The Effects of O- and N-Linked Glycosylation on the Secretion and Bile Salt-Stimulation of Pancreatic Carboxyl Ester Lipase Activity (43850)

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Abstract. **Pancreatic carboxyl ester lipase is a glycoprotein that requires millimolar concentrations of trihydroxy bile salts, such as cholate, for maximal catalytic activity against cholesteryl esters and triglycerides. Binding of cholate, with subsequent activation, has been proposed to occur in the carboxy-terminal region of carboxyl ester lipase, which contains multiple sites for O-linked glycosylation (1). To investigate the role of O- and N-linked glycosylation in the secretion of carboxyl ester lipase by cells and its activation by cholate, rat carboxyl ester lipase cDNA was transfected into the mutant chinese hamster ovary cell line, *ldlD*, and the ability of the cells to modify the expressed carboxyl ester lipase by N- and O-linked glycosylation was modulated by using various incubation conditions and metabolic inhibitors. The results showed that, similar to other lipases, maximal secretion of carboxyl ester lipase activity required N-linked glycosylation. In contrast, O-linked glycosylation did not affect the secretion of carboxyl ester lipase activity. In addition, the cholate stimulation of hydrolysis was also independent of O-linked glycosylation.** [P.S.E.B.M. 1995, Vol 208]

The carboxyl ester lipase (CEL) (Enzyme Committee # 3.1.1.13) secreted by the pancreas is a glycoprotein which can catalyze the hydrolysis of not only dietary cholesteryl esters (CE), but also triglycerides (TG) and retinyl esters (2). The maximal activity *in vitro* against these substrates in bulk phase is dependent on millimolar concentrations of trihydroxy bile salts (cholate and its derivatives), which are found in the upper small intestine at such concentrations. Interestingly, this enzyme has also been found in cells in hepatic origin (rat liver [3], human HepG2 cells [4, 5]) and in the milk of some mammalian species (2).

Despite its potentially wide role in the lipid metabolism in a number of tissues, there are many gaps in our understanding of the structure-function relationships of CEL. In particular, for maximal activity there is the unusual requirement for trihydroxy bile salts, which must play a role in addition to solubilizing substrates, since catalytic activity in the presence of dihydroxy bile salts, equally good detergent molecules, is typically quite low (for example, see Ref. 6). Binding of cholate to purified CEL has been shown to induce conformational changes which correlated with enzyme activation (7). Based on the sequence of cloned CEL, which revealed a number of potential O-linked glycosylation sites clustered at the carboxy terminal end of the protein in all animal species studied to date, it was hypothesized that this region may be the site of bile salt binding and catalytic activation (1). To test this hypothesis and to examine more general effects of glycosylation on CEL secretion and activity, rat pancreatic CEL cDNA was transfected into chinese hamster ovary (CHO) cells with reversible defects in O-linked glycosylation (*ldlD* cells) (8). In some experiments, we also used the standard inhibitor of N-linked glycosylation, tunicamycin. In this way, we were able to study the effects of both types of glycosylation on CEL se-

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cretion and on the bile salt dependence of its catalytic activity.

Materials and Methods

All reagents were of the highest grade commercially available.

Cell Culture. The *ldlD* cells were maintained in 5% CO_2 at 37°C in Ham's F-12 medium supplemented with 2 m*M* glutamine and 5% v/v fetal calf serum (FCS).

