Phosphorylation of the Oncofetal Variant of the Human Bile Salt-dependent Lipase

IDENTIFICATION OF PHOSPHORYLATION SITE AND RELATION WITH SECRETION PROCESS*  

Received for publication, September 21, 2000, and in revised form, January 5, 2001  
Published, JBC Papers in Press, January 8, 2001, DOI 10.1074/jbc.M008658200

Alain Verine‡, Josette Le Petit-Thevenin, Laurence Panicot-Dubois, Annick Valette, and Dominique Lombardo  
From INSERM Unité 260, Faculté de Médecine, 27 Blvd. Jean Moulin, 13385 Marseille Cedex 5, France

In this paper, we report, for the first time, the localization of the phosphorylation site of the fetoacinar pancreatic protein (FAPP), which is an oncofetal variant of the pancreatic bile salt-dependent lipase. Using Chinese hamster ovary (CHO) cells transfected with the cDNA encoding FAPP, we radiolabeled the enzyme with 32P, and then the protein was purified by affinity chromatography on cholate-immobilized Sepharose column and submitted to a CNBr hydrolysis. Analysis of peptides by high pressure liquid chromatography, associated with the radioactivity profile, revealed that the phosphorylation site is located at threonine 340. Site-specific mutagenesis experiments, in which the threonine was replaced by an alanine residue, were used to invalidate the phosphorylation of FAPP and to study the influence of the modification on the activity and secretion of the enzyme. These studies showed that CHO cells, transfected with the mutated cDNA of FAPP, kept all of their ability to synthesize the protein, but the loss of the phosphorylation motif prevented the release of the protein in the extracellular compartment. However, the mutated enzyme, which was sequestered in the transfected CHO cells, remains active on bile salt-dependent lipase substrates.

The bile salt-dependent lipase (BSDL); EC 3.1.1.13) is an enzyme implicated in the duodenal hydrolysis of cholesteryl esters (1–4). This enzyme is found in the pancreatic secretions of all species examined up to now, from fishes to humans (5, 6). BSDL is synthesized within the endoplasmic reticulum of acinar cells and follows the secretory pathway of these cells before its release into the pancreatic juice. To be secreted, the enzyme experiences co- and post-translational modifications. The first one is the N-glycosylation at Asn187, which occurs in the endoplasmic reticulum (7). The second post-translational modification is the O-glycosylation of each tandemly repeated sequence present in the C-terminal domain of BSDL (8). During its intracellular traffic from the endoplasmic reticulum to the trans-Golgi network, BSDL is associated with intracellular membranes (9, 10), from which the enzyme is dissociated upon the phosphorylation of a hydroxylated amino acid residue of the protein, such as threonine or serine, by the action of a protein kinase casein kinase II (11). This third post-translational modification remains poorly documented, but it appeared essential to the secretion of the enzyme (12) and should occur once the sequential glycosylation of the protein was achieved. We have further determined that the stoichiometry of the phosphorylation is about 1.2 ± 0.5 mol of phosphorus/mol of secreted BSDL (12). Sequence comparison of BSDL (5, 13–16) indicated that this protein differs from species to species at the level of the C-terminal domain that encompasses a variable amount of tandemly repeated identical sequences. The number of these repeated sequences varies from none in salmon (5) up to 39 in gorilla (17). Furthermore, the fetoacinar pancreatic protein (FAPP) is a phosphorylated oncofetal variant of the human BSDL (18) that has only six repeated sequences instead of the 16 normally present in human enzyme (8). As a consequence of the variability of the C-terminal domain of the protein, the phosphorylation site should be located within the N-terminal domain of BSDL. Sequence analysis of BSDL, using the ExPASy Prosite program of the Swiss Institute of Bioinformatics, suggests that as many as eight putative CK II phosphorylation sites are present on BSDL sequence, all located within the N-terminal domain of the protein.2

The aim of these studies was to investigate the role of the phosphorylation step in the BSDL behavior and most particularly (i) to determine the nature and the location of the amino acid involved in phosphorylation process, (ii) to analyze the influence of the phosphorylation step vis-à-vis of the secretion. For this purpose, the phosphorylation site was invalidated by site-directed mutagenesis. Due to the low amount of identical motifs coding for repeated sequences of the protein, FAPP cDNA sequence was used instead of that of BSDL. The former cDNA would be easier to manipulate for site-directed mutagenesis experiments. Consequently, we used a vector including the cDNA encoding FAPP that leads to a secreted protein upon transfection in CHO cells (19).

EXPERIMENTAL PROCEDURES

Materials—Unless otherwise stated, all A grade chemicals were purchased from Sigma. Culture medium Ham F-12 was from Life Technologies, Inc. Taq polymerase was purchased from CLONTECH (Palo Alto, CA) and was a part of the GC-rich PCR kit. [32P]Orthophosphoric acid was from PerkinElmer Life Sciences. Polyclonal antibodies (pAbL64) against BSDL purified from human pancreatic juice were raised in our.

