O-Glycosylation of C-terminal Tandem-repeated Sequences Regulates the Secretion of Rat Pancreatic Bile Salt-dependent Lipase*

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Amino acid sequences rich in Pro, Glu, Ser, and Thr (PEST) are common to rapidly degraded proteins (Rogers, S., Wells, R. & Rechsteiner, M. (1986) Science 234, 364–368). On pancreatic bile salt-dependent lipase (BSDL), PEST sequences are present in the C-terminal region of the enzyme to which is associated the O-glycosylation. We have postulated that the O-glycosylation of BSDL may contribute to mask PEST sequences and to trigger the secretion of this enzyme instead of its delivery into a degradative pathway (Brunéau, N., and Lombardo, D. (1995) J. Biol. Chem. 270, 13524–13523). To further examine the role of the O-linked glycosylation on BSDL metabolism, rat pancreatic BSDL cDNA was stably transfected into two Chinese hamster ovary (CHO) cell lines, the CHO K1 wild-type line and the O-glycosylation defective CHO IdID line. In these latter cells, O-glycosylation can be reversibly modulated by culture conditions. Results indicate that the rate of BSDL synthesis by transfected CHO K1 or CHO IdID cells reflects, independently of culture conditions, the amount of mRNA specific for BSDL present in these transfected cells. Nevertheless, the rate of secretion of the enzyme depends upon cell culture conditions and increases with the cell capability to O-glycosylate C-terminal tandem-repeated sequences. Immunoprecipitation experiments performed on cell lysates suggested that a rapid degradation of BSDL occurred particularly when transfected CHO IdID cells were cultured under non-permissive conditions. We further showed that BSDL secreted by CHO IdID cells grown under non-permissive conditions that normally prevent O-glycosylation incorporated galactose and was reactive with peanut agglutinin, which recognizes the core structure of O-linked glycans. We concluded that the BSDL expressed by CHO IdID cells grown under non-permissive conditions was rapidly degraded but a fraction of the enzyme was allowed to O-glycosylate and consequently was secreted.

1 The abbreviations used are: BSDL, bile salt-dependent lipase (EC 3.1.1.30); CHO, Chinese hamster ovary (cell line); Grp94, glucose-regulated protein of 94 kDa; PCS, fetal calf serum; apo, apolipoprotein; kh, kilo base pair(s); ER, endoplasmic reticulum; PAGE, polyacrylamide gel electrophoresis; DFP, diisopropyl fluorophosphate; PNA, peanut agglutinin; PNGase F, peptide N-glycosidase F.

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hypothesis and to further examine the role of the glycosylation on BSDL metabolism, rat pancreatic BSDL cDNA was stably transfected into Chinese hamster ovary (CHO) cells defective for O-linked glycosylation (ldID line) (16) and into wild-type CHO cells (K1 line).

**EXPERIMENTAL PROCEDURES**

**Materials—**Monensin and peanut agglutinin immobilized on agarose (PNA-agarose) were from Sigma, Brefeldin A, sialidase, and leupeptin came from Boehringer (Mannheim, Germany). Phenylmethylsulfonyl fluoride, benzamidine, and β-phenyl propionate were from Fluka (Buchs, Switzerland). Ham’s F-12 medium, RPMI medium, fetal calf serum, and Geneticin (G418) were from Life Technologies Inc. [3H]DFP was from NEN Life Science Products (Les Ulis, France). Peptide N-glycosidase F (PNGase F) was from Oxford GlycoSystems (Oxford, United Kingdom) and used according to the manufacturer’s protocol.

**Antibodies—**Polyclonal antibodies raised using purified secretory rat BSDL were obtained in our laboratory and isolated by affinity chromatography on protein A-Sepharose (17). These antibodies were able to immunoprecipitate non-glycosylated BSDL obtained by in vitro translation using rabbit reticulocyte lysates (data not shown). Dot blots were performed using antibodies specific for BSDL as primary antibodies and the BM chemiluminescence Western blotting kit (Boehringer) was used to detect the antigen-antibody complexes.

**Protein and Enzyme Assays—**Proteins were routinely assayed with the bicinchoninic acid method (Pierce) using bovine serum albumin as standard. The activity on 4-nitrophenyl hexanoyloxy was measured at 410 nm and pH 7.4 in a thermostated cell at 30 °C as described elsewhere (18).