Transfection of *ldlD* Cells. An expression vector (pCEH) containing full-length rat pancreatic CEL cDNA under the control of the SV40 promoter (kindly provided by Drs. W. Rutter and J. Han and described in Ref. 9) was transiently expressed in *ldlD* cells. Briefly, cells were plated at a density of 270,000 cells/100 mm dish and 2 days later transfected with pCEH using the calcium phosphate method (10). Cells were subsequently maintained in Ham's F-12 medium containing 3% FCS for 24 hr and then transferred to a 12-well plate and incubated 18 hr in medium containing 0.5% FCS and the appropriate sugar supplements (none, 20 μ*M* galactose [Gal], 200 μ*M* N-acetylgalactosamine [GalNAc], or both Gal and GalNAc) that prevent or allow O-linked glycosylation (see Results and Discussion for more details). An aliquot of medium was taken to assay for CEL enzymatic activity (as described below) to confirm expression. Cell monolayers were then washed three times with medium containing 0.2% BSA and incubated for 6 hr (unless otherwise indicated) in medium containing 0.2% BSA and the appropriate sugar supplements. To determine whether the transfected CEL underwent the normal secretory pathway, in some experiments, brefeldin A (BFA), a fungal metabolite standardly used to block intracellular transport and processing of numerous proteins, was added at 5 μg/ml. To inhibit N-linked glycosylation, the transfected cells were cultured 6 hr (unless otherwise indicated) in the presence of 5 μg/ml of tunicamycin. At the conclusion of all of the incubation conditions, aliquots of media were taken for assays of CE and TG hydrolysis. None of the treatment conditions were associated with general toxicity, as judged by comparable cell protein masses among the wells.

Enzyme Assays. The hydrolytic activity of CEL against two substrates, CE and TG, were assayed as follows. Cholesteryl esterase (CEsterase) activity was determined by a radiometric assay previously described (11). Briefly, the reaction mixture (0.2 ml) contained 50 μl of conditioned medium, 50 m*M* Tris maleate (pH 7.0), and sodium cholate (50 m*M*, unless otherwise indicated). The reaction was initiated by adding 2 nmol cholesteryl-[1-^{14}C]oleate (specific activity 25 μCi/umol) in 0.01 ml ethanol and allowed to proceed at 37°C for 1 hr (Fig. 1), 3 hr (Fig. 2), or 20 min (Fig. 3). The reaction was terminated by the addition of 3.25 ml of methanol/chloroform/heptane (1.41:1.25:1.00). After mixing, 1.05 ml of 0.05 *M* borate buffer (pH 10) was added. The tubes were mixed again and the phases separated by centrifugation. These steps result in the partitioning of the remaining substrate (cholesteryl oleate or trolein [see below]) into the lower organic phase and the released oleate into the upper aqueous phase. An aliquot of the aqueous phase was taken for scintillation counting to quantitate the amount of released labeled oleate. One unit of activity was defined as 1 pmol labeled oleate released/hr.

The TG lipase activity was determined also as previously described (11). Briefly, the reaction was initiated by adding 2 nmol [^{14}C]triolein (specific activity 25 μCi/umol) in 0.01 ml ethanol and allowed to proceed at 37°C for 1 hr or the indicated time. After termination as above, the amount of released labeled oleate was determined by scintillation counting. One unit of activity was defined as 1 pmol labeled oleate released/hr.

For both substrates, assays were performed in duplicate and the coefficient of variation was less than 10%. The conditioned medium from nontransfected cells did not contain any detectable cholesteryl esterase or TG lipase activities.

Statistical Analyses. To make comparison among the results for the controls in Figure 1, one-way ANOVA was performed. Within each group (None, Gal, GalNAc, Both), *t* tests were performed to compare the results for brefeldin A or tunicamycin treatment to the control for that group. To make comparisons among the results for groups at time points in Figure 2, one-way ANOVA was performed. All statistical testing was done with the Instat program (GraphPAD Software, San Diego).

Results and Discussion

To study the role of glycosylation in the secretion of CEL and its activation by trihydroxy bile salts, a plasmid containing the SV40 promoter and rat CEL cDNA was used to transiently express CEL in the CHO cell line, *ldlD*. This line is deficient in the activity of UDP-Gal/UDP-GalNAc 4-epimerase (8). Consequently, UDP-Gal and UDP-GalNAc cannot be synthesized with glucose as the sole sugar source in culture medium. Since these sugars are required for the synthesis of fully mature O- and N-linked oligosaccharide chains (12), in the absence of both sugars, no O-linked glycosylation occurs; N-linked oligosaccharide chains can be synthesized under these conditions, but because neither galactose nor sialic acid can be added to such chains, only truncated species are formed. When provided with Gal, but not GalNAc, *ldlD* cells can synthesize glycoproteins with mature N-linked chains but still cannot add mucin-type O-linked chains. Addition of GalNAc to galactose-free culture medium permits the synthesis of glycoproteins with truncated, immature O-linked chains. Finally, addition of both Gal and GalNAc restores the ability to synthesize glycroproteins with mature O-linked chains (8). Thus, by manipulating the sugar sources in the medium, changes in the glycosylation characteristics of the expressed CEL were accomplished.

