"Adult Only" Esterase/Phospholipase A of the Small-Intestine Brush Border Membrane: Isolation, Identification of the Catalytic Site, and Biosynthesis[†,‡]

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Received September 23, 1996; Revised Manuscript Received December 11, 1996[⊗]

ABSTRACT: We have isolated and investigated an esterase/phospholipase A (EC 3.1.1) which is an intrinsic protein of the small-intestine brush border membrane of adult but not of preweaning rabbits. This enzyme had been referred to previously as AdRabB [Boll, W., Schmid-Chanda, T., Semenza, G., & Mantei, N. (1993) *J. Biol. Chem. 268*, 12901−12911]. Its amino acid sequence shows four extensive, homologous repeats between the signal sequence and a hydrophobic stretch in the C-terminal region. We have identified a serine residue (Ser^{400}) in the (unexpectedly) *single* catalytic site and indicate that this esterase is likely to operate by way of a Ser-His-Asp/Glu triad. This esterase/phospholipase A is synthesized as a single polypeptide chain of 170−180 kDa, agreeing well with the size deduced from its cognate cDNA sequence [Boll, W., et al. (1993) *J. Biol. Chem. 268*, 12901−12911], and it undergoes (at least) *N*-glycosylation. Once it has reached the brush border membrane, it is subjected in vivo to two types of proteolytic processing, presumably by pancreatic protease(s): a split after Arg^{263}, with loss of the first N-terminal repeat, and two or more cleavages much farther downstream but still located in the luminal bulk of the protein; these splits lead to multiple bands of approximately 140, 125, 100, and 90 kDa.

Recently, we have reported a previously unrecognized esterase/phospholipase of the small intestinal brush border membrane (Boll et al., 1993). The approach we used was unusual in the sense that we constructed a differential cDNA library, adult minus baby rabbit small intestine, and isolated and identified a number of clones. One of these clones (AdRabB) was found to correspond to a protein of unexpected structure; i.e., it had a conspicuously long 4-fold repeat between the presumed N-terminal signal sequence (cleavage "score" of 8.2) and the predicted C-terminal membrane anchor, followed by a short cytosolic stretch. This unusual structure, presumably arising from two cycles of partial gene duplication, reminded us of that of pre-pro-lactase-phlorizin hydrolase (Mantei et al., 1988).

The sequence determined had no homology with any other in either nucleic acid or protein sequence data banks. The protein isolated had esterase/phospholipase A activity. In our previous publication, this enzyme was named AdRabB, for protein encoded by the differential cDNA clone B, present in adult but not in baby rabbit small intestine. In the present paper, it will be referred to as esterase/phospholipase A (EC 3.1.1) and abbreviated as Estr/PLase.[1]

In the following, we report the localization of the active site. We find that one single serine residue reacts with DFP with complete loss of the enzymatic activities. A single DFP molecule is incorporated per polypeptide chain, strongly suggesting that this protein, in spite of its extensive 4-fold repeats, has only one single functional active site.

Further, we have studied the in vitro biosynthesis of this enzyme in small-intestine explants and in transfected COS 7 cells and compared the band patterns obtained with that of the enzyme as prepared from the brush border membranes. We find that the biosynthesis product has a size corresponding to that predicted from the cognate cDNA, indicating the absence of intracellular proteolytic processing [compare instead that of the other known 4-fold repeat polypeptide, pro-LPH, which is processed intracellularly to "mature" LPH corresponding to only 60% of the size of pro-LPH (Mantei et al., 1988; Danielsen et al., 1984; Büller et al., 1987; Lottaz et al., 1992; Keller et al., 1992, 1995)]. Estr/PLase, as isolated from the brush border membrane, shows instead a more complicated band pattern, indicating that it undergoes proteolytic processing after it has reached the brush border membrane, in vivo, presumably by luminal pancreatic proteases.

