INTRODUCTION

The bile salt-dependent lipase (BSDL, EC.3.1.1.-), also called cholesterol esterase, is a lipolytic enzyme, which upon activation by the primary bile salts catalyzes the hydrolysis of a wide range of substrates (Lombardo et al., 1980; Lombardo and Guy, 1980). The actual function of BSDL remains controversial. Several data suggest, however, that the physiological roles of BSDL are the facilitation of intestinal hydrolysis of cholesterol esters into free cholesterol and fatty acids (Howles, 1996) and its transport to the small intestine where this enzyme is thought to exert its physiological function. Both BSDL and Grp94 antigenic sites were localized and found to be associated all along the pancreatic acinar cell secretory pathway. Grp94 and BSDL remain associated from leaving the pancreas until arriving at the intestinal lumen. In pancreatic juice, both proteins appear as a complex of high molecular mass (180 kDa) containing at least one each of p94 and BSDL molecules, interacting by hydrophobic forces. At the intestinal level, associated Grp94 and BSDL were detected on microvilli and in the endosomal compartment of enterocytes. The BSDL mRNA, however, was not expressed by the intestinal mucosa. The pancreatic Grp94-BSDL complex was internalized through the endosomal compartment of enterocytes. Finally, the two proteins dissociated in this compartment and BSDL, but not Grp94, was transferred to the basolateral membrane.

SUMMARY

In previous studies on the AR4-2J cell line, we have shown that secretion of bile salt-dependent lipase (BSDL) involves a multiprotein complex, including a protein of 94 kDa (p94) that is immunologically related to the chaperone Grp94, which seems to play essential roles in the folding process of BSDL. Combined biochemical and immunocytochemical investigations were carried out to study the secretion of BSDL by normal pancreatic cells and its transport to the small intestine where this enzyme is thought to exert its physiological function. Both BSDL and Grp94 antigenic sites were localized and found to be associated all along the pancreatic acinar cell secretory pathway. Grp94 and BSDL remain associated from leaving the pancreas until arriving at the intestinal lumen. In pancreatic juice, both proteins appear as a complex of high molecular mass (180 kDa) containing at least one each of p94 and BSDL molecules, interacting by hydrophobic forces. At the intestinal level, associated Grp94 and BSDL were detected on microvilli and in the endosomal compartment of enterocytes. The BSDL mRNA, however, was not expressed by the intestinal mucosa. The pancreatic Grp94-BSDL complex was internalized through the endosomal compartment of enterocytes. Finally, the two proteins dissociated in this compartment and BSDL, but not Grp94, was transferred to the basolateral membrane.

Key words: Pancreatic bile salt-dependent lipase, Glucose-regulated protein 94, Immunocytochemistry

INTRODUCTION

The bile salt-dependent lipase (BSDL, EC.3.1.1.-), also called cholesterol esterase, is a lipolytic enzyme, which upon activation by the primary bile salts catalyzes the hydrolysis of a wide range of substrates (Lombardo et al., 1980; Lombardo and Guy, 1980). The actual function of BSDL remains controversial. Several data suggest, however, that the physiological roles of BSDL are the facilitation of intestinal hydrolysis of cholesterol esters into free cholesterol and fatty acids (Howles, 1996) and its transport to the small intestine where this enzyme is thought to exert its physiological function. Both BSDL and Grp94 antigenic sites were localized and found to be associated all along the pancreatic acinar cell secretory pathway. Grp94 and BSDL remain associated from leaving the pancreas until arriving at the intestinal lumen. In pancreatic juice, both proteins appear as a complex of high molecular mass (180 kDa) containing at least one each of p94 and BSDL molecules, interacting by hydrophobic forces. At the intestinal level, associated Grp94 and BSDL were detected on microvilli and in the endosomal compartment of enterocytes. The BSDL mRNA, however, was not expressed by the intestinal mucosa. The pancreatic Grp94-BSDL complex was internalized through the endosomal compartment of enterocytes. Finally, the two proteins dissociated in this compartment and BSDL, but not Grp94, was transferred to the basolateral membrane.

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contrast to other secretory pancreatic enzymes, are associated with intracellular membranes during their secretory process. This association with intracellular membranes involves a multimeric folding complex, including a chaperone molecule immunologically related to the glucose-regulated protein of 94 kDa (Grp94). These data, together with previous evidence for other pancreatic secretory proteins (Le-Gall and Bendayan, 1996; Vélez-Granell et al., 1994), demonstrate that the folding and secretion of pancreatic enzymes could be under the control of chaperones.

The Grp94 chaperone is a member of the glucose-regulated protein family and shares 50% amino acid identity with the 90 kDa heat shock protein (Hsp90) (Little et al., 1994). Grp94, also known as Erp99 or endoplasmic, is a 94 kDa glycoprotein. Grp94 was first identified as a gene product whose synthesis is regulated upon glucose starvation and in response to various experimental treatments that induce the accumulation of unfolded proteins in the endoplasmic reticulum (ER) (Little et al., 1994). It has been shown to contribute to protein trafficking and appears to associate with folding intermediates of a number of oligomeric proteins. In the endoplasmic reticulum (ER), Grp78 and Grp94 act sequentially to fold intermediates of the newly synthesized immunoglobulin chains (Melnick et al., 1992, 1994). The Grp94 chaperone, as protein disulfide isomerase (PDI) and Grp78, is located in the ER lumen of various cells. However, despite possessing the ER retention signal KDEL (Lys-Asp-Glu-Leu) at their C termini, Grp94 as well PDI and Grp78 have also been localized in the Golgi apparatus and secretory granules of pancreatic cells (Takemoto et al., 1992).