2 A. Verine, J. Le Petit-Thevenin, L. Panicot-Dubois, A. Valette, and D. Lombardo, unpublished observations.
laboratory in rabbit (20) and were purified on protein A-Sepharose. These antibodies also recognized FAPP (21).

Cell Cultures—Transfected CHO cells were routinely cultured in Ham’s F-12 medium, supplemented with 10% (by volume) fetal calf serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. Cells, in 100-mm tissue culture dishes, were maintained under 5% CO₂ atmosphere at 37 °C.

Transfection—Transcripts of FAPP, obtained by reverse transcriptase-PCR (19), were digested by HindIII and EcoRI restriction enzymes and ligated into pSecTag (Invitrogen), an expression vector that carries the V-J2-C region of the mouse Igκ chains driving expression of the FAPP cDNA in E. coli. The plasmid, referred to as pSec-FAPPw ("w" represents "wild"), was then transfected into CHO-K1 cell line using LipofectAMINE according to the manufacturer’s procedure (Life Technologies). Transfected cells were first stabilized in Ham’s F-12 medium supplemented with zeocin (500 μg/ml). The different clones were then isolated by end dilution procedure and maintained under zeocin selection for at least 6 weeks. Control cells were transfected, according to the same protocol, with the empty pSecTag vector, and corresponding positive clones (CHO-control) were selected as indicated previously.

Cell Protein Preparation—Transfected cells were grown to about 80% confluence, and then they were washed twice with incomplete PBS buffer (10 mM sodium phosphate buffer at pH 7.4 with 0.15 M NaCl and without Ca²⁺ and Mg²⁺ ions) and scraped with a rubber policeman. Cells were resuspended in this buffer and pelleted by low speed centrifugation. The supernatant was removed, and the sedimented cell pellet was homogenized in the complete PBS buffer (0.5 ml for cells obtained from a 100-mm diameter dish culture) by sonication (15 s, 4 watts, 4 °C). Homogenates were quickly cleared by centrifugation at 14,000 × g for 30 min at 4 °C, and the supernatants were immediately used for enzymatic assays or frozen and stored at −80 °C until use. Under these conditions, no loss of esterolytic activity was observed for at least 4 weeks.

Metabolic ³²P Labeling of FAPP and Purification on Cholate-immobilized Sepharose Affinity Column—CHO cells transfected with the cDNA of FAPP (19) were grown until 80% confluence in Ham’s F-12 medium. Cells were washed twice at room temperature with incomplete PBS and cultured for 3 h in phosphorus-free Ham’s F-12 medium. After this preincubation was used to deplete CHO cells of phosphorus, the medium was removed and replaced by 3 ml of the phosphorus-free fresh Ham’s F-12 medium in which was added 0.33 mCi/ml of sodium ³²P orthophosphate, and cells were incubated overnight at 37 °C. At the end of the incubation time, the cell culture medium, containing free radioactive and radiolabeled secreted proteins, was pelleted, and plasmid cDNA was isolated using a miniprep kit method (19). Under these conditions, no loss of esterolytic activity was observed for at least 4 weeks.

The ³²P-radiolabeled FAPP was then isolated from concentrated cell culture medium by affinity chromatography on a cholate-immobilized Sepharose Affinity Column—CHO cells transfected with the cDNA coding for FAPP (19). The enzyme was eluted by competition using the equilibrating buffer, supplemented with sodium cholate (2% w/v). Eluted material was then extensively dialyzed against cold ammonium hydrogenocarbonate solution (5 mM, pH 8.0) and finally concentrated by lyophilization. Unlabeled FAPP was also isolated according to the same protocol.

Cyanogen Bromide Digestion and Amino Acid Sequence Analysis—The purified radiolabeled material was mixed with 500 μg of pure nonradiolabeled enzyme, used as a vector, and dissolved into 0.2 ml of 70% formic acid. The reaction was started by adding CNBr (12 mg), and the mixture was incubated, under slow agitation, for 6 h at room temperature and for an additional 12 h at 4 °C. The reaction was stopped by adding 1 ml of distilled water and 0.2 ml of ethanol. The mixture was then evaporated to dryness under vacuo.

Peptide fragments were dissolved in 0.17 ml of guanidinium HCl (6 M) in 0.1% trifluoroacetic acid, were separated by HPLC using a C8 reversed-phase column and eluted with a gradient of acetonitrile from 0 to 100% in 0.1% trifluoroacetic acid. Peptide collection was monitored by recording the absorbance at 214 nm (Applied Biosystems absorbance detector model 785A). The radioactivity of each fraction was measured by liquid scintillation. The fraction, containing the radioactive peptide, was once again chromatographed under identical conditions using the same column and lyophilized, and its amino acid sequence was determined.