MATERIALS AND METHODS

Methionine-free RPMI1640 medium was purchased from Amimed (Muttenz, Switzerland); [^{35}S]methionine (in vivo cell-labeling grade) was from Amersham (Amersham, U.K.), and protein A-Sepharose CL4B and CNBr-activated Sepharose 4B were from Pharmacia (Dübendorf, Switzerland). Cell

† The financial support of the Wolfermann-Nägeli-Stiftung, Zurich, and of the Swiss National Science Foundation, Berne, is gratefully acknowledged.

‡ Dedicated to Prof. D. Seebach on the occasion of his 60th birthday.

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⊗ Abstract published in *Advance ACS Abstracts,* March 1, 1997.

[1] Abbreviations: BNPS-skatole, 2-[2-(nitrophenyl)sulfenyl]-3-methyl-3′-bromoindolenine; DFP, diisopropyl fluorophosphate; DMEM, Dulbecco's modified Eagle's medium; DOC, sodium deoxycholate; Estr/PLase, esterase/phospholipase A (EC 3.1.1); FCS, fetal calf serum; LPH, lactase-phlorizin hydrolase; SI, sucrase-isomaltase; MEM, minimum essential medium (Eagle's) with Earle's salts; NP-40, Nonidet P40; PBS, phosphate-buffered saline; PMSF, phenylmethanesulfonyl fluoride; PVDF, poly(vinylidene difluoride); SDS−PAGE, SDS−polyacrylamide gel electrophoresis; Tricine, *N*-[tris(hydroxymethyl)-methyl]glycine.

S0006-2960(96)02398-7 CCC: $14.00

culture media were from Gibco BRL (Life Technologies, Basel, Switzerland). All other chemicals were from Fluka (Buchs, Switzerland) and were of the highest possible purity. Polyclonal antibodies against LPH, SI, and Estr/PLase were prepared as previously described: W905D (anti-rabbit LPH; Wacker et al., 1992), MS72 (anti-rabbit SI; Wacker et al., 1981), Gst AdRabB5 (anti-rabbit Estr/PLase amino acids 39−178; Boll et al., 1993), and AdRabB6-MBP (anti-rabbit Estr/PLase amino acids 703−1138; Boll et al., 1993).

Organ Culture. Explants from the small intestine of 6-month-old male New Zealand white rabbits were cultured as previously described (Keller et al., 1992). The tissue pieces were pulse labeled for 60 min at 37 °C in labeling medium (methionine-free RPMI1640 medium supplemented with 10% dialyzed and heat-inactivated FCS) containing 150 μCi [^{35}S]methionine and then chased for 1 or 5 h at 37 °C in the same medium, supplemented with 2.5 mM cold methionine. After being rinsed with 25 mM Tris (pH 8.0) and 50 mM NaCl, the tissue pieces were directly used for immunoprecipitations in the presence of protease inhibitors (see below) as described in Lottaz et al. (1992).

Transient Transfection of COS 7 Cells. COS 7 cells (cultured in DMEM/10% FCS) were transiently transfected with the plasmid pSCT-AdRabB (Boll et al., 1993) as described by Chen and Okayama (1987). One day before transfection, the cells were seeded into six-well plates (10 cm^2/well) so that they were approximately 80% confluent the next day. During the last 30 min prior to transfection, the cells were incubated in 1.5 mL of fresh DMEM/10% FCS and 3.5% CO_2 at 37 °C. The calcium phosphate/DNA mixture was prepared by mixing 5 μg of plasmid DNA with 100 μL of 250 mM $CaCl_2$ and 100 μL of 2X BBS [50 mM *N*,*N*-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid (BES), 280 mM NaCl, and 1.5 mM Na_2HPO_4, with NaOH adjusted to exactly pH 6.95] and incubating for 10−15 min at room temperature. This mixture (150 μL) was then slowly added to the cells, and incubations were continued for 24 h with 3.5% CO_2 and at 37 °C. On the next day, the cells were washed twice with PBS, re-fed with 2 mL of fresh medium, and incubated for 24 h with 5% CO_2 at 37 °C.