Since the biosynthesis of pancreatic BSDL seems to be dependent upon the presence of the Grp94-related protein (Bruneau and Lombardo, 1995), we attempted to determine by immunocytochemical and biochemical approaches whether the Grp94-related protein was associated with BSDL in normal pancreatic acinar cells and secreted. The present study has shown that Grp94 and BSDL antigenic sites are indeed associated all along the secretory pathway and, upon secretion, remain associated in the pancreatic juice when transported from the pancreas to the intestinal lumen. We have further shown that the Grp94-related protein-BSDL complex is internalized by enterocytes. Finally, both proteins dissociate in the late endosomal compartment and the BSDL, but not the Grp94, is transferred to the basolateral membrane.

MATERIALS AND METHODS

Biological material

Pancreatic and duodenal tissues were sampled from normal 100 g Sprague-Dawley male rats and processed for biochemical and morphological studies.

Pancreatic juice was collected upon cannulation of the pancreatic duct from various anaeasthetized rats for 6 hour periods.

cDNA probes

cDNA probes specific for BSDL and for Grp94 were obtained by chemical synthesis (Life Technologies, Burlington, ON, Canada). One probe corresponded to a sequence complementary to nucleotides 1041-1071 of pancreatic BSDL mRNA (Kissel et al., 1989), and the other to a sequence complementary to nucleotides 1798-1828 of murine Grp94 mRNA (Mazzarella and Green, 1987). cDNA probes were labeled with digoxigenin (Dig-) as described previously (Gingras and Bendayan, 1995).

Antibodies

Affinity-purified rabbit polyclonal antibodies against rat pancreatic BSDL (Abouakil et al., 1988) and monoclonal antibodies specific for Grp94 (clone 9G10) from StressGen Biotechnology Corp. (Victoria, BC, Canada) were used for immunocytochemistry, immunoblotting and immunoprecipitation. These antibodies are well characterized and have previously been used in various experiments (Nauseef et al., 1995; Kuznetsov et al., 1994; Bruneau and Lombardo, 1995). The monoclonal antibody directed against the Grp94 was prepared by immunization of rats. Rat spleen cells were then fused with mouse plasmacytoma cell lines (StressGen Biotechnology Corp., Victoria, BC, Canada). Thus secondary antibodies to rat or mouse IgG molecules were used for the immunochemical and immunocytochemical techniques.

Protein assays

Proteins were routinely assayed by the bicinechonic acid method (Pierce, Rockford, IL) using bovine serum albumin as a standard.

Polyacrylamide gel electrophoresis and western blotting

Gel electrophoresis (SDS-PAGE) was performed in slab gels of polyacrylamide (7.5%) and sodium dodecyl sulfate (1%) in reducing conditions according to Laemmli (1970), using a BioRad mini-protein II apparatus. After SDS-PAGE, proteins were electrophoretically transferred to a nitrocellulose membrane in 0.2 M Tris/HCl buffer pH 9.2 (10% methanol), at 150 mA for 3 hours in a cold room (Burnette, 1981). Completeness of transfer was assessed by staining polyacrylamide gels with Coomassie Blue R250. Destaining was performed in ethanol/acetic acid/water (2/3/35 by volume). Western blots were performed using the specific antibodies for BSDL and Grp94. The antigen-antibody complexes were revealed either by the BM chemiluminescence Western-blotting kit (Boehringer-Mannheim, Germany) or by the alkaline-phosphatase technique.

Immunoprecipitation

Pancreatic juice proteins were incubated overnight at 4°C with 10 μg of antibodies. Pre-washed Protein A-Sepharose (10 mg) was added to antibody-antigen complexes and incubated for 4 hours at 4°C under agitation. At the end of the incubation, the antigen-antibody-Protein-A complex was recovered by centrifugation (10,000 g, 20 minutes). The pellet was then washed twice with the 10 mM Tris/HCl buffer, pH 7.4, 25 mM EDTA and 1% Triton X-100 (washing buffer), twice with the washing buffer supplemented with 1 M NaCl, twice with the last buffer supplemented with 0.1% SDS and twice again with 10 mM Tris/HCl buffer, pH 7.4, containing 5 mM EDTA. The pellet was then transferred into Laemmli’s sample buffer for the SDS-PAGE, warmed for 5 minutes at 95°C, centrifuged and electrophoresed on SDS-PAGE.

In situ hybridization

Rat tissues were fixed with Bouin’s solution for 24-48 hours, washed in water, dehydrated through increasing concentrations of ethanol and embedded in paraffin. 5 µm thick sections were mounted in diethyl pyrocarbonate (DEPC)-treated water (RNase-free water) on Superfrost/Plus slides (Fisher Scientific, Montreal, Quebec, Canada). The sections were deparaffinized and rinsed in DEPC-treated water. They were then washed twice in 2× SSC (1× SSC = 150 mM sodium chloride, 15 mM sodium citrate) for 10 minutes at 60°C, dipped in DEPC-treated water, then in 0.05 M Tris/HCl buffer, pH 7.6, at room temperature incubated with Proteinase K (5 μg/ml in 50 mM Tris/HCl, buffer pH 7.6) for 5 minutes at 37°C, and finally rinsed with 50 mM Tris/HCl buffer, pH 7.6. The sections were fixed in 0.4% paraformaldehyde in PBS at 4°C for 20 minutes, rinsed in DEPC-
treated water and incubated for 1 hour at 37°C in the hybridization buffer (R&D systems, Minneapolis, MN, USA). Dig-labeled probes were diluted to the required concentration in the hybridization buffer. The hybridization with the specific probe was then performed in a humid chamber at 37°C overnight. After hybridization, the sections were washed at 37°C twice for 5 minutes successively with: 4× SSC/30% formamide, 2× SSC/30% formamide and 0.2× SSC/30% formamide. They were incubated in 50 mM Tris/HCl buffer, pH 7.6, 150 mM NaCl, 2 mM MgCl₂ (TBS) containing 0.1% bovine serum albumin and the Dig-labeled hybrids were detected with the anti-Dig-alkaline phosphatase antibodies diluted 1/500 in TBS containing 0.1% BSA. The sections were then incubated twice for 5 minutes in the revelation buffer (100 mM Tris/HCl, pH 9.5, 150 mM NaCl, 50 mM MgCl₂) and finally incubated with the revelation buffer containing NBT and X-phosphate (Boehringer-Mannheim, Germany). They were mounted with 50% glycerol in PBS and examined with a Leitz Orthoplan microscope (Montreal, Quebec, Canada). Control experiments were performed following exactly the same protocol but using the Dig-labeled sense probes.