**Polyacrylamide Gel Electrophoresis—**Gel electrophoreses (SDS-PAGE) were performed on a slab gel of polyacrylamide (7.5%) and 0.1% sodium dodecyl sulfate under reducing conditions according to Laemmli (19).

**Cell Culture—**The Chinese hamster ovary cell line CHO K1 (wild-type) and CHO ldID were supplied by the American Type Culture Collection (Rockville, MD; ATCC designation CCL 61 and SD 1401). The CHO ldID cells were used with the kind permission of Dr. M. Krieger (Massachusetts Institute of Technology, Cambridge, MA). Each CHO cell line was maintained in 5% CO2 at 37 °C in Ham’s F-12 medium, RPMI medium, fetal calf serum, and Geneticin (G418) (20) as described by Mas et al. (23). Briefly, immunoprecipitated BSDL was routinely assayed using the bicinchoninic acid method (Pierce) using bovine serum albumin as standard.

**Inhibitor Treatment—**Confluent transfected CHO cells were treated with 10 μg/ml of β-phenyl propionate. For this purpose, the drug was added from a stock solution of monensin, 10 μg/ml in PBS: ethyl alcohol (1:1 by volume) to dishes at the appropriate final concentration. After the required time of incubation, the cell culture medium was withdrawn and saved for further analyses. The corresponding cell layer was washed twice with PBS, scraped with a rubber policeman, and lysed in a 10 mM Hepes (pH 7.4) buffer (lysis buffer, 200 mM NaCl, 2 mM CaCl2, 2 mM MgCl2, 1.5% Triton X-100, 10 μg/ml leupeptin, 2 mM benzamidine, 2 mM phenylmethylsulfonyl fluoride, 2 mM soybean trypsin inhibitor, and 2 mM β-phenyl propionate). The homogenate was then clarified by centrifugation (20,000 g, 10 min × g, 4 °C). The BSDL activity was determined in the cell culture medium and the cell lysate.

**Pulse-chase Protocol—**After 45 min of starvation of CHO cells in methionine-free RPMI medium, dishes were pulse-labeled with [35S]methionine (20 or 40 μCi/ml). The pulse medium was removed after the required time of incubation, followed by two quick washes with the chase medium, and cells were chased in Ham’s F-12 medium with the required amount of FCS for different time intervals. The chase was stopped by aspirating the medium and cells were washed twice with ice-cold PBS. Immunoprecipitation was performed as indicated, cell-free chase medium was also used for immunoprecipitation.

**Immunoprecipitation of BSDL—**One ml of clarified cell lysate or cell-free medium was incubated overnight at 4 °C with 25 μg of antibodies to rat BSDL. Protein A (10 μg/ml) was added to the antigen-specific antibodies (16). The antigen-antibody Protein A complexes were recovered by centrifugation (10,000 g, 15 min, 4 °C). The final pellet was then washed twice with the washing buffer (10 mM Tris/HCl (pH 7.4) buffer, 5 mM EDTA, and 0.5% Triton X-100), twice with the washing buffer supplemented with 1 mM NaCl and 0.1% SDS, and finally washed with 10 mM Tris/HCl (pH 7.4), 5 mM EDTA buffer. The pellet was then transferred into the SDS-PAGE Laemmli’s sample buffer, warmed for 2 min at 95 °C, centrifuged, and electrophoresed on SDS-PAGE. When required, a two-cycle immunoprecipitation procedure slightly modified from Doolittle et al. (22) was used. Gels were stained with Coomassie Blue R250 and destained as above, immersed (30–60 min in Amplify (Amersham), and autoradiographed using Kodak Bio Max films.

**Isolation of the C-terminal Peptide of BSDL—**The basic protocol for the isolation of the O-linked C-terminal tail of BSDL has been extensively described by Mas et al. (23). Briefly, immunoprecipitated BSDL was radiolabeled with [3H]DFP on the serine residue (Ser-194) involved in the catalytic site (8). This residue is close to Asn-187, which contains the neo-
A total cellular RNA extracted from CHO-K1, CHO ldlD, CHO K1–3B, and CHO ldlD-6B were separated on 1% agarose gel, transferred to a nylon membrane and hybridized to a 32P-labeled probe specific for BSDL (left panel) or specific for actin (right panel). B, dot-blot quantitation of mRNA specific for BSDL and Grp94. Decreasing amounts of RNA extracted from CHO K1–3B and CHO ldlD-6B clones were dotted on nitrocellulose filter and analyzed using 32P-labeled probes for BSDL and Grp94. Graphs represent the regression analysis of dot-blot signal intensities (dark intensity (pixel) in arbitrary units) versus µg of mRNA (2) (the amount of dotted RNA was normalized with the actin probe).