For immuno-overlays (see below), the cells were collected 48 h after transfection by detachment with a rubber policeman. They were solubilized in 5X Laemmli sample buffer, boiled for 2 min, and then used for SDS−PAGE analysis (Laemmli, 1970).

For metabolic labeling studies (48 h after transfection), the cells were washed with MEM and then incubated in 2 mL of labeling medium (MEM/10% dialyzed FCS) for 60 min with 5% CO_2 at 37 °C. After depletion of methionine, the cells were pulse labeled for 3 h in 1 mL of labeling medium, containing 50 μCi [^{35}S]methionine. The labeled cells were collected and solubilized in 400 μL of lysis buffer [25 mM Tris (pH 8.0), 50 mM NaCl, 1% DOC, 1% NP-40, 0.01 volume of 100 mM PMSF, and 0.01 volume of inhibitor mixture [0.25 mg/mL pepstatin, 0.06 mg/mL aprotinin, 1.1 mg/mL leupeptin, 4.7 mg/mL benzamidine, 0.24 mg/mL bestatin, 0.3 mg/mL E-64 [*N*-(*trans*-epoxysuccinyl)-L-leucine-4-guanidinobutylamide], and 38.3 mg/mL *o*-phenanthroline]]. Immunoprecipitations were performed as described previously (Lottaz et al., 1992).

Protein Determination. Protein was determined by a modified Lowry procedure (Peterson, 1977).

Enzyme Assays. To determine the esterase activity, *p*-nitrophenyl esters of fatty acids were dissolved in 60% 2-propanol/40% NP-40 (v/v) to 20 mM. The assay mixture (800 μL) contained 50 mM Hepes/NaOH (pH 8.0), 1% DOC, and 20 μL of substrate (final concentration of 0.5 mM; 1% NP-40). The reaction was started by addition of a 50 μL enzyme aliquot. After 30 min at 37 °C, the absorbance was determined at 410 nm. The standard substrate was *p*-nitrophenyl palmitate. Phospholipase activity was determined as described (Boll et al., 1993).

Phase Separation in Triton X-114. Triton X-114 was precondensed before use (Bordier, 1981). Enzyme samples were brought to 200 μL with buffer [0.5 mM EDTA or 10 mM $NaPO_4$ and 500 mM NaCl (pH 7.4)] in 2% Triton X-114, vortexed, put on ice for 5 min, and subjected to phase separation at 30 °C for 3 min. The detergent-rich and detergent-poor phases were separated through 0.3 mL of 6% (w/v) sucrose in the respective buffers containing 0.06% Triton X-114 and 0.2 mg/mL cytochrome *c*[2] by centrifugation for 3 min at 3000*g* and assayed for enzyme activity.

SDS−PAGE and Electroblotting. Electrophoresis of Estr/PLase was performed using a discontinuous sulfate/borate system (Neville, 1971) as described (Wacker et al., 1981) or the system of Laemmli (1970). Electrophoresis of peptides was carried out using the Tricine system (16.5% T, 3% C) of Schägger and von Jagow (1987). Proteins or peptides from polyacrylamide gels were transferred onto PVDF membranes (Immobilon P; Millipore, Volketswil, Switzerland) using semidry blotting in a Sartoblot II-S apparatus (Sartorius) with the buffer system of Kyhse-Andersen (1984).

Immuno-Overlays. After electrotransfer, the membrane was incubated in TTW5 [50 mM Tris-HCl (pH 7.4), 500 mM NaCl, 0.04% NaN_3, and 0.05% Tween-20] for 20 min at room temperature and then with the first antibody (diluted 2000 times in TTW5) for 60 min, then was washed four times for 5 min each with TTW5, incubated with the secondary antibody (goat anti-guinea pig IgG, alkaline phosphatase-conjugated, Inotech/KPL; diluted 1000 times in TTW5) for 60 min, and then washed again four times for 5 min with TTW5. The blot was developed with 5-bromo-4-chloro-3-indolylphosphate/nitroblue tetrazolium (Inotech/KPL) in 100 mM Tris-HCl (pH 9.5).