**Immunocytochemistry**

**Immunofluorescence**

Rat pancreatic and duodenal tissues were fixed in Bouin’s fixative and embedded in paraffin. Tissue sections were deparaffinized and washed twice in 10 mM phosphate buffer, pH 7.4, 150 mM NaCl (PBS) and incubated with the anti-BSDL (dilution 1/10, 2 hours) or the anti-Grp94 (dilution 1/20, overnight) antibodies. For revealing BSDL antigenic sites, the sections were washed twice in the PBS and then incubated with the FITC-goat anti-rabbit IgG antibody (Sigma Chemicals, St Louis, MO, USA) for 1 hour. For revealing Grp94 antigenic sites, the sections were incubated successively with biotinylated anti-mouse IgG antibody (1/200, 1 hour), streptavidin-HRP (1/500, 30 minutes) and FITC-tyramide (1/50, 10 minutes) in ×1 amplification diluent (NEN Life Science products, Boston, MA, USA) as described previously (Mayer and Bendayan, 1997). The sections were observed on a Leitz DM RB microscope using regular and oil-immersion PL Fluotar objectives.

**Immunoelectron microscopy**

Rat pancreatic and duodenal tissues were fixed by immersion with 1% (v/v) glutaraldehyde in 0.1 M sodium phosphate buffer, pH 7.4, for 2 hours at 4°C. The tissue samples were washed in the phosphate buffer, dehydrated by a series of graded ethanol solutions and embedded in Unicryl or Lowicryl (British Biocell, Cardiff, UK) at −20°C as described previously (Bendayan, 1995). Thin sections were mounted on Parlodion and carbon-coated nickel grids. The grids were successively incubated by floating them on a drop of 1% ovalbumin in PBS for 30 minutes at room temperature, a drop of the primary antibody (anti-BSDL, at 1/200 dilution for 3 hours at room temperature or anti-Grp94 at 1/20 dilution at 4°C overnight), and rinsed with PBS. They were then transferred to 1% ovalbumin solution and incubated for 30 minutes on a drop of a goat anti-rabbit IgG-gold complex for the BSDL or a goat anti-mouse IgG-gold complex for the Grp94. The grids were then thoroughly washed with PBS and distilled water. The thin sections were stained with uranyl acetate and examined with a Philips 410 electron microscope.

For sequential double labeling, the tissue sections, mounted on nickel grids, were incubated with the monoclonal antibody specific for Grp94 (dilution 1/20) at 4°C overnight and the goat anti-mouse IgG-gold complex (10 nm in size) for 30 minutes. Upon rinsing with PBS and distilled water, the same protocol was repeated with the polyclonal antibody specific for BSDL (dilution 1/200) and the goat anti-rabbit IgG-gold complex (15 nm in size).

**Control experiments**

Control experiments, for light and electron microscopy immunocytochemistry, were performed by omitting the primary antibody and by using the antigen-adsorbed-antibody complexes.

**Quantitative evaluation and determination of percentage of association**

The labeling intensities obtained by electron microscopy for BSDL and Grp94, defined as the number of gold particles per μm², were established over different cellular compartments on thin sections of pancreatic and duodenal tissues. Direct planimetry and counting of gold particles were carried out as described previously (Bendayan, 1995) using a Videoplan 2 image processing system (Carl Zeiss Inc., Toronto, Ontario, Canada). 30 micrographs were recorded and analyzed for each protein in each cellular compartment and in each tissue. The percentage of association, BSDL-Grp94 revealed by the double-labeling experiments was evaluated as follows: only gold particles revealing Grp94 (10 nm gold) that were separated by less than 20 nm from a gold particle representing BSDL (15 nm gold) were taken into account and both particles were considered associated. The percentage of association was calculated by dividing the number of associated particles by the total number of Grp94 particles. For the evaluation of the density of labeling along the basolateral membrane of the enterocyte, we measured the length of the membrane and the density was defined as the number of gold particles per μm.

**RESULTS**

**Intracellular localization of BSDL in rat pancreas**

In order to determine the precise localization of BSDL and Grp94 in the rat pancreatic acinar cells and their association, we used different morphological and biochemical approaches. In situ hybridization was performed on rat pancreatic tissue by using Dig-tagged synthetic oligonucleotides complementary to BSDL cDNA. This led to a black positive reaction restricted to the basolateral region of the acinar cells (Fig. 1A). Cells of the islet of Langerhans were negative, as were all nuclei. No signal was found when sections were processed under control conditions, i.e. using synthetic sense oligonucleotides to BSDL cDNA (result not illustrated). Therefore, the acinar cells appear to be the only pancreatic cells expressing BSDL mRNA.

Immunofluorescence for detecting BSDL antigenic sites, performed on pancreatic tissue, revealed a positive signal in acinar cells. The staining was particularly intense in the apical region of the cells (Fig. 1B), known to contain large amounts of secretory granules. No specific labeling was observed in islet endocrine cells.