The CEL secreted by all mammalian species studied to date is a glycoprotein (2). In those CEL species already cloned (cow, man, rat, rabbit), there is a conserved region of amino acids clustered in the carboxy terminal end with multiple sites for O-linked glycosylation. In addition, in the amino terminal portion of the molecule, there is at least one site for N-linked glycosylation (13, 14). To evaluate the effects of O- and N-linked glycosylation on the secretion of CEL activity, the transfected *ldlD* cells were incubated under a variety of conditions. The results are summarized in Figure 1. As shown, under all supplemental sugar conditions, BFA essentially abolished ($P < 0.01$) the secretion of CEL activity, as assayed by hydrolysis of either CE or TG. This indicated that the CEL expressed from the transfected cDNA traversed the normal secretory pathway and did not bypass the Golgi apparatus.

Under conditions in which full O-linked glycosylation was not allowed (i.e., cells in media lacking both Gal and GalNAc), the secreted activities were not statistically different ($P > 0.16$) from those observed under the permissive (Gal and GalNAc) culture condition (Fig. 1, A and B). In contrast, when the first step of the synthesis of the core oligosaccharide of N-linked chains was blocked by tunicamycin, the amount of CEL activity directed against either CE or TG was only 25%–50% of control ($P < 0.02$), independent of sugar supplementation (Fig. 1, A and B). These results are consistent with the recent report showing reductions of 50% and 45% in the secretion of CEL enzyme activity and enzyme protein, respectively, by tunicamycin-treated pancreatoma cells (14). Interestingly, the dependence on N-linked glycosylation for maximal secretion of CEL activity did not require fully mature oligosaccharide chains, as the truncated N-linked oligosaccharide chains synthesized in the absence of supplementation with Gal and GalNAc were sufficient for the secretion of abundant enzymatic activity (Fig. 1, A and B). Overall, then, the results summarized in Figure 1 suggest that N- and not O-linked glycosylation is important for the secretion of CEL, similar to findings reported for two other lipases, lipoprotein lipase (15) and hepatic lipase (16).

It has been speculated that O-linked glycosylation can influence the rate at which a glycoprotein is secreted (8). To investigate this possibility for CEL, the accumulated activity over time secreted by transfected *ldlD* cells maintained in different media was determined. At each time point, the absence of O-linked glycosylation did not affect ($P > 0.4$) the amount of CEL activity, whether measured with radiolabeled cholesteryl oleate (Fig. 2) or triolein (data not shown), similar to results reported for another glycoprotein, human chorionic gonadotropin (17). Taken with the above results, neither the amount of CEL activity secreted nor the rate of its secretion was affected by O-linked glycosylation.