Enzyme Activity and Protein Determinations—Enzyme activity was determined on p-nitrophenyl hexanoate as already described (22) and standardized for purity as described previously. The specific activity was performed without (control) and with added bile salts (sodium taurocholate, 4 mM).

The lactate dehydrogenase activity was determined as described by Goldberg (23), and protein content was determined with the bicinchoninic acid test from Pierce using BSA as a standard.

SDS-PAGE—SDS-PAGE was performed in 10% polyacrylamide and 0.1% SDS as described by Laemmli (24), using a Bio-Rad Mini Protein II apparatus. After electrophoretic migration, proteins were electrotransferred onto nitrocellulose membranes at 4 mA/cm² for 18 h. The efficiency of the electrotransfer was checked by staining the nitrocellulose membrane with 2% Ponceau S solution.

Nitrocellulose membranes were air-dried, and transferred proteins were detected by autoradiography for 24 h at −70 °C (BioMax MR, Eastman Kodak Co.) and/or by immunodetection using polyclonal antibodies pAbL64 specific to human pancreatic BSDL/FAPP. Autoradiograms were analyzed by densitometric scanning and quantified using the NIH Image program (National Institutes of Health, Bethesda, MD).

For immunodetection assays, membranes were blocked for 1 h in Tris-HCl buffer (5 mM, pH 8.0) containing 150 mM NaCl and 3% bovine serum albumin. The immunodetection was carried out for 1 h using pAbL64 (1 μg/ml). After incubation for 1 h in blocking buffer containing 0.05% Tween 20, membranes were rinsed and incubated for another 1 h in a solution containing alkaline phosphatase-conjugated goat anti-rabbit IgG. After several washings with PBS supplemented with 0.05% Tween 20, membranes were developed for 10 min with a mixture of nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (0.5 mM each) in 0.1 M Tris/HCl buffer (pH 9.5), 100 mM NaCl, and 1 mM MgCl₂.

Site-directed Mutagenesis—The pSec-FAPPw transcript, initially used for the transfection of the CHO cells with the cDNA coding for FAPP (19), was subcloned in Escherichia coli cells and mutated to prevent the expression of the threonine residue bearing the phosphoryl motif.

For this purpose, a pair of primers, designed to cover the sequence encoding this threonine, was used according to the method described by Ansaldi et al. (25). Their sequences were modified to replace the threonine codon (ACG) with the alanine codon (GCC). The two primers had the following sequences: 5′-AAC AAG GCC GAC AAG AAA GTA GGC GAG-3′ and 5′-CAT TCC GAG CAG-3′. The two primers had the following sequences: 5′-AAC AAG GCC GAC AAG AAA GTA GGC GAG-3′ and 5′-CAT TCC GAG CAG-3′. Underlined letters indicate the modified bases. The oligonucleotide primers, each complementary to opposite strands of the pSec-FAPPw vector, were extended using the GC-rich PCR kit from CLONTECH. The amount of the initial substrate was maintained below 100 ng. The amplification was performed on a PerkinElmer Life Sciences 2400 GeneAmp PCR system using a 15-mer oligonucleotide primer pair as described previously. An annealing temperature of 52 °C, 0.5 min, and extension (68 °C, 4 min) and the reaction was terminated by an incubation at 68 °C for 8 min. A treatment with DpnI endonuclease was used to eliminate the methylated parental DNA template. After digestion by DpnI, only the newly synthesized DNA containing the desired mutation remained. About 5 μl of the digested DNA was used to transform competent E. coli cells (Top10 F strain), which were spread and incubated at 37 °C overnight on agarose plates containing the appropriate antibiotic (ampicillin) and the isopropyl-1-thio-β-D-galactopyranoside/5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside mixture to perform the blue/white selection. The white and blue colonies were picked and cultured in Luria-Bertani medium supplemented with 50 μg/ml ampicillin. Cells were then pelleted, and plasmid DNA was isolated using a miniprep kit method (Qiagen). The presence of the desired PCR product within the plasmid was checked by digestion with NotI restriction enzyme. Positive plasmids were totally sequenced to detect any undesired mutation within the FAPP cDNA. The plasmid, bearing the desired mutation, was then transfected into CHO-K1 cells and selected, as described previously.
supplemented with 0.5% Triton X-100 and 6-fold with TBE buffer alone. Sepharose beads were sedimented and then added to 30 μl of Laemmlis sample buffer and boiled for 3 min. After a rapid centrifugation, the supernatant was submitted to SDS-PAGE. Proteins were transferred onto nitrocellulose membranes. Each assay was performed in duplicate on the same nitrocellulose membrane, which was divided into two parts. Proteins present on the first half of the membrane were revealed by Western blot using pAbL64 as primary antibody. The second half was treated for 6 h at 37°C with 0.1 unit/ml of neuraminidase (Sigma) in acetate buffer (100 mm, pH 5.2) and probed with biotin-conjugated peanut agglutinin *Arachis hypogaea* (PNA) lectin (10 μl/ml) and antibodies to biotin linked to alkaline phosphatase and developed as Western blottings.