At the electron microscope level, the labeling obtained by using specific antibodies to BSDL in conjunction with the immunogold approach, indicated the presence of BSDL antigenic sites in the various compartments along the secretory route, rough endoplasmic reticulum (RER) → Golgi apparatus → condensing vacuoles → zymogen granules (Fig. 1C). These data agreed with previous results obtained with human pancreas (Lechène de La Porte et al., 1987). Quantitation of the labeling showed that BSDL was preferentially present in those compartments, with an increasing gradient from ER to zymogen granules (Table 1). This reflects the process of protein concentration that occurs along the RER → Golgi → granules secretory pathway (Bendayan et al., 1980). Immunofluorescence and immunogold control experiments resulted in absence of labeling. The acinar cell is thus able to express the specific mRNA for BSDL and...
Fig. 1. Expression and detection BSDL in rat pancreatic tissue. (A) In situ hybridization with Dig-labeled BSDL probes on a paraffin section of rat pancreatic tissue. The black positive reaction is present over the exocrine acinar cells, particularly in the basolateral region. The islet of Langerhans (IL) is negative. (B) Paraffin section of rat pancreatic tissue processed by immunofluorescence to demonstrate the distribution of BSDL. The reaction product is mainly over the apical area rich in secretory granules. (C) Immunocytochemical detection of BSDL antigenic sites in rat pancreatic tissue, using the immunogold approach. The rough endoplasmic reticulum (rer), the Golgi apparatus (G) and the zymogen granules (zg) are labeled by the gold particles. The nucleus (n) and the mitochondria (m) are almost devoid of labeling. Bars, 10 µm (A, B), 1.0 µm (C).
to translate it. In its fundamental attributes, the secretory route of BSDL was similar to that of other pancreatic secretory proteins and it followed a regulated pathway of secretion.

### Intracellular localization of Grp94 in the rat pancreas

The expression of Grp94 was assessed in pancreatic tissue. In situ hybridization was carried out by using Dig-tagged synthetic oligonucleotides complementary to Grp94 cDNA. A black positive staining was detected in the basolateral area of the acinar cells while a weak staining was present in the islet cells (Fig. 2A). No signal was found under control conditions, i.e. using synthetic sense oligonucleotides to Grp94 cDNA (data not shown). This indicates that the mRNA of Grp94 is transcribed by exocrine and, to a lesser extent, by endocrine pancreatic cells.

Immunofluorescence for Grp94 antigenic sites revealed a strong positive signal in pancreatic acinar cells, whereas the signal was much lower in endocrine cells. The staining in acinar cells was localized around the nuclei and in the apical region of the cells. Nuclei were not labeled (Fig. 2B).

At the electron microscope level, specific immunocytochemical labeling for Grp94 was found over different cellular compartments of the acinar cells (Fig. 2C). Indeed, the presence of Grp94 antigenic sites was detected in the RER, the Golgi apparatus, the condensing vacuoles and the zymogen granules. Quantitation of the labeling was performed (Table 1) and confirmed that the Grp94 is expressed by the acinar cells, this chaperone being present in all the compartments of the regulated secretory pathway. Control experiments confirmed the specificity of the results.

### Co-distribution of BSDL and GRP94 in the pancreas

In pancreatic acinar cells, BSDL and Grp94 appear to follow a regulated pathway of secretion. Previous studies have demonstrated that a multiprotein complex, including the Grp94-related protein, plays an essential role in the folding of BSDL (Bruneau and Lombardo, 1995).

To demonstrate the association of BSDL with Grp94, double-labeling experiments were performed with antibodies against BSDL and Grp94 combined to different-sized gold particles. Results indicate that the association of BSDL with Grp94 takes place all along the secretory pathway of acinar cell, from the RER to the zymogen granules (Fig. 3). Indeed, a high number of small (10 nm) and large (15 nm) gold particles, representative of Grp94 and BSDL antigenic sites respectively, were found within less than 20 nm distance of each other, suggesting that they are associated. However, not all gold particles revealing BSDL or Grp94 were found to be associated; a significant number remained free. The degree of association between both proteins varied according to the cellular compartment and increased from the endoplasmic reticulum to the zymogen granules (Table 1). This suggests that the Grp94-related protein is associated mainly with BSDL in the late compartment of the secretory process.

### Secretion of BSDL and Grp94 by pancreatic acinar cells

Based on these results, we predicted that pancreatic acinar cells would secrete both proteins. Therefore, we checked for the presence of BSDL and Grp94 in pancreatic secretion. At the electron microscope level, the immunogold labeling obtained by using the specific antibodies to BSDL indicated the presence of BSDL antigenic sites in discharging zymogen granules and in the content of the acinar lumen (pancreatic juice) (Fig. 4A). The proteins of the pancreatic juice were separated by SDS-PAGE, electrotransferred to a nitrocellulose membrane and finally developed with the anti-BSDL antibody. As shown in Fig. 4A(inset), a single band was recovered, indicating that one protein reacts with the antibody to rat BSDL with a molecular mass of 74 kDa, compatible with the mass of mature BSDL in rat pancreatic juice (Abouakil et al., 1993).

For the Grp94, gold particles revealing antigenic sites were also found in discharging zymogen granules and over the dense flocculent material present in the acinar lumen (Fig. 4B). To confirm the secretion of this Grp94-related protein, the proteins of the pancreatic juice were separated by SDS-PAGE, electrotransferred to a nitrocellulose membrane and finally developed with the monoclonal antibody to Grp94 and a goat anti-rat IgG antibody conjugated with peroxidase. As shown in Fig. 4B(inset), antibodies to Grp94 revealed two proteins, with molecular masses of 94 kDa and 180 kDa. This high molecular mass for Grp94-related protein, which is normally 94 kDa, is compatible with either an homodimer of p94 or a heterodimer containing at least one p94 protein. These data suggest that two forms of Grp94-related protein could be present in pancreatic secretion, one being in a monomeric form and the other in a complex form.

