of aberrant molecules to the ubiquitin-dependent degradation pathway.

We have recently shown that BSDL dissociates from intracellular membranes (i.e. from the folding complex) after phosphorylation of the enzyme [35]. This phosphorylation occurs within a genistein-sensitive compartment of AR4-2J cells [36]. Because genistein inhibits the formation and the release of cargo vesicles in the *trans*-Golgi network [47] one may suspect that the BSDL dissociates from the membrane folding complex in this latter compartment. This, in part, corroborates the fact that the membrane-associated BSDL was N- and O-glycosylated and appeared to be released from membranes after its glycosylation was completed in the *trans*-Golgi compartment [3,4]. Altogether, these results suggested that the dissociation of secretion-competent molecules of BSDL from the folding complex occurs after the *trans*-Golgi compartment, probably in the *trans*-Golgi network, which is already known to segregate vesicle populations in cells [48]. However, the phosphorylation of BSDL and its release from intracellular membranes does not signify that all BSDL molecules dissociate from Grp94 [36], and two populations of BSDL seem to be secreted in the pancreatic juice [14]. The first population consists of monomeric BSDL, while the second population consists of BSDL complexed to Grp94. Thus BSDL, secreted in association with Grp94, represents some 30 % of the secreted BSDL mass, and is internalized by intestinal cells [14]. How Grp94 (which was detected in exocrine pancreatic secretions [49]) segregates between these two forms of BSDL is unknown. What makes these forms of enzyme different has also to be determined. It is possible that a fraction of BSDL that cannot dissociate from Grp94, is maintained in a state compatible with its capture by the enterocyte, as observed with human growth hormone [50], or that BSDL complexed to Grp94 is protected against proteolytic degradation in the intestinal lumen. In this sense, Grp94 could be involved in the sorting and transport of some specific proteins or peptides, such as BSDL and antigenic peptides [51], to their final destination, which can be the extracellular compartment or the cell surface.

In conclusion, our data suggested that an intimate relationship between Grp94 and BSDL exists in AR4-2J pancreatic cells. Only the fraction of BSDL that is structured by Grp94 can be sorted towards secretion. Furthermore, BSDL molecules that are not accommodated by the chaperone are not secreted and are targeted to a compartment where ubiquitin-dependent degradation occurs.

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