### RESULTS

**Localization of the Phosphorylated Site of FAPP**

Proteins of CHO cells transfected with the pSecFAPPw vector were metabolically labeled with [32P]orthophosphoric acid. After an overnight incubation, the cell-free medium containing about 1.1 ± 0.4 mg (n = 32) of radioactive protein was recovered. The radiolabeled secreted [32P]FAPP was purified by affinity chromatography. After exhaustive washings, only 2% of the total loaded radioactive material was eluted with competitive sodium cholate (−40 μg of [32P]-labeled FAPP). The eluted material was then analyzed, and after SDS-PAGE followed by electrotransfer onto nitrocellulose membrane, only one band can be detected after Ponceau S development or autoradiography. This protein is associated with a molecular mass of 78 kDa, which correlates with that of FAPP (19). After concentration by lyophilization, the affinity-purified [32P]FAPP was subjected to CNBr hydrolysis. When the digested peptides were separated by reverse phase HPLC, the elution profile revealed many peaks (Fig. 1). Major peaks, detected by UV absorption at 214 nm, were eluted near the end of the gradient under weak polarity conditions. The radioactivity of each fraction was quantitated, and the radioactivity profile was superimposed to the peptide elution pattern. It appeared that the radioactivity was mainly recovered under a single peak, associated with the fraction number 47 (Fig. 1, arrow). Several analyses were performed, under the same chromatographic conditions, and all fractions, containing the radioactive peptide, were pooled and once again chromatographed on the same HPLC column. This allows to collect enough radioactive material for further sequencing. One major sequence of 16 amino acids, PAINKGNKKVTEEDFY, was determined. Comparison of this sequence with the amino acid sequence deduced from the FAPP cDNA (19) or BSDL cDNA (8), revealed a high degree of homology with the sequence PAINKGNKKVTEEDFY, located between Pro330 and Tyr345 in the N-terminal domain of FAPP and BSDL. The missing amino acid residue, X, in the sequence of the radioactive peptide can be identified as Thr340, which therefore could represent the phosphorylation site. Furthermore, the sequence around this Thr residue is representative of a consensus motif that can be phosphorylated by a protein kinase casein kinase II (11). This motif is present in the BSDL sequence of all species examined up to now except salmon enzyme (16) and in that of FAPP (19). Amino acid numbering is given according to Reue et al. (8).

**Site-directed Mutagenesis**

The definitive proof that Thr340 is the phosphorylation site of FAPP was obtained by site-directed mutagenesis. In this experiment, a pair of primers was designed to create a mutation at the threonine 340 codon. The pair of primers was used in a PCR experiment to amplify the pSecFAPPw vector and to replace the threonine 340 residue by alanine. At the end of the PCR experiment, the methylated parental vector was digested by DpnI. The remaining material was used to transform the Top 10 F’ *E. coli* strain to obtain enough material for sequencing and expression in CHO cells. The sequence of the vector does not differ from that given by the manufacturer. The sequence of the reverse transcriptase-PCR fragment inserted into the vector matches that of FAPP (19), except at the level of the codon encoding Thr340 (not shown). This mutated vector will be referred to as pSecFAPPT340A. CHO cells were then stably transfected with the pSecFAPPw, pSecFAPPT340A, and empty pSecTag vector. After selection in zeocin, the transfected cells were cultured until confluence, and the expression of FAPP was determined by Western blotting performed on cell lysates. As many as 12 pSecFAPPw and pSecFAPPT340A clones were selected in the presence of zeocin, and all expressed FAPP, which migrates as a 78-kDa protein. However, cells transfected with the empty pSecTag did not express the protein (Fig. 2, upper panel). A clone representative of each transfection experiment was selected to give CHO-FAPPw, CHO-FAPPT340A and CHO-control cell clones, respectively. As also shown on Fig. 2 (lower panel), FAPP expressed either by CHO-FAPPw or CHO-FAPPT340A is quantitatively retained on, and conse-
Orthophosphate-supplemented medium. At the end of the FAPP expression, CHO-control cells were radiolabeled in the N-terminal domain of FAPP. CHO-FAPPw, CHO-FAPPT340A, and CHO-control clones. These clones were allowed to grow and finally lysed. Cell lysates were then separated on SDS-PAGE (60 μg of cell protein/lane) and analyzed by Western blotting using antibodies to human BSDL. Alternatively, FAPP expressed by either clone was purified by affinity chromatography on a cholate-immobilized Sepharose column. For this purpose, 250 μl of cell lysate was loaded onto the gel and incubated overnight at 4 °C under gentle agitation. Unbound proteins were then washed with the loading buffer (25 mM Tris-HCl, pH 9.0, buffer, 2 mM EDTA, and 1 mM benzamidine), and then bound material was eluted with the loading buffer supplemented with 2% (w/v) sodium cholate. Bound and unbound proteins were then concentrated and analyzed by SDS-PAGE and Western blotting.