### Association between BSDL and Grp94 in pancreatic juice

Double-immunogold labeling revealed the simultaneous presence of BSDL and Grp94 antigenic sites in discharging zymogen granules and in the content of the acinar lumen (Fig. 5A). Moreover, we have found that the Grp94 remains associated with the BSDL in both compartments. The amount of Grp94 labeling associated with that of BSDL in acinar lumen is similar to that found for the zymogen granules (about 60%; Table 1).

We have shown (Fig. 5A(inset)) that Grp94 displays a molecular mass compatible with the formation of a complex. We thus attempted to determine whether this complex encompasses both the BSDL and the Grp94. The pancreatic juice proteins were subjected to immunoprecipitation with antibodies specific for Grp94 on the one hand and for BSDL on the other. The immunoprecipitated material with anti-Grp94 was separated by SDS-PAGE and electrotransferred onto a nitrocellulose membrane. The Ponceau Red staining indicated that, essentially, not just one but several proteins were co-immunoprecipitated with the antibody to Grp94 (data not shown). This indicates that Grp94 is associated to a few pancreatic proteins. This membrane

### Table 1. Densities of labeling obtained with the anti-BSDL and the anti-Grp94 over different cellular compartments of the rat pancreatic acinar cells

<table>
<thead>
<tr>
<th>Cellular compartments</th>
<th>BSDL*</th>
<th>Grp94*</th>
<th>Association (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RER</td>
<td>7.5±0.4</td>
<td>2.9±0.2</td>
<td>28.8±1.4</td>
</tr>
<tr>
<td>Golgi apparatus</td>
<td>84.8±2.8</td>
<td>14.5±0.9</td>
<td>37.0±1.4</td>
</tr>
<tr>
<td>Condensing vacuoles</td>
<td>239.5±7.4</td>
<td>36.4±2.6</td>
<td>55.5±1.6</td>
</tr>
<tr>
<td>Zymogen granules</td>
<td>235.8±3.7</td>
<td>31.8±1.8</td>
<td>61.9±1.6</td>
</tr>
<tr>
<td>Acinar lumen</td>
<td>135.9±7.8</td>
<td>19.5±1.3</td>
<td>58.7±1.8</td>
</tr>
<tr>
<td>Mitochondria</td>
<td>2.9±0.4</td>
<td>0.9±0.2</td>
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*Gold particles/μm² (mean values ± s.e.m.).

% Data show the percentage of association of Grp94 with BSDL.
Fig. 2. Expression and detection of Grp94-related protein on rat pancreatic tissue. (A) In situ hybridization with Dig-labeled Grp94 probes on a paraffin section of pancreatic tissue. The black positive reaction is present mainly in the basolateral region over the exocrine acinar cells. The islet of Langerhans (IL) is weakly labeled. (B) Paraffin section of rat pancreatic tissue processed by immunofluorescence to demonstrate the distribution of Grp94-related protein. The reaction product is present in the acinar cells, mainly in the apical region. (C) Immunocytochemical detection of Grp94 antigenic sites on rat pancreatic tissue using the immunogold approach. The rough endoplasmic reticulum (rer), the Golgi apparatus (G) and the zymogen granules (zg) appear labeled by gold particles. Bars, 10 μm (A,B), 0.5 μm (C).
was then subjected to western blotting using antibodies to BSDL and developed with secondary antibodies conjugated to peroxidase (Fig. 5B, lane 2). One protein with a molecular mass of 74 kDa (lane 2), compatible with the BSDL found in rat pancreatic juice (lane 1), was detected.

In a second experiment, the proteins of the pancreatic juice were immunoprecipitated with antibodies to BSDL, separated by SDS-PAGE and electrotransferred onto a nitrocellulose membrane. Again the Ponceau Red staining indicated that essentially a few proteins were co-immunoprecipitated with the antibody to BSDL (data not shown). The membrane was therefore subjected to western blotting using antibodies to Grp94 and developed with secondary antibodies conjugated to alkaline phosphatase. One protein was detected and displayed a high molecular mass of about 180 kDa (Fig. 5C, lane 2) as found by direct western blotting of pancreatic juice (Fig. 5C, lane 1). On SDS-PAGE and in the presence of 8 M urea, Grp94-related protein as well as BSDL behave as proteins of high molecular mass in the 180 kDa range (data not shown). These data demonstrate that BSDL and Grp94 are secreted as a complex by acinar cells and remain associated in the pancreatic juice, as found by electron microscopy (Fig. 5A).

Localization of BSDL in rat intestine

Once in the presence of bile salts, the BSDL transported towards the intestinal lumen catalyzes the hydrolysis of lipid-soluble vitamin esters and cholesterol esters (Wang and Hartsuck, 1993). BSDL enhances the uptake of cholesterol by intestinal epithelial cells (Howles et al., 1996) and could be involved in the transfer of cholesterol within the enterocyte (Myers-Payne et al., 1995).

At the intestinal level, we have revealed BSDL-antigenic sites...
Fig. 4. Immunocytochemical detection of BSDL (A) and Grp94 (B) antigenic sites in pancreatic acinar lumina (L), and in the discharging zymogen granules (arrows). Bars, 0.5 μm. (Inset) Western immunoblotting analysis of rat pancreatic juice. The proteins were separated by SDS-PAGE (30 μg of loaded proteins) and electrotransferred onto a nitrocellulose membrane. BSDL and Grp94-related protein were localized on the replicates by immunodetection using antibodies to rat BSDL (A) and Grp94 (B), respectively. The positions of molecular mass markers are indicated on the left in kDa.
by immunocytochemistry using antibodies to rat pancreatic BSDL. Immunofluorescence showed a positive and diffuse staining in enterocytes, indicating its presence in these cells (Fig. 6B). At the electron microscope level, specific immunogold labeling was found on microvilli and endosomal compartments (Fig. 6C). These data suggest that pancreatic BSDL, recognized by our antibody, is adsorbed on microvilli membranes and internalized in the endosomal compartment of the enterocyte. The