**RESULTS**

**Secretion of Bile Salt-dependent Lipase by Transfected CHO Cells**—The wild-type CHO K1 and the O-glycosylation-defective CHO ldlD cells were stably transfected with the full-length cDNA of the rat BSDL. Two clones expressing BSDL activity were selected from each cell line and used for these studies. They were referred to as the CHO K1–3B and the CHO ldlD-6B clone, respectively. No enzyme activity above background was expressed in either non-transfected or pMAMneo transfected (control) cell lines. Northern blot analyses were used to assess the mRNA abundance in both CHO K1–3B and CHO ldlD-6B clones. The BSDL mRNA level in stably transfected CHO K1–3B and CHO ldlD-6B cells were separated on 1% agarose gel, transferred to a nylon membrane and hybridized to a 32P-labeled probe specific for BSDL (left panel) or specific for actin (right panel). B, dot-blot quantitation of mRNA specific for BSDL and Grp94. Decreasing amounts of RNA extracted from CHO K1–3B and CHO ldlD-6B clones were dotted on nitrocellulose filter and analyzed using 32P-labeled probes for BSDL and Grp94. Graphs represent the regression analysis of dot-blot signal intensities (dark intensity (pixel) in arbitrary units) versus µg of mRNA (2) (the amount of dotted RNA was normalized with the actin probe).

**Fig. 1. Northern blot analysis of RNA in transfected cells.** A, total cellular RNA extracted from CHO-K1, CHO ldlD, CHO K1–3B, and CHO ldlD-6B were separated on 1% agarose gel, transferred to a nylon membrane and hybridized to a 32P-labeled probe specific for BSDL (left panel) or specific for actin (right panel). B, dot-blot quantitation of mRNA specific for BSDL and Grp94. Decreasing amounts of RNA extracted from CHO K1–3B and CHO ldlD-6B clones were dotted on nitrocellulose filter and analyzed using 32P-labeled probes for BSDL and Grp94. Graphs represent the regression analysis of dot-blot signal intensities (dark intensity (pixel) in arbitrary units) versus µg of mRNA (2) (the amount of dotted RNA was normalized with the actin probe).

**Fig. 2. Synthesis of BSDL by transfected CHO cells.** CHO ldlD-6B transfected cells, grown under non-permissive conditions ( ), 0.5% FCS) or permissive conditions ( ), 10% FCS) and CHO K1–3B transfected cells ( ), were depleted of methionine with Ham’s F-12 methionine-free medium for 45 min, pulsed with [35S]methionine for 15 min, and chased for the required time. Cells were then quickly washed with the medium and then with PBS buffer and harvested. Cells were lysed, and 1 ml of lysate was immunoprecipitated with antibodies to rat BSDL and analyzed by SDS-PAGE and autoradiography. Bands migrating at 75 kDa and corresponding to BSDL were quantitated by scanning. The dark intensity (pixel) at each time of chase was subtracted from that at time 0, and values thus obtained were plotted with time.

**Fig. 3.** Secretion of BSDL by CHO K1–3B and CHO ldlD-6B cells. Discrete CHO K1–3B and CHO ldlD-6B cells were pulse-labeled by [35S]methionine and immunoprecipitated. The signal intensities were quantitated by scanning (9), the dark intensity (pixel) at each time of chase was subtracted from that at time 0, giving the amount of neo-synthesized BSDL with time. The slopes thus obtained suggested that the BSDL synthesis was 5 times faster in CHO K1–3B than in CHO ldlD-6B. These slopes, normalized for the amount of mRNA specific for BSDL, gave the translational rate (amount of protein produced per min and unit of mRNA), which was, within experimental errors, identical in CHO K1–3B cells and in CHO ldlD-6B cells cultured under permissive or non-permissive conditions. These results showed that culture conditions did not affect either transcriptional or translational processes in transfected CHO cells.