Fig. 2. Expression of FAPP by CHO-FAPPw, CHO-FAPPT340A, and CHO-control clones. Cho cells transfected with pSecFAPPw, pSecFAPPT340A, or the empty vector were selected to give CHO-FAPPw, CHO-FAPPT340A, and CHO-control clones. These clones were allowed to grow and finally lysed. Cell lysates were then separated on SDS-PAGE (60 μg of cell protein/lane) and analyzed by Western blotting using antibodies to human BSDL. Alternatively, FAPP expressed by either clone was purified by affinity chromatography on a cholate-immobilized Sepharose column. For this purpose, 250 μl of cell lysate was loaded onto the gel and incubated overnight at 4 °C under gentle agitation. Unbound proteins were then washed with the loading buffer (25 mM Tris-HCl, pH 9.0, buffer, 2 mM EDTA, and 1 mM benzamidine), and then bound material was eluted with the loading buffer supplemented with 2% (w/v) sodium cholate. Bound and unbound proteins were then concentrated and analyzed by SDS-PAGE and Western blotting.

Threonine 340 is the Phosphorylation Site of BSDL—By using the chemical method, we have previously shown that 1.2 ± 0.5 mol of phosphorus was present per mol of BSDL. If this result is very interesting, it may appear also somewhat ambiguous and may indicate the presence of one or perhaps two phosphoryl group(s) on FAPP. The addition of sodium [32P]orthophosphate in culture medium has been used to confirm that threonine 340 is the unique phosphorylatable site on the N-terminal domain of FAPP. CHO-FAPPw, CHO-FAPPT340A, and CHO-control cells were radiolabeled in [32P]orthophosphate-supplemented medium. At the end of the incubation, cell-free medium was withdrawn, dialyzed, and concentrated, whereas cells were harvested and lysed. Same amounts of proteins, contained in the cell-free medium and in the cleared cell lysate, were loaded on the cholate-immobilized Sepharose column. After washing of the affinity gel, retained FAPP was eluted from beads by boiling in Laemmli sample buffer and separated on SDS-PAGE, electrotransferred on nitrocellulose membrane. The nitrocellulose membrane was dried and autoradiographed overnight at -70 °C. The left and right panels show the cell-free medium and the cleared cell lysate, respectively. Analyses were performed using material isolated from CHO cells transfected with empty pSecTag vector (CHO-control), with cDNA of FAPP (CHO-FAPPw), and with cDNA of FAPP where the threonine 340 was mutated (CHO-FAPPT340A).

The Phosphorylation of Threonine 340 Is Essential for the Secretion of FAPP—As shown above, phosphorylated FAPP cannot be detected in cell-free medium of CHO-FAPPT340A cells. This result indicated that FAPP secretion could be dependent upon phosphorylation. Therefore, the influence of the phosphorylation of the threonine 340 on the secretion of FAPP was analyzed by immunodetection of the protein in cell-free medium of CHO-FAPPw and CHO-FAPPT340A cells. For this purpose, nitrocellulose membranes, used in Fig. 3 to determine the phosphorylation state of FAPP and FAPPT340A, were submitted to an immunodetection with pAbL64. As shown on Fig. 4, left panel, no band was immunodetected in the cell-free medium of CHO control transfected with the empty pSecTag vector. However, a protein, migrating at 78 kDa and reactive with pAbL64, was isolated by affinity chromatography on the cholate-immobilized Sepharose column from the cell-free medium of the CHO-FAPPw clone, which ascertained that this clone had the capacity to synthesize and secrete FAPP. On the other hand, mutated FAPP, expressed by the CHO-FAPPT340A clone, cannot be immunodetected in the cell-free medium of this clone. This suggests that mutated FAPP was absent of the cell-free medium of CHO-FAPPT340A cells and, consequently, cannot be isolated by affinity chromatography from this medium. A Western blotting performed directly on the cell-free medium of CHO-FAPP and CHO-FAPPT340A cells indicated that the protein can be detected in the culture medium of the former cells and was absent in that of the latter clone (see Fig. 2).

Cell homogenates showed a pattern somewhat different (Fig.
Phosphorylation of FAPP

Fig. 4. Immunodetection of enzyme in the intra- and extracellular medium of transfected CHO cells. The nitrocellulose membrane used for autoradiography in Fig. 3 was used for immunodetection using pAbL64, specific antibodies to the human BSDL. The presence of the protein was detected in CHO cells transfected with empty pSecTag vector (CHO-control), with cDNA of normal FAPP (CHO-FAPPw), and with cDNA of FAPP where the phosphorylation site threonine 340 was mutated (CHO-FAPPT340A), respectively.