**Fig. 5.** Co-detection of BSDL and Grp94-related protein in pancreatic acinar cells and lumina. (A) Double-immunocytochemical detection of BSDL (IgG-gold 15 nm) and Grp94 (IgG-gold 10 nm) in the rat pancreatic acinar lumen (L). Some of the labeling appears to be associated and is present over the flocculent material of the lumen (L) and in the discharging zymogen granule (arrow). Some of the associations are encircled. Bar, 0.5 μm. (B) Lane 1, pancreatic juice proteins were separated by SDS-PAGE and transferred onto nitrocellulose membrane. The development of the membrane was performed with polyclonal antibodies to BSDL using the chemiluminescence western-blotting kit. One major band was recovered with an apparent mass of 74 kDa. Lane 2, pancreatic juice proteins were immunoprecipitated using the monoclonal antibody to Grp94, the antigen-antibody complex was separated by SDS-PAGE and transferred onto a nitrocellulose membrane. Development of the membrane was performed with the polyclonal antibody to BSDL using the chemiluminescence western-blotting kit. Again a major band with an apparent mass of 74 kDa was recovered. (C) Lane 1, pancreatic juice proteins were separated by SDS-PAGE and transferred onto nitrocellulose membrane. Development of the membrane was performed with the monoclonal antibody to Grp94 and anti-rat IgG labeled with alkaline phosphatase. One band was recovered with an apparent mass of 180 kDa. Lane 2, pancreatic juice proteins were immunoprecipitated using polyclonal antibody to BSDL, the antigen-antibody complex was separated by SDS-PAGE and transferred onto nitrocellulose membrane. Development of the membrane was performed with the monoclonal antibody to Grp94 and antibodies to rat IgG labeled with alkaline phosphatase. Again one major band at 180 kDa was recovered.
Fig. 6. Detection of BSDL in rat duodenal mucosa. (A) In situ hybridization with Dig-labeled BSDL probes on a paraffin section of rat intestine tissue. No reaction was generated over the duodenal mucosa. (B) Paraffin sections of rat intestinal tissue processed by immunofluorescence to demonstrate the distribution of BSDL. The enterocytes show a positive reaction for BSDL within their cytoplasm. The nuclei and the mucus cells are negative. (C) Immunocytochemical detection of BSDL antigenic sites on rat intestinal tissue using the immunogold approach. The microvilli (mv), the endosomal compartment (e) and the basolateral interdigitations (blm) are labeled. Mitochondria (m) are almost devoid of labeling. Bars, 10 µm (A,B), 0.5 µm (C).
BSDL antigenic sites were also detected on the basolateral plasma membrane interdigitations of the enterocyte (Fig. 6C) and in the intercellular space of the lamina propria (data not shown). These observations were confirmed by quantitative evaluations (Table 2) and support our previous suggestion that BSDL is internalized by the enterocyte (Lechêne de la Porte et al., 1987).

In view of the presence of BSDL in enterocytes, we felt compelled to determine whether the detected BSDL is effectively of pancreatic origin or synthesized by enterocytes. In situ hybridization was performed on intestinal tissue using Dig-tagged synthetic oligonucleotides complementary to BSDL cDNA sequences. Fig. 6A shows that using this BSDL probe, no positive reaction was detected over the duodenal mucosa. No signal was found either under control conditions. Therefore, BSDL present in enterocytes is of exogenous origin, most likely synthesized and secreted by pancreatic acinar cells.

**Localization of Grp94 in rat intestine**

The presence of Grp94-related protein in pancreatic juice and its association with BSDL suggested that this Grp94 not only...
participates in the pancreatic secretion of BSDL, but also in the transport of the enzyme to the intestine. Morphological experiments were performed to determine whether the Grp94-related protein is also involved in the internalization of BSDL by enterocyte. The detection of Grp94 antigenic sites by immunofluorescence revealed a positive signal in enterocytic cells (Fig. 7B). By electron microscopy, specific immunogold labeling for Grp94-related protein was found over certain cellular compartments of the enterocyte (Fig. 7C). The quantitation indicated that Grp94-related protein is present in the same compartments as BSDL, i.e. in microvilli, endocytic vesicles and multivesicular bodies. It is not, however, present in the basolateral plasma membrane interdigitations. When in situ hybridization was performed with the Grp94 probe, a specific positive staining was detected in the duodenal mucosa (Fig. 7A), indicating that Grp94 mRNA is actually transcribed by enterocytes. Although Grp94 may be endogenously expressed by enterocytes, we suggest that the Grp94 detected on microvilli and endosomal compartments of enterocytes could originate from the pancreas.

Co-detection of BSDL and Grp94 in the intestine

Finally we attempted to determine whether the BSDL-Grp94 association remains during internalization by the enterocyte. By double labeling, we found that BSDL stays associated with Grp94 on microvilli and endocytic vesicles (Fig. 8). The quantitation indicates that the fraction of Grp94-related protein associated with BSDL is similar in pancreatic juice and on microvilli (Table 2), suggesting that the complex that
encompasses BSDL and Grp94-related protein in the juice is preferentially internalized by the intestinal mucosa and both proteins dissociate in the late endosomal compartment.