The time dependence of the secretion of BSDL was next examined. For this purpose, CHO K1–3B and CHO ldlD-6B cells were pulse-labeled by [35S]methionine and chased for time intervals up to 240 min. Aliquots of cell culture medium were collected and immunoprecipitated. As shown in Fig. 3A, after a pulse of 15 min, BSDL was detected in the culture medium of CHO K1–3B cells after only 5 min of chase. CHO ldlD-6B cells also secreted BSDL; however, the enzyme was detectable by immunoprecipitation after a 30-min chase. Quantitation of the precipitated material by scanning showed that the rate of BSDL secretion by CHO K1–3B cells was about 50 times faster than that of CHO ldlD-6B grown under permissive conditions. The latter clone appeared to secrete 4-fold less BSDL when cultured under non-permissive conditions. The time dependence of BSDL secretion by either CHO K1–3B and CHO ldlD-6B cells was also determined from the enzyme activity recorded in culture medium. For this purpose, CHO K1–3B and CHO ldlD-6B cells were washed in PBS and incubated with fresh medium. At time intervals up to 4 h, aliquots of the medium were collected and assayed for BSDL activity. The
activity of BSDL increased with time in the culture medium of both cell lines, but the rate of BSDL secretion by CHO K1–3B cells (36.6 ± 1.3 milliunits/h/mg of cell protein) was approximately 30 times higher than that of CHO ldlD-6B cells grown under permissive conditions (1.2 ± 0.2 milliunits/h/mg of cell protein). Additionally, as above, and consistent with the estimates made by scanning, the rate of secretion was approximately 5-fold slower (0.22 milliunits/h/mg of cell protein) when CHO ldlD-6B cells were cultured under non-permissive conditions.

These data showed that, even though the rates of BSDL translation in CHO K1–3B and CHO ldlD-6B clones were similar, the rate of secretion of the enzyme significantly differed between these clones. Overall, the rate of BSDL secretion by CHO ldlD-6B cells was dependent upon the culture, permissive versus non-permissive, conditions. Taken together, these data suggested that post-translational modification of BSDL by O-glycosylation could regulate the rate at which the enzyme is secreted.

**BSDL Degradation in CHO ldlD-6B Cells**—As shown above, the rate of BSDL synthesis, which reflects the abundance of BSDL mRNA in each clone, was independent of the culture conditions. Nevertheless, the rate of secretion of the enzyme by CHO ldlD cells clearly depends upon culture conditions. Therefore, it was tempting to address the question of whether, in CHO ldlD cells grown under non-permissive conditions, BSDL was partly subjected to intracellular degradation. The CHO ldlD-6B cells grown to confluence were pulsed for 30 min with [35S]methionine. After a 3-h chase, cell culture medium and cell lysate were immunoprecipitated. The CHO K1–3B cells were pulsed for 5 min and chased for 20 min before immunoprecipitation. The immunoprecipitated material was then separated by SDS-PAGE and autoradiographed. The material immunoprecipitated from the cell lysate and culture medium of CHO K1–3B cells (data not shown) displayed an electrophoretic migration corresponding to a doublet at 72–75 kDa, compatible with the Mr of the rat BSDL (9, 26–28). The cell culture medium of CHO ldlD-6B cells, independently of culture conditions, also contained material associated with a Mr of 75 kDa. In contrast, analysis of the CHO ldlD-6B cell lysates indicated that the radioactive material migrating at approximately 75 kDa was largely depleted; instead, predominant bands with Mr values of 43–46 and 55 kDa were detected. An immunoprecipitation of cell lysate performed immediately after a short pulse (≤5 min) also displays sub-bands at about 60 and 55 kDa (data not shown). This, a priori, indicated that BSDL expressed in transfected CHO ldlD-6B cells may be rapidly degraded and that only a fraction of non-degraded enzyme can be secreted. Therefore, we wished to assess the relative amounts of BSDL (75 kDa) and the possible degradation products (43–46 and 55 kDa). For this purpose, CHO ldlD-6B cells grown in 0.5% FCS-supplemented medium were pulse-labeled for 15 min with [35S]methionine. After the pulse, cells were washed with PBS and chased in fresh medium for intervals up to 240 min, then cells were harvested and lysed. Cell lysates corresponding to each time of chase were immunoprecipitated, analyzed by SDS-PAGE and autoradiography, and quantitated by scanning. The low Mr material migrating at approximately 43–46 and 55 kDa was already present at time 0 of chase. The 75-kDa BSDL signal decreased over time, correlating with the secretion of the enzyme. The intensity of the signal migrating at 55 kDa slowly increased with the duration of the chase period, whereas that of bands migrating at 43–46 kDa was quite stable. The same pattern was obtained when CHO ldlD-6B cells were grown under permissive conditions, during which time no low Mr sub-bands appeared in CHO K1–3B cell lysates. These data
Increased the secretion of the enzyme (Fig. 4, upper panel). For CHO ldlD-6B cells, the signal detected after immunoprecipitation and analyzed on SDS-PAGE and autoradiography. However, these data suggested that degradation of BSDL may occur in the transfected CHO ldlD cells.