Amino Acid Sequence Analysis. Amino acid sequences were determined using an Applied Biosystems 470A gas phase sequencer with an on-line 120 A PTH amino acid derivative analyzer. Peptides were separated by SDS−PAGE and blotted onto a PVDF membrane. Radioactive bands were excised and directly sequenced. Radioactivity in each cycle was determined by collecting the (nonconverted) anilinothiazolinone amino acid derivatives.

Purification of Estr/PLase from Rabbit Small Intestine by Immunoadsorption Chromatography. The IgG fraction was prepared from guinea pig serum (Boll et al., 1993) by chromatography with Affi Gel Blue CM (BioRad) followed by precipitation with ammonium sulfate (Gee et al., 1979) and was coupled to BrCN-activated Sepharose 4B (Cuatrecasas et al., 1969).

Brush border membrane vesicles prepared from frozen intestines by the Mg-precipitation method (Wacker et al.,

[2] Cytochrome *c* serves to ease in distinguishing the phases.

1992) were diluted with column buffer [10 mM $NaPO_4$ (pH 7.4), 500 mM NaCl, 1 mM EDTA, 0.02% NaN_3, and 1% Triton X-100] to 12 mg/mL protein, brought to 2% Triton X-100, and solubilized with stirring overnight at 4 °C in the presence of protease inhibitors (benzamidine, 1 mM; leupeptin, 2 μM; chymostatin, 12 μM; antipain, 1.7 μM; pepstatin, 20 μM; and aprotinin, 0.15 μM). After centrifugation (100000*g*, 1 h), the supernatant was applied to the anti-Estr/PLase column (column volume of 10 mL) at 2 cm h^{-1}. The column was washed with 10 volumes of column buffer, and the Estr/PLase was desorbed by hypotonic elution (Danielsen et al., 1982) using 0.5 mM EDTA (pH 7.4) and 0.1% Triton X-100. Estr/PLase-containing fractions were concentrated by ultracentrifugation (Wacker et al., 1992).

Inactivation of Estr/PLase with DFP. The enzyme (40 μg/mL) in 5 mM sodium maleate (pH 6.0), 50 mM NaCl, 0.5 mM EDTA, 0.02% NaN_3, and 0.1% Triton X-100 was incubated with DFP (0.1 mM) at 37 °C. At different times, aliquots were withdrawn and enzyme activities determined. A sample incubated under identical conditions, but without DFP, served as a control.

Incorporation of [^{14}C]DFP into Estr/PLase. Estr/PLase (1.01 mg) in 5.4 mL of 23 mM sodium-maleate (pH 6.0), 0.5 mM EDTA, and 0.1% Triton X-100 was incubated with 480 nmol of 1,3-[^{14}C]DFP (NEN; 160 μCi/μmol, final concentration of 89 μM) at 37 °C. After 20 min, an aliquot was withdrawn (remaining activity of approximately 45%). Non-protein-bound DFP was removed by gel filtration through a Sephadex G-25 column equilibrated in PBS containing 0.05% Triton X-100. The labeled protein ([^{14}C]DFP−Estr/PLase) eluted in the void volume. Following determination of enzyme activity, radioactivity, and protein content, the fraction was concentrated and washed with the same buffer by Amicon filtration. Specific enzyme activity and radioactivity were again determined.

After 21 h of incubation with DFP, less than 1% of enzyme activity remained. The reaction mixture was processed as above but was passed twice over the Sephadex G-25 column.

Cleavage of [^{14}C]DFP−Estr/PLase with Formic Acid, BNPS-Skatole, and CNBr. Estr/PLase, typically 40−80 μg in 300 μL, was precipitated with trichloroacetic acid (final concentration of 10%). After standing on ice for 30 min, the precipitate was washed with 5% trichloroacetic acid, three times with ethanol/ether (1:1), and with ether and dried.