4, right panel); as expected, no band corresponding to FAPP was immunodetected in CHO-control cells. However, following affinity chromatography, FAPP can be immunodetected as a doublet of protein migrating around 78 kDa in CHO-FAPPw and CHO-FAPPT340A clones. This doublet probably corresponds to different states of maturation of the glycosylation of this protein (10). Another band was immunodetected in the lower molecular mass range and might correspond to a degradation product. When the amount of FAPP expressed by each clone was quantitated by densitometric scanning, it appeared that the amount of FAPP expressed by the CHO-FAPPT340A clone was decreased by some 40% when compared with that expressed by the CHO-FAPPw clone. All of these results indicate that FAPPT340A, which cannot be phosphorylated, might not be secreted by CHO-FAPPT340A cells.

FAPP Expressed by CHO-FAPPT340A Remains Active—The levels of esterase activity were determined in the intra- and extracellular media obtained from CHO-FAPPw and CHO-FAPPT340A cells (Table I). In cell culture medium of CHO-FAPPw, once corrected for the esterase activity present in culture medium of CHO control cells, the esterase activity was about 10-fold the level recorded in the cell-free medium of CHO-FAPPT340A cells. The amount of BSDL activity detected in cell-free medium of CHO-FAPPT340A cells represents some 3.5 ± 1.3% of the total intracellular and extracellular activities. This value is close to that of lactate dehydrogenase activity present in the cell-free medium of CHO-FAPPT340A cells that accounts for 4.4 ± 2.1% of the total lactate dehydrogenase activity. Consequently, BSDL activity detected in cell culture medium of the CHO-FAPPT340A clone might be due to some cell lysis. Furthermore, the amount of BSDL activity recorded in cell culture medium of CHO-FAPPw clone is close to that already found (19). Thus, it is clear that the invalidation of the phosphorylation site of FAPP inhibited the release of T340A mutated protein in the cell-free medium.

In the cleared cell homogenate of CHO cells either transfected with pSecFAPPw or with pSecFAPPT340A vectors, a significant level of bile salt-dependent lipase activity can be detected (Table I). However, a lower activity, compared with that of CHO-FAPPw clone, was detected in the homogenate of the CHO-FAPPT340A clone. This lower activity could be due to the expression of a partially inactive enzyme, consecutive to the mutation of the phosphorylation site. However, the decrease in activity of some 40% observed, after correction with the control clone activity, paralleled the decrease in expression level of the mutated protein by the CHO-FAPPT340A clone (see Fig. 4). From this observation, it can be concluded that FAPP and mutated FAPP displayed the same specific activity. Consequently, the activity of FAPP did not depend upon phosphorylation of the threonine 340 residue. Furthermore, the mutation T340A did not alter the activity of the enzyme and its modulation by bile salts.

FAPPT340A Is O-Glycosylated—We have shown that, during its maturation, BSDL was successively N- and O-glycosylated. We have further hypothesized that O-glycosylation of the protein masks PEST sequences (Pro-, Glu-, Ser-, and Thr-rich sequences) that are a signal for rapid degradation (26), and, in fine, this glycosylation, regulates the secretion of the enzyme (27). The protein that is not accurately glycosylated should be degraded. Nevertheless, in this maturation process, the timing of phosphorylation step with regard to the glycosylation remained uncertain. To clarify this point, we examined the glycosylation state of mutated FAPP. For this purpose, the mutated protein produced by the CHO-FAPPT340A clone was isolated by a cholate-immobilized Sepharose column. The retained material was then submitted to SDS-PAGE, transferred onto nitrocellulose membrane, and probed with pAbL64 and with biotin-labeled PNA lectin. As shown in Fig. 5, pAbL64 and PNA lectin detected material migrating with the same molecular mass suggesting that, albeit not phosphorylated, mutated FAPP is O-glycosylated.

This result may be interpreted in two ways; first, phospho-

![Table I](image)

**Table I. Effect of mutagenesis on esterase activity**

Transfected CHO cells were grown overnight in a medium without calf fetal serum. At the end of the incubation time, the cell-free medium was removed. The cells were harvested and lysed. Cell lysate was cleared by centrifugation. Activity levels were recorded as indicated. Values are means ± S.D. of at least three independent experiments. Controls represent CHO cells transfected with the empty vector. Corrected values correspond to the difference between raw and control values.

| Esterase activity     | Raw  | Corrected |
|-----------------------|------|-----------|
| Extracellular activities | 10⁻³ units/mg cell protein | 10⁻³ units/mg cell protein |
| CHO-control           | 6.7 ± 0.7 | 5.6 ± 0.6 |
| CHO-FAPPw             | 1.6 ± 0.5 | 0.5 ± 0.2 |

**Fig. 5. Glycosylation of mutated FAPP.** Mutated FAPP from CHO-FAPPT340A was extracted, from cleared cell homogenate, by affinity on cholate-immobilized Sepharose beads. After several washings, Sepharose beads were added to Laemmli buffer and boiled. After centrifugation, proteins present in the supernatant were loaded on SDS-PAGE and transferred onto nitrocellulose membrane. The membrane was divided into two identical parts. The first half (A) was revealed with BSDL antibodies (pAbL64), and the second half (B) was probed with biotin-labeled PNA lectin and alkaline phosphatase-conjugated antibodies to biotin.
Phosphorylation of FAPP

Phosphorylation occurs after the O-glycosylation of the protein, and second, O-glycosylation may proceed independently of the phosphorylation.