In previous studies, we have shown that the ionophore monensin and brefeldin A inhibited the BSDL secretion; the ionophore also induced the retention of the enzyme in a membrane compartment of AR 4–2J pancreatic cells (15). These drugs, which impair trans-Golgi to secretory vesicles and the endoplasmic reticulum to Golgi vesicular traffic, respectively, also inhibited the secretion of BSDL by either CHO K1–3B and CHO ldlD-6B cells. They also induced a concomitant increase of the BSDL activity within cell lysate of both transfected cell lines. The increase in activity was higher in CHO K1–3B cells supplemented with monensin and brefeldin A (170 ± 10% and 310 ± 20%, respectively) than in CHO ldlD-6B cells grown under non-permissive conditions (130 ± 5% and 140 ± 4%, respectively). However, these data suggested that brefeldin A and monensin may have blocked the degradative process of BSDL. Pulse-chase experiments were performed to analyze the effects of monensin and brefeldin A on BSDL expressed by CHO K1–3B cells present in cell culture medium and in cell lysate. As expected, these drugs led to increased retention and secretion of BSDL in CHO K1–3B cells and decreased the secretion of the enzyme (Fig. 4, upper panel). For CHO ldlD-6B cells, the signal detected after immunoprecipitation of the culture medium was also significantly decreased by monensin and brefeldin A treatment (Fig. 4, lower panel). In lysates of latter cells, grown under non-permissive conditions, multiple signals were detected after immunoprecipitation, and accumulation of material at 75 kDa, corresponding to undegraded BSDL, was evident (arrow). Intense bands presenting with M, of approximately 55 and 43–46 kDa (arrowheads), lower than that of full-length BSDL, were also detected. As with the CHO K1–3B cells, brefeldin A and monensin led to intracellular accumulation and decreased secretion of BSDL. When compared with the amount of undegraded BSDL, it appeared that the relative quantity of low M, material was much higher in lysate of CHO ldlD-6B cells cultured in the absence than in the presence of drugs. The same pattern was obtained with CHO ldlD-6B cells cultured under permissive conditions (data not shown), whereas a minute amount of this material was detected in CHO K1–3B cell lysate (Fig. 4, upper panel). Hence, brefeldin A and monensin induced the intracellular accumulation of undegraded enzyme. These data indicated that low M, bands can be generated by a breakdown process of BSDL. Although unlikely, it is possible that low M, material resulted from a co-precipitation of proteins associated with BSDL (15).

To overcome the possible co-precipitation of proteins associated with BSDL, lysate from either CHO K1–3B and CHO ldlD-6B cells was subjected to a two-step immunoprecipitation procedure (22) with polyclonal antibodies to rat BSDL. After the two-step immunoprecipitation, BSDL can be specifically detected in cell lysate of CHO K1–3B cells, as observed after the one-step immunoprecipitation procedure, whereas in CHO ldlD-6B cell lysate the low M, material, although decreased, can still be precipitated. Therefore, it is likely that the low M, material immunoprecipitated in CHO ldlD-6B cells with antibodies to rat BSDL reflects some degradation of BSDL.