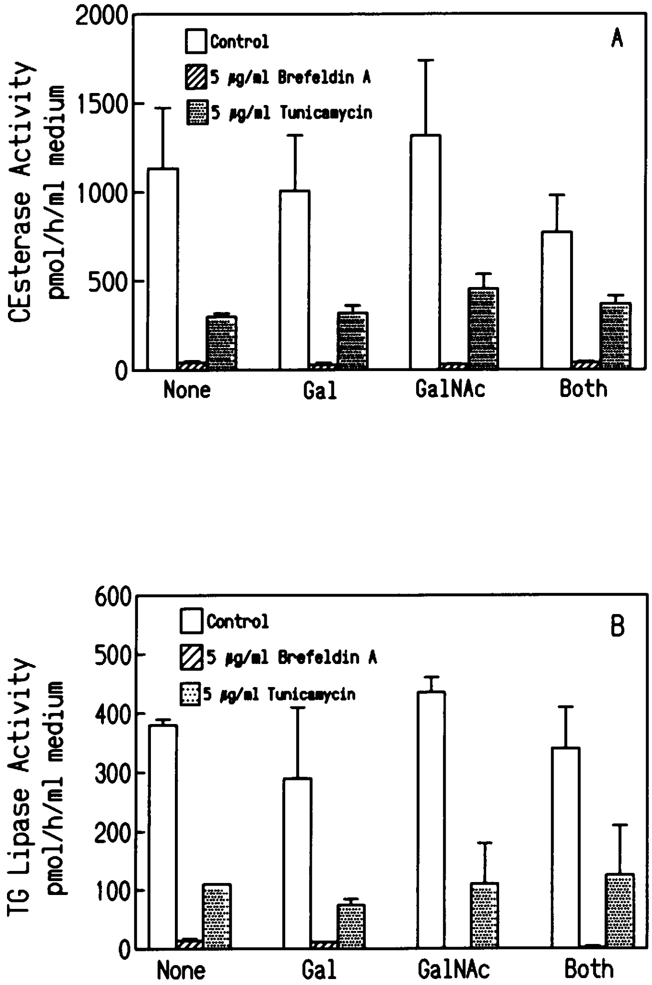

Figure 1. Effects of glycosylation on the secretion of CEL activity. CHO *ldlD* cells transfected with CEL cDNA were incubated for 6 hr in the presence of the sugar supplements and inhibitors shown. The hydrolysis of CE (Panel A) or TG (Panel B) by the conditioned media was then assayed as described in Materials and Methods. Values are means ± SD, $n = 3$.

Finally, the relationship between O-linked glycosylation and the activation of CEL by cholate was probed. As shown in Figure 3, under all media conditions, at millimolar concentrations of cholate there was activation of CE and TG hydrolysis. As previously noted (6, 11), there was also an optimal concentration range of cholate for the stimulation of CE hydrolysis. Thus, the characteristics of CEL activation

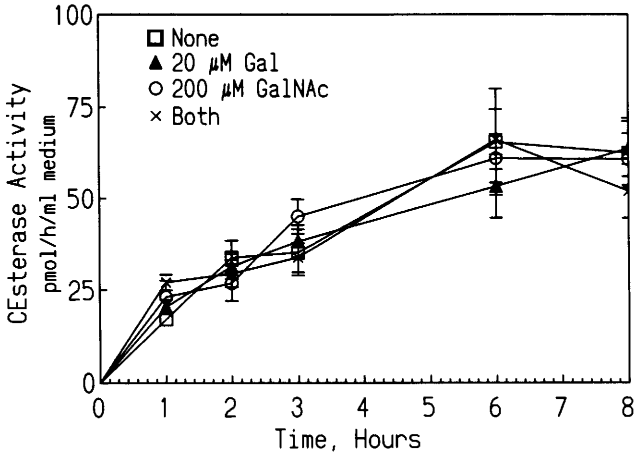

Figure 2. Effects of glycosylation on the rate of secretion of CEL activity. CHO *ldlD* cells transfected with CEL cDNA were incubated in maintenance medium. At the beginning of the time course, the cells were washed and then incubated in experimental media containing the indicated sugar supplements. At each hour thereafter, up to 8 hr, 50 µl aliquots of conditioned medium were withdrawn from each well for measurement of CE hydrolysis. Values are means ± SD, $n = 3$.

by cholate resembled those of the native enzyme and were independent of the glycosylation status of the enzyme.

In summary, by expressing CEL in a cell line in which the ability to modify proteins by O- and N-linked glycosylation could be easily manipulated, we have evaluated the effects of these post-translational modifications on two aspects of CEL metabolism—its secretion (the rate and amount of activity accumulated) and its stimulation by cholate. The former process depended on N-linked glycosylation; neither process required O-linked glycosylation. While this report was in preparation, results consistent with ours, but obtained from different approaches, were published. Hansson *et al.* (18) expressed cDNAs encoding human breast milk CEL and showed that deletion of the carboxy-terminal region containing the cluster of O-linked glycosylation sites did not affect the catalytic activity or bile salt activation of the recombinant enzyme. Also, as noted earlier, Abouakil *et al.* (14) using the rat pancreatoma cell line AR42J, which expresses pancreatic CEL, showed that at 5–7.5 µg/ml of tunicamycin, there were 50% and 45% reductions in secreted enzyme activity and mass, respectively.