DISCUSSION

Recent studies (9, 10) on bile salt-dependent lipase have demonstrated that, during its secretion route in the pancreatic cell, the enzyme is associated with intracellular membranes. This association allowed a correct and complete glycosylation of the enzyme or participated in the inhibition of the enzyme activity that could be harmful to the cell. Therefore, it has been shown that the enzyme is phosphorylated by a protein kinase cascade. For instance, it is known that this protein kinase system was insensitive to protein kinase A, C, and G inhibitors. The phosphorylation process is favored by okadic acid, which is known to inhibit protein phosphatases 1 and 2A. Nevertheless, the phosphorylation of BSDL was inhibited by the 5,6-dichloro-1β-d-ribofuranosylbenzimidazole, a specific inhibitor of protein casein kinase II. This impairment of BSDL phosphorylation is associated with a significant decrease of enzyme secretion (11). We have further shown that the phosphorylation process appeared as an essential step for the dissociation of the enzyme from intracellular membranes (12). The importance of this step is ascertained by the fact that there is only [1.2 ± 0.5 mol of phosphorus/mol of enzyme. The nature of the amino acid bearing the phosphorylation motif remained unknown and might be a threonine or serine residue (11). On BSDL, and on its oncotic variant FAPP, eight putative casein kinase II phosphorylation sites were located at the N-terminal domain of the protein. Despite this important number of potential sites, it appeared that only one site could be phosphorylated and sufficient for the secretion of the enzyme (12). In the present study, we show that the threonine residue, at position 340, is the phosphorylation site of BSDL. The replacement of this threonine residue by an amino acid of similar size did not prevent the expression of enzyme, albeit that it cannot be phosphorylated. It appeared that invalidation of the phosphorylation site at Thr340 preserved the esterase activity of BSDL but abolished the secretion of the enzyme. All of these data confirm our previous studies that have proposed that the release of BSDL from intracellular membranes was consecutive to its phosphorylation (11), an event that is essential to the enzyme secretion. A direct link between phosphorylation and enzyme secretion is established in this study. It appeared now that, before its secretion, the enzyme must be successively N-, trans-glycosylation, probably in the trans-Golgi network, and O-glycosylated and phosphorylated. The question that now arises is how these different processes are organized in time. This point may be resolved by the study of O-glycosylation of the mutated FAPP. Our results, showing the presence of O-glycosylated motifs on mutated FAPP, and the demonstration that the phosphorylation occurs in a genistin-sensitive compartment, which could be the trans-Golgi network (11), and that this modification also releases the enzyme from intracellular membranes (11, 12) strongly suggest that the phosphorylation step takes place after glycosylation processes. Consequently, phosphorylation could be the ultimate step driving the enzyme toward the secretion pathway. One possibility is that only secretion-competent BSDL molecules are phosphorylated and targeted toward secretion, whereas others could be degraded.

It has been shown that the inhibition of the phosphorylation of BSDL by genistin induces an accumulation of enzyme in a cell compartment where it was colocalized with the 58-kDa Golgi protein (11). Confocal microscopy suggests that the cellular retention compartment may be the endoplasmic reticulum (ER) of human pancreatic tumoral cells (30). However, FAPP expressed by these latter cells carries out the glycine oncotic J28 epitope. The building of this epitope requires fucosyltransferase activities located in the trans-Golgi compartment (31). Furthermore, FAPP is phosphorylated. In this study, we suggested that the phosphorylation of FAPP (and probably that of BSDL) occurred after O-glycosylation, probably in the trans-Golgi network, where the protein should accumulate upon phosphorylation deficiency. This apparent discrepancy may be explained by a retrograde transport pathway of the nonphosphorylated BSDL from the trans-Golgi network back to the ER. We have, recently, shown that BSDL secretion is depending upon Rab6 cycling (28). The fact that Rab6 retrieves secretory cargo vesicles from the trans-Golgi back to the ER (29) supports the possible retrograde transport of nonphosphorylated BSDL to ER. This route may be used to transport nonphosphorylated BSDL molecules back to the ER, where the enzyme could be degraded by the proteasome machinery (32).

Acknowledgments—We thank M. Bain for expert assistance and fruitful discussions and Dr. E. Pasqualini for the release of the pSec-FAPP construct.