Glycosylation of BSDL Expressed in CHO ldlD-6B Cells—At this point, we needed to take into account one fundamental fact, which concerns the ability of CHO ldlD cells to glycosylate the fraction of BSDL to be secreted. We first examined the incorporation of galactose and mannose in BSDL expressed by CHO ldlD-6B cells cultured under permissive conditions. For this purpose, CHO ldlD-6B cells were cultured in glucose-depleted RPMI, and then glycoproteins were metabolically radiolabeled with [14C]galactose or [14C]mannose (10 μCi/ml for 4 h). At the end of the incubation time, cell-free medium was withdrawn and cells were extensively washed with fresh medium, harvested, lysed, and clarified as above described. Cell-free medium and cell lysate (1 ml each) were subjected to immunoprecipitation and analyzed by SDS-PAGE. As shown in Fig. 5, radioactive material corresponding to BSDL can be precipitated either after mannose or galactose labeling. Immunoprecipitation obtained from cell lysate indicated the presence of three bands migrating at 68, 72, and 75 kDa; within this heterogeneous material, the 72 kDa band was dominantly radiolabeled by [14C]galactose and [14C]mannose (lane 2). In cell-free medium, only the two higher forms could be detected with equal intensity (lane 1). These two bands correlated with those already detected after [35S]methionine metabolic labeling (see figures herein) and might corre-
bound with Tris/HCl buffer containing SDS. Both bound were washed, and the material specifically bound to beads was eluted by centrifugation, the supernatant contained unbound material. Beads incubated overnight at 4 °C under agitation. Beads were then decanted 0.5 ml of cell-free medium or cell lysate (adjusted to pH 7.4) and again treated with 10 mM Tris/HCl (pH 7.4), 3% Triton X-100, and 10% glycerol, boiled 2 min, and centrifuged for 15 min at 10,000 × g, 4 °C, 30 min. Under these conditions, ~58% of the starting radioactivity was found in the supernatant. This result suggested that O-linked glycans, represented by labile material, had incorporated some 60% of the [14C]galactose that labeled BSDL. The remaining [14C]galactose coprecipitates with BSDL and was likely incorporated into N-linked glycans. However, when BSDL was labeled with [14C]mannose and treated under the same conditions, the material released represented less than 20% of the starting radioactive material. In another set of experiment, [14C]mannose or [14C]galactose-labeled BSDL was immunoprecipitated and boiled in the presence of 0.5% SDS and 5% β-mercaptoethanol and finally treated with 20 units/ml PNGase F (37 °C, 18 h), precipitated with trichloroacetic acid, and centrifuged as above. Under these conditions, approximately 63% of mannose and 40% of galactose radioactivity was released. In controls where PNGase F was omitted, more than 80% of the radioactive material was found in the trichloroacetic acid pellet, which corresponded to the trichloroacetic acid precipitation yield (90% as determined by [35S]methionine-labeled BSDL). Taken together, these data suggested that mannose was, as expected, mainly incorporated in N-linked structures. Additionally, galactose partitioned between N- (approximately 40%) and O-glycosylation (approximately 60%). Obviously, binding to PNA and galactose incorporation are good evidence for the O-glycosylation of BSDL. Therefore, we next investigated the glycosylation of BSDL expressed by CHO ldlD-6B cells grown under non-permissive conditions. For this purpose, CHO ldlD-6B cells were incubated for 4 h with [14C]galactose in glucose-depleted RPMI after adapting them for 24 h to non-permissive conditions. Labeled proteins present in the cell-free medium were then examined before and after immunoprecipitation. Three proteins present in the cell-free medium were detected at approximately 34–36 kDa (Fig. 7, lane 1); only BSDL (75 kDa, arrow) and trace of the lower Mr protein were immunoprecipitated by antibodies specific for rat BSDL (lane 2). The same data were obtained when cells were incubated with [14C]mannose under the same conditions (data not shown). The presence of O-linked glycans on the BSDL secreted by CHO ldlD-6B cells grown in 0.5% FCS was further investigated. For this purpose, CHO ldlD-6B cells, accustomed to non-permissive conditions, were starved in methionine-free RPMI and incubated for another 24 h in the presence of [35S]methionine (10 μCi/ml) while still in the presence of 0.5% FCS. At the end of the incubation,
BSL of cell-free medium was immunoprecipitated (Fig. 8, lane 1) or treated with sialidase (0.5 unit) and subjected to the PNA-agarose fractionation as described above. The unbound and bound fractions were then analyzed on SDS-PAGE and autoradiography. Results of a typical experiment are given in Fig. 8 (lanes 2 and 3). As shown in this figure, radioactive material corresponding to BSDL ($M_\text{r}$ = 75 kDa, arrow; the low $M_\text{r}$ material may be a degradation product) was detected in the bound fraction but could not be visualized in the unbound fraction. These data indicated that BSDL was totally adsorbed on the PNA-agarose beads and consequently appeared O-glycosylated. Therefore, we can suggest that the fraction of BSDL secreted by CHO ldld-6B cells, grown under conditions that did not normally allow glycosylation of proteins, was indeed O-glycosylated.