Previous studies of a number of glycoproteins have shown that N- or O-linked oligosaccharides can influence many different aspects of protein metabolism, such as intracellular sorting (19), stability (20), and biological function (21). Thus, it certainly remains possible that these post-translational modifications have important roles in aspects of CEL metabolism other than those investigated in the present studies. Future experiments will evaluate this possibility.

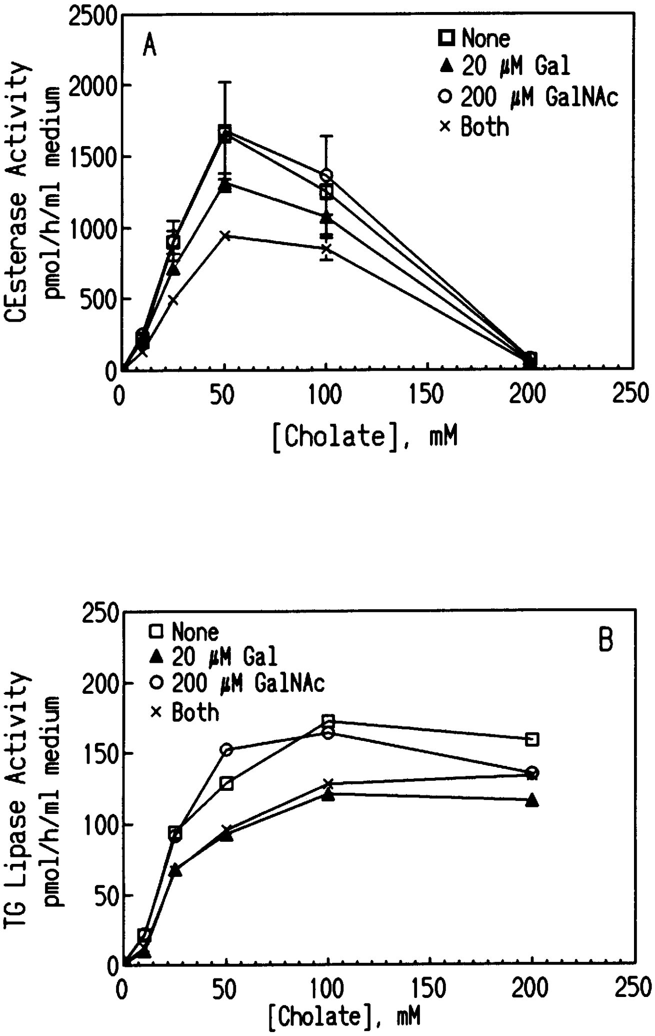

Figure 3. Effects of glycosylation on cholate activation of secreted CEL activity. CHO *ldlD* cells transfected with CEL cDNA were incubated for 6 hr in the presence of the indicated sugar supplements. Aliquots of conditioned media were withdrawn and cholate added to the concentrations indicated on the *x* axis. The hydrolysis of CE (Panel A) or TG (Panel B) by the conditioned media was then measured. Values are means ± SD, $n = 3$.

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1. Baba T, Downs D, Jackson KW, Tang J, Wang C. Structure of human milk bile salt activated lipase. Biochemistry **30**:500–510, 1991.
2. Wang C, Hartsuck JA. Bile salt-activated lipase. A multiple function lipolytic enzyme. Biochim Biophys Acta **1166**:1–19, 1993.
3. Camulli ED, Linke MJ, Brockman HL, Hui DY. Identity of a cytosolic neutral cholesterol esterase in rat liver with the bile salt stimulated cholesterol esterase in pancreas. Biochim Biophys Acta **1005**:177–182, 1989.
4. Hui DY, Wang Y. (abstract) Cholesterol esterase mediated uptake of high density lipoprotein-associated cholesteryl esters by HepG2 cells. Circulation **80**:II334, 1989.
5. Winkler KE, Harrison EH, Marsh JB, Glick JM, Ross AC. Characterization of a bile salt-dependent cholesteryl ester hy-

drolase activity secreted from HepG2 cells. Biochim Biophys Acta **1126**:151–158, 1992.