**DISCUSSION**

Previous and present results have established that, as with all digestive enzymes in rat pancreas, the BSDL follows the regulated secretory pathway (RER$\rightarrow$Golgi$\rightarrow$zymogen granules) originally defined by Palade (1975). Our recent data on AR4-2J pancreatic acinar cells line allowed us to propose a model for the specific transport of BSDL along the secretory pathway (Abouakil et al., 1993; Bruneau et al., 1995). A particular feature in BSDL secretion by AR4-2J cells is its association with intracellular membranes. The membrane-associated BSDL is O- and N-glycosylated and consequently the enzyme should be associated with membranes until the trans-Golgi compartment. We have shown that this association involves a multiprotein complex, including the chaperone Grp94-related protein, which is linked directly to BSDL (Bruneau and Lombardo, 1995). The chaperone Grp94-related protein seems to play an essential role in the folding and transport of BSDL. Multiple reasons may be advocated to explain the particular secretory process of BSDL. The most likely would reflect the fact that BSDL is a unique secretory pancreatic protein with mucin-like, tandem-repeated sequences on which O-linked oligosaccharides are clustered. Recently, we have shown that the rate of secretion of BSDL depends upon the ability of the cell to O-glycosylate the C-terminal repeats of the protein and that only O-glycosylated BSDL is secreted (Bruneau et al., 1997).

In this study, using morpho-biochemical techniques, we have defined the secretory pathway of pancreatic BSDL, its association with Grp94-related protein and its internalization by duodenal enterocytes. Morphological data have revealed the presence of both BSDL and the chaperone Grp94-related protein, together with a BSDL-Grp94-related protein complex, in different compartments along the RER$\rightarrow$Golgi$\rightarrow$zymogen granules secretory pathway (Figs 1-3). However not all the proteins revealed appear to be associated. This could reflect either the existence of associated and free BSDL molecules within the cells and the pancreatic juice, or an underestimation of the amount of association due to technical limitations of the morphological approach. These morphological data, together with previous results, suggested that post-translational modifications of BSDL are required for the release of the multiprotein complex from the membrane but not for its dissociation from the chaperone Grp94-related protein. Indeed, we have shown that the association between both proteins takes place in the RER up to the Golgi and the secretory granule, and remains in the pancreatic juice present in the acinar lumen (Figs 3, 5). This association appears to increase and to reach its highest percentage in the last compartments of the secretory pathway (Table 1), suggesting that the Grp94-related protein within the multiprotein complex is required for the quality control of BSDL folding in the RER and Golgi apparatus, and must intervene in the transport of BSDL until the lumen.

Several chaperones have been shown to participate in the process of the synthesis and secretion of pancreatic enzymes (Le-Gall and Bendayan, 1996, Vélez-Granell et al., 1994). Furthermore, some of them were found to be associated to pancreatic secretory proteins, to follow them along the RER$\rightarrow$Golgi$\rightarrow$granules secretory pathway and to be secreted into the pancreatic juice (Le-Gall and Bendayan, 1996). Grp94 is a glycoprotein that belongs to the Hsp90 family, which is implicated in the transport of important biological molecules within the cell (Koyasu et al., 1986) and carries the KDEL C-terminal sequence for the retention of proteins in the lumen of the endoplasmic reticulum (Sorger and Pelham, 1987). Despite its KDEL sequence, the protein is secreted. Several hypotheses can be put forward to account for the secretion of Grp94 in the pancreatic juice. (1) Secretion could result from an ‘overflow’ of the KDEL-bearing proteins from the ER cisternae, as shown by Hensel et al. (1994) for other chaperones. (2) It would reflect a defect in the sorting mechanism taking place in the ER of pancreatic cells (Takemoto et al., 1992). (3) We could suggest the existence of a homologous p94 protein detected by the antibody directed against Grp94, and which would not bear the KDEL sequence, or in which this sequence would not be accessible to its receptor, allowing this protein to proceed along the secretory pathway.

Another role ascribed to Grp94 is its ability to associate with a population of immunogenic peptides and to elicit CD8+ cytotoxic T-cell responses. It has been observed that a fraction of Grp94 is localized to the cell surface (Nicolitta, 1998). The mechanistic basis of this unusual localization remains to be determined and the pancreatic secretion of Grp94 may well result from similarly unusual processing.

By immunolabelling and immunoblotting, we have shown that the pancreatic Grp94-related protein is secreted in the pancreatic juice. One peculiarity of this Grp94-related protein is its two forms: one form of 94 KDa, corresponding to its conventional molecular mass, and a second displaying an unusual apparent mass of approximately 180 KDa, compatible with either a homodimer of p94 or a dimer containing at least one p94 protein. We have further shown by morphological and biochemical techniques that the main fraction of Grp94-related protein was associated with the BSDL in the acinar lumen (see Table 1) and, as a consequence, could remain as a dimer in pancreatic juice (Fig. 5B, lane 2). Grp94-related protein and BSDL behave as proteins of high molecular mass on SDS-PAGE in the presence of 8 M urea, which suggests that the formation of dimers involves hydrophobic forces. Hydrodynamic studies of Grp94 allowed the identification of a discrete domain, defined by residues 676-719, which mediates the dimeric assembly of the protein (Wearsch and Nichitta, 1996). The hydrophobicity profile of BSDL has also

<table>
<thead>
<tr>
<th>Cellular compartments</th>
<th>BSDL</th>
<th>Grp94</th>
<th>Association (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Microvilli</td>
<td>12.8±1.0*</td>
<td>5.2±0.4*</td>
<td>67.8±4.0</td>
</tr>
<tr>
<td>Endocytic vesicles</td>
<td>104.4±16.4*</td>
<td>43.8±9.3*</td>
<td>42.0±14.7</td>
</tr>
<tr>
<td>Multivesicular bodies</td>
<td>25.9±3.5*</td>
<td>4.4±1.2*</td>
<td>n.d.</td>
</tr>
<tr>
<td>Basolateral membrane</td>
<td>3.0±0.3‡</td>
<td>0.3±0.03‡</td>
<td>n.d.</td>
</tr>
<tr>
<td>Mitochondria</td>
<td>0.9±0.08*</td>
<td>0.4±0.1*</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

*Gold particles/μm² (mean values ± s.e.m.).
‡Gold particles/μm (mean values ± s.e.m.).

n.d., not determined.