Glycosylation of C-terminal Repeats of BSDL Expressed in CHO ldld-6B Cells—Although it is known that the O-linked glycosylation of BSLD strictly locates within the C-terminal tandem-repeated sequences that comprise PEST sequences (33, 24), we next attempted to demonstrate that the O-glycosylation of BSDL expressed by CHO ldld-6B cells was effectively located on the C-terminal tail of the enzyme. For this purpose, we applied the procedure described by Mas et al. (23) to characterize the O-glycosylated peptide that carries the J28 epitope of the oncoketal variant of BSDL (30). Therefore, BSDL expressed by CHO ldld-6B cells was immunoprecipitated, labeled with $[^3\text{H}]$DFP, and cleaved by cyanogen bromide as described by Mas et al. (23). The peptides were desialylated and separated on a PNA-agarose column equilibrated and eluted with PBS + 0.5% Triton X-100, washed with PBS + 0.1% Triton X-100, and finally the O-glycosylated peptide was eluted with PBS containing 0.3 M lactose. The radioactivity was determined in each fraction (□), and bound material eluted by lactose was detected by dot blot using antibodies specific for BSDL and quantitated by scanning (●). B, BSDL expressed by CHO ldld-6B cells was metabolically labeled with $[^1\text{C}]$galactose and immunoprecipitated. BSDL was then treated with cyanogen bromide; peptides were desialylated and analyzed as in A. The $[^1\text{C}]$ radioactivity was determined in every eluted fraction (△).

**DISCUSSION**

The structures of the rat and human BSDL genes were determined a few years ago (31, 32). The organization of these genes indicated the presence of 11 exons interrupted by 10 introns. Each exon may encode an unique structural or functional domain of the enzyme. The largest exon, number 11, encodes the signal sequence for degradation (11) (PEST sequences) and the mucin-like tandem repeated sequences (33). Recent studies (14, 15) allowed us to postulate that the C-terminal domain of BSDL may be responsible for the interaction of the enzyme with a membrane folding complex including a 94-kDa protein (p94) immunologically related to Grp94. The release of BSDL from membranes occurs once terminal sugars are added to glycans, and the Grp94-related protein was assumed to assist the proper sorting of BSDL from the endoplasmic reticulum (ER) to the trans-Golgi (15). Because no intracellular degradation of
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BSDL occurred in pancreatic cells upon treatment with drugs that affect secretion (9), the association of BSDL with microsome membranes by means of the Grp94-related protein may be important for the O-glycosylation of the C-terminal tandem-repeated sequences of BSDL which includes PEST sequences (15). Thus, we hypothesized that the O-glycosylation of this C-terminal domain can divert BSDL from entering into a degradative route.