6. Zolfaghari R, Harrison EH, Ross AC, Fisher EA. Expression in Xenopus oocytes of rat liver mRNA coding for a bile salt-dependent cholesteryl ester hydrolase. Proc Natl Acad Sci USA **86**:6913–6916, 1989.
7. Jacobson PW, Wiesenfeld PD, Gallo LL, Tate RL, Osborne JC Jr. Sodium cholate-induced changes in the conformation and activity of rat pancreatic cholesterol esterase. J Biol Chem **265**:515–521, 1990.
8. Krieger M, Reddy P, Kozarsky K, Kingsley D, Hobbie L, Penman M. Analysis of the synthesis, intracellular sorting, and function of glycoproteins using a mammalian cell mutant with reversible glycosylation defects. Methods Cell Biol **32**:57–84, 1989.
9. Han JH, Stratowa C, Rutter WJ. Isolation of full-length putative rat lysophospholipase cDNA using improved methods for mRNA isolation and cDNA cloning. Biochemistry **26**:1617–1625, 1987.
10. Sambrook J, Fritsch EF, Maniatis T. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor, NY: Cold Spring Harbor Press, pp16.32–16.36, 1989.
11. Harrison EH. Bile salt-dependent, neutral cholesteryl ester hydrolase of rat liver: Possible relationship with pancreatic cholesteryl ester hydrolase. Biochim Biophys Acta **963**:28–34, 1988.
12. Schachter H, Roseman S. In: Lennarz WJ, Ed. The Biochemistry of Glycoproteins and Proteoglycans. New York: Plenum, pp85–160, 1980.
13. Kissel JA, Fontaine RN, Turck CW, Brockman HL, Hui DY. Molecular cloning and expression of cDNA for rat pancreatic cholesterol esterase. Biochim Biophys Acta **1006**:227–236, 1989.
14. Abouakil N, Mas E, Bruneau N, Benajiba A, Lombardo D. Bile salt-dependent lipase biosynthesis in rat pancreatic AR 4-2 J cells. J Biol Chem **268**:25755–25763, 1993.
15. Masuno H, Schultz CJ, Park JW, Blanchette-Mackie EJ, Mateo C, Scow RO. Glycosylation, activity and secretion of lipoprotein lipase in cultured brown adipocytes of newborn mice. Biochem J **277**:801–809, 1991.
16. Stahnke G, Davis RC, Doolittle MH, Wong H, Schotz MC, Will H. Effect of N-linked glycosylation on hepatic lipase activity. J Lipid Res **32**:477–484, 1991.
17. Matzuk M, Krieger M, Corless CL, Boime I. Effects of preventing O-glycosylation on the secretion of human chorionic gonadotropin in Chinese hamster ovary cells. Proc Natl Acad Sci USA **84**:6354–6358, 1987.
18. Hansson L, Bläckberg L, Edlund M, Lundberg L, Strömqvist M, Hernell O. Recombinant human bile salt-stimulated lipase. J Biol Chem **268**:26692–26698, 1993.
19. Korzarsky KF, Call SM, Dower SF, Krieger M. Abnormal intracellular sorting of O-linked carbohydrate-deficient interleukin-2 receptors. Mol Cell Biol **8**:3357–3363, 1988.
20. Collet X, Fielding CJ. Effects of inhibitors of N-linked oligosaccharide processing on the secretion, stability, and activity of lecithin:cholesterol acyltransferase. Biochemistry **30**:3228–3234, 1991.
21. Sly WS. In: Horowitz MI, Ed. The Glycoconjugates. New York: Academic Press, Vol **4**:3–25, 1982.