Table 2. Densities of labeling obtained with the anti-BSDL and the anti-Grp94 over different cellular compartments of the enterocyte cells
allowed for the characterization of a hydrophobic domain located between residues 98 and 121 (Sbarra et al., 1998). It is possible that these hydrophobic domains of Grp94 and BSDL are involved in forming the complex, which contains at least one Grp94-related protein and one BSDL molecule. Analysis of Grp94 structure by hydrodynamic and electron microscopic approaches indicated that the molecule is a tri-nodular rod comprised of two subunits oriented in a direct, antiparallel array (Nichitta, 1998).

The orientation is conferred by a C-terminal oligomerization domain that maintains the N termini at opposing ends of the molecule (Nichitta, 1998). The antibody directed against the Grp78, which recognized the KDEL sequence in both the Grp78 and the Grp94, was used in western blots on rat pancreatic juice and no protein was detected (our unpublished observation). Therefore, the Grp94 KDEL sequence is either absent or not accessible. The BSDL-Grp94 complexes, which are not dissociated in ER, are thus secreted via the regulated secretory pathway. Furthermore, it has recently been demonstrated that, once bound, the peptide-Grp94 complex is SDS-resistant (Nichitta, 1998). Our electrophoresis data show that in the pancreatic juice, the BSDL-Grp94 complex is also SDS-resistant and involves the hydrophobic interactions.

By a morphological approach, we have shown (Figs 5 and 8) that the Grp94-related protein remains associated with the BSDL until reaching the microvilli at the level of duodenal enterocytes. It has been shown that biological activities of pancreatic enzymes decrease during transit from the proximal to the distal gut, the rates of degradation differing, however, from one enzyme to the other. As to the collapse-dependent lipase, its activity decreases rapidly in contrast to proteases and amylase, which are relatively more stable. The mechanism by which the collapse-dependent lipase activity is destroyed reflects chymotrypsin-catalyzed proteolysis. Less than 1% of lipolytic activity secreted into the duodenum reaches the ileum, as compared to 22% for the trypsin activity (Holtmann et al., 1997). As with the collapse-dependent lipase, the BSDL may be extremely sensitive to proteolysis and be degraded by chymotrypsin. In such a case, the Grp94-related protein could, by its association with BSDL, protect this enzyme from degradation in the duodenal lumen and allow for its binding and internalization by enterocytes.

Once in the vicinity of microvilli and upon binding to the cell surface, the complex is internalized (see Fig. 8 and Table 2) via endocytic vesicles by the enterocytes. This is in agreement with previous immunolocalization studies on human intestine (Lechêne de la Porte et al., 1987), showing the presence of BSDL in enterocyte. In addition, our in situ hybridization has established that the enterocyte is devoid of BSDL mRNA and thus unable to synthesize this protein. Therefore any BSDL detected in enterocytes is of exogenous origin and must derive from the pancreas.

In the intestinal lumen, the role of BSDL is to catalyze the hydrolysis of cholesterol esters, which cannot be absorbed without prior hydrolysis to free cholesterol, and to increase the free-cholesterol concentration gradient between the micellar and plasma membrane pools as well as enhancing the passive uptake of free cholesterol (Huang and Hui, 1990; Shamir et al., 1995; Howles et al., 1996). Whereas its role within the enterocyte remains to be established, BSDL could act by functioning either as a carrier for cholesterol (Lopez-Candales et al., 1993) or as an intracellular esterifying enzyme (Gallo et al., 1984). However, recent in vitro studies seem to invalidate this hypothesis (Shamir et al., 1995). In addition, BSDL taken up by CaCo-2 cells was found to have a short residence time and is either degraded or rapidly secreted (Huang and Hui, 1990).

Recently, it has been shown that both phospholipase A2 and BSDL are required for efficient cholesterol uptake by the intestinal mucosa (Mackay et al., 1997). We suggest that one other secreted pancreatic protein, namely the Grp94-related protein, is important in promoting the enterocytic absorption of BSDL.

Finally one question remains unanswered and concerns the mechanism by which BSDL interacts with the membrane and is internalized by the intestinal cells. Both proteins could be recognized by corresponding receptors or binding sites. Indeed, Bosner et al. (1988) have demonstrated the interaction of BSDL with heparin-like molecules at the surface of intestinal cells. Using a heterologous system (CaCo-2 cells and porcine enzyme), Huang and Hui (1990) showed that the initial interaction of BSDL with these cells was mediated by the binding of BSDL to low-affinity and high-capacity binding sites on the cell surface. Heparin, a component of the brush border membrane, binds pancreatic triglyceride lipase in a receptor-like manner to promote the absorption of fatty acids (Bosner et al., 1989). By analogy, BSDL has been shown to possess a functional heparin binding site, which does not interfere with the BSDL-Grp94 complex (Bruneau and Lombardo, 1995). On the other hand, the presence of Grp94 at the plasma membrane of dentritic cells (Nichitta, 1998) suggests that a receptor to this protein may also exist. The nature of these sites and/or receptors remains to be determined.

In summary, we have demonstrated for the first time that the transport of BSDL all along the pancreatic secretory route occurs via an association with a Grp94-related protein. The complex is secreted in the pancreatic juice and reaches the duodenal lumen, where it is internalized by enterocytes. Finally, the dissociation of the Grp94-BSDL complex seems to take place in the endosomal compartment and the BSDL is transferred to the basolateral membrane of the enterocyte. The function of BSDL within the enterocyte was not elucidated; however, we propose that BSDL could act in a concerted mechanism with acyl-coA cholesterol acyltransferase in the formation of chylomicrons, as suggested by Gallo et al. (1984).

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