To investigate this possibility, two CHO cell lines, the glycosylation-defective ldId line and the wild-type K1 line, were transfected with the full-length cDNA of the rat BSDL. The CHO-IldID cell line is markedly deficient in UDP-Gal and UDP-GalNac 4-epimerase activity, leading to a defect in O-glycosylation when these cells are grown in 0.5% FCS-supplemented medium (16). The abnormal glycosylation of proteins could be completely reversed by providing the cells with exogenous sources of galactose and N-acetylgalactosamine (free sugars or 10% FCS) (34). In this study, we showed that the rate of secretion of BSDL by CHO IldID-6B cells was 1/30 to 1/50 that observed with CHO K1–3B cells. The difference was even more significant when CHO IldID-6B cells were grown under non-permissive conditions. This large difference cannot be explained by modification of translational rate, although the amount of BSDL mRNA present in CHO IldID-6B cells was 5-fold lower than that of CHO K1–3B. Therefore, it is possible that the low amount of BSDL secreted by CHO IldID-6B cells can reflect the intracellular degradation of BSDL as a result of its O-glycosylation defect. We have further examined the behavior of BSDL within CHO K1–3B and CHO IldID-6B cells by pulse-chase experiments followed by immunoprecipitation of cell lysates with antibodies to BSDL. The results suggested that low material can be precipitated; however, it increased slowly with time, whereas that of full-length BSDL decreased rapidly. Moreover, low material was detectable after one- and two-step immunoprecipitation procedures performed with CHO IldID-6B cell lysate. This material was even present after a short pulse-chase experiment, but was absent in CHO K1–3B cell lysate. Treatment of transfected cells with brefeldin A and monensin resulted in an accumulation of BSDL in CHO K1–3B and in CHO IldID-6B cells. Consequently, rapid degradation of BSDL expressed in CHO IldID-6B cells grown under conditions that prevent O-glycosylation was strongly suspected. The site for BSDL degradation remains unknown; however, the effect of brefeldin A, which blocked the degradation of the protein, suggested the involvement of a post-ER compartment. Memensin also blocked the degradative process of BSDL, implying that a post-Golgi compartment could be involved. Therefore, the diversion of BSDL to a post-Golgi compartment for degradation can be compared with that of apolipoprotein (apo) E and B, which are secreted by hepatocytes. Ye et al. (35) reported that apoE may be degraded in a post-Golgi compartment by lysosomal proteases and cytosolic Ca2+-dependent cysteine proteases. In addition, apoB degradation occurred in a post-ER location, which is associated with a Golgi membrane fraction via the action of cysteine proteases (36). Similarly, BSDL could also be degraded in lysosomes or in cytosol. Degradation of misfolded ER luminal proteins by the cytosolic ubiquitin-proteasome pathway is now well documented (37). Nevertheless, one may consider that BSDL is a PEST sequence-containing protein. It is supposed that PEST sequences target proteins for degradation in the cytosol, possibly by calpains (38). However, many reports have suggested that PEST regions may constitute the recognition sites for ubiquitination and degradation by the 26 S proteasome (39). PEST motifs are often conditional proteolytic signals, and there are a number of ways to expose or mask them to proteolytic factors (39). Therefore, a model for BSDL degradation could be a route to cytosol that involves the diversion of post-Golgi vesicles to an intracellular location in which the contents of the vesicles are degraded. It is known that the targeting of secretory vesicles is sensitive to the state of cargo. Consequently, when the C-terminal domain of BSDL is not O-glycosylated, the protein could be included in vesicles diverted from secretion and targeted to a post-trans-Golgi compartment for degradation; this route is invalid when BSDL is normally O-glycosylated. Conceivably, the membrane complex to which BSDL is associated during its transport from ER to a post-Golgi compartment (14, 15) may be involved in the routing of the enzyme either to secretion or to degradation according to the glycosylation state of the enzyme. Accordingly, when BSDL is fully glycosylated, it is released from membrane (15) and routed to secretion. Otherwise, BSDL could be kept associated with the complex and targeted to degradation.

Because O-linked glycosylation can influence the rate at which a glycoprotein is secreted (16, 40), this would provide a clear explanation as to why the secretion rate of BSDL increased with the cell ability to O-glycosylate in the following rank order: CHO IldID-6B cells grown under non-permissive conditions < CHO IldID-6B cells grown under permissive conditions < CHO K1–3B cells. We further showed that BSDL secreted by CHO IldID-6B cells was O-glycosylated; the protein incorporated alkaline-sensitive galactose on C-terminal tandem-repeated sequences and carried out Galβ1–3GalNac-O-T/S structures, which were recognized by the PNA lectin. Incorporation of mannose also demonstrated the presence of an N-linked structure (9). The fraction of BSDL secreted by CHO IldID-6B cells cultured under non-permissive conditions had also incorporated galactose and was recognized by the PNA lectin. Therefore, these data indicated that O-linked glycans are present on BSDL secreted by CHO IldID-6B cells independently of culture conditions.

In conclusion, CHO IldID-6B cells, when grown under conditions that should prevent O-glycosylation, secreted a low amount of BSDL. This protein was apparently rapidly degraded intracellularly, but a small fraction of BSDL was able to be O-glycosylated and secreted. Taken together, these data suggest that the rate of secretion of BSDL depends upon the ability of the cell to O-glycosylate C-terminal repeats of the protein.

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