REFERENCES

1. Lindstrom, M. B., Sternby, B., and Bogrstro ¨m, B. (1988) Biochim. Biophys. Acta 959, 178–184
2. Lombardo, D., and Guy, O. (1980) Biochim. Biophys. Acta 611, 147–155
3. Howles, P. N., Carter, C. P., and Hui, D. Y. (1996) J. Biol. Chem. 271, 7196–7202
4. Shamir, R., Johnson, W. J., Zolfaghari, R. L., Lee, H. S., and Fisher, E. A. (1995) Biochemistry 34, 6531–6538
5. Gjellesvik, D. R., Lombardo, D., and Walthier, B. T. (1992) Biochim. Biophys. Acta 1124, 123–134
6. Lombardo, D., Guy, O., and Figarella, C. (1978) Biochim. Biophys. Acta 527, 142–149
7. Sugio, T., Mas, E., Abouakil, N., Endo, T., Etsrabo, M. J., Kobata, A., and Lombardo, D. (1993) Eur. J. Biochem. 216, 799–805
8. Reue, K., Zambaux, J., Wang, H., Lee, G., Leete, T. H., Honk, M., Shively, J. E., Sternby, B., Borgstrom, B., Ameis, D., and Schotz, M. C. (1991) J. Lipid Res. 32, 267–276
9. Bruneau, N., and Lombardo, D. (1995) J. Biol. Chem. 270, 13524–13533
10. Bruneau, N., Leech n de la Porte, P., Sharra, V., and Lombardo, D. (1995) Eur. J. Biochem. 233, 209–218
11. Pasqualini, E., Caillol, N., Valette, A., Lloubes, R., Verine, A., and Lombardo, D. (2000) Biochem. J. 345, 121–128
12. Pasqualini, E., Caillol, N., Mas, E., Bruneau, N., Lloubes, R., and Lombardo, D. (1997) Biochem. J. 327, 527–535
13. Han, J. H., Stratowa, C., and Rutter, W. J. (1987) J. Biol. Chem. 262, 1617–1625
14. Kyger, E. M., Wiegen, R. C., and Lange, L. G. (1989) Biochim. Biophys. Res. Commun. 164, 1302–1309
15. Colwell, N. S., Alemam-Gomez, J. A., and Kumar, B. V. (1993) Biochim. Biophys. Acta 1172, 175–180
16. Bruneau, N., Vabre, N., Mas, E., Hamosh, M., Lombardo, D., and Hamosh, P. (1998) Biochim. Biophys. Acta 1393, 80–89
17. Madeszky, K., Lidberg, U., Bjursell, G., and Nilson, J. (1999) Gene (Amst.) 239, 273–282
18. Etsrabo, M. J., and Imperial, S. (1989) J. Biol. Chem. 264, 21865–21871
19. Pasqualini, E., Caillol, N., Panicot, L., Mas, E., Lloubes, R., and Lombardo, D. (1998) J. Biol. Chem. 273, 28208–28218
20. Abouakil, N., Rogalska, E., Bonize, J., and Lombardo, D. (1988) Biochim. Biophys. Acta 961, 299–308
21. Mas, E., Abouakil, N., Roudani, S., Milla lles, F., Guy-Crotte, O., Figarella, C., Etsrabo, M. J., and Lombardo, D. (1995) Biochem. J. 299, 609–615
22. Verine, A., Bruneau, N., Valette, A., Le Petit-Thervenin, J., Pasqualini, E., and Lombardo, D. (1999) Biochim. Biophys. Acta 1424, 179–187
23. Goldberg, E. (1972) J. Biol. Chem. 247, 2044–2048
24. Laemmli, U. K. (1970) Nature 227, 680–685
25. Ansalldi, M., Lepelleterier, M., and Mejean, V. (1996) Anal. Biochem. 234, 110–111
26. Reichstein, M., and Rogers, S. W. (1996) Trends Biochem. Sci. 21, 267–271
27. Bruneau, N., Nganga, A., Fisher, E. A., and Lombardo, D. (1997) J. Biol. Chem. 272, 27633–27641
28. Caillol, N., Pasqualini, E., Lloubes, R., and Lombardo, D. (2000) J. Cell. Biochem. 79, 626–647
29. White, J., Jacques, L., Mallard, F., Gerot, A., Grill, S., Reinsch, S., Keller, P., Tschaschel, B., Ehrard, A., Goud, B., and Stelzer, E. H. K. (1999) J. Cell Biol. 147, 743–759
30. Milla lles, F., Langa, F., Mazo, A., and Etsrabo, M. J. (1993) Eur. J. Cell Biol. 61, 115–121
31. Panicot, L., Mas, E., Pasqualini, E., Zerfaoui, M., Lombardo, D., Sadoulet, M-O., and El Battari, A. (1999) Glycobiology 9, 935–946
32. Le Petit-Thervenin, J., Verine, A., Nganga, A., Nohiti, O., Lombardo, D., and Bruneau, N. (2001) Biochim. Biophys. Acta 1530, 184–198