For formic acid cleavage (Landon, 1977), the pellet was resuspended in 100 μL of 75% formic acid and incubated for 24−72 h at 37 °C. The reaction was terminated by addition of water and subsequent drying in a Speed-Vac concentrator. The residue was taken up in water several times and dried again.

BNPS-skatole cleavage (Fontana, 1972) was performed by resuspending the pellet in 100 μL of 50% acetic acid followed by the addition of 100 μg of tyrosine dissolved in 10 μL of acetic acid and 200 μg of BNPS-skatole (recrystallized from acetone before use) in 20 μL of acetic acid. The mixture was rotated for 48 h at room temperature in the dark. The reaction was stopped by addition of water, extraction with ethyl acetate, and drying (Speed-Vac).

For CNBr cleavage (Gross & Witkop, 1961), the sample was treated with 2 μL of β-mercaptoethanol at 37 °C for 2 h prior to trichloroacetic acid precipitation. The pellet was resuspended in 100 μL of 70% formic acid; 2 mg of CNBr in 20 μL of 70% formic acid was added, and the sample was incubated for 24 h under N_2 in the dark. The reaction was terminated by dilution with water and drying.

Deglycosylation of Cleavage Products. After cleavage by either BNPS-skatole, formic acid, or BrCN, the residue was taken up in 10 μL of 0.1% SDS, boiled for 1 min, diluted to 0.06% SDS by adding 1.2 units of *N*-glycosidase F (Boehringer, Mannheim) in 6 μL of 100 mM $NaPO_4$, 25 mM EDTA, 5 mM NaN_3, and 50% glycerol (pH 7.6), and incubated for 24 h at 37 °C. The reaction was terminated by the addition of SDS−PAGE sample buffer and boiling.

RESULTS

Estr/PLase as isolated from rabbit small-intestinal brush border membranes with a polyclonal antibody yielded protein bands in the 140−115 kDa range, whereas when expressed in transfected COS cells, it only yielded a single protein band of approximately 175 kDa (Boll et al., 1993). An antibody directed against a part (amino acids 38−178) of the N-terminal repeat of the Estr/PLase recognized the latter, largest band but failed to recognize any of the bands in SDS−PAGE of brush border membranes. This indicated that after biosynthesis the protein had undergone proteolytic processing in the N-terminal region. In order to study this processing and to identify the processing site(s), it was necessary to purify the enzyme from the brush border membrane in sizable amounts. This was achieved by immunoadsorption chromatography.

The purified enzyme could also be used to investigate the enzymatic properties further; previously, this had only been done using a protein A−antibody−enzyme complex (Boll et al., 1993). Finally, the active site could be localized using DFP, a covalent active site-directed inhibitor.

Estr/PLase Shows Multiple Bands When Immunoisolated from the Brush Border Membrane. After the antibody-Sepharose column was washed extensively with high-ionic strength buffer, the Estr/PLase protein could be desorbed by hypotonic elution with a purification factor of 2500. SI, the main protein of the brush border membrane, was absent in the preparation. A second round of purification over the same column did not increase the specific enzyme activity. In SDS−PAGE, Coomassie Blue staining revealed bands of approximately 140, 125, 100, and 90 kDa. Western blot analysis using an antibody directed against a C-terminal portion (amino acids 702−1138) showed, in addition to the bands stained by Coomassie Blue, one more, very faint of approximately 170 kDa; this band was also decorated by an antibody directed against an N-terminal part (amino acids 39−178), while the others were not (Figure 1). That is, all the bands stained with Coomassie Blue were decorated by one or both antibodies; they all reacted with [^{14}C]DFP (see below). Thus, they all were Estr/PLase.

N-Terminal amino acid sequence analysis yielded identical sequences for the 140, 125, 100, and 90 kDa bands, i.e., EGTEIRXP. The N-terminal amino acid corresponds to residue 364 in the Estr/PLase sequence as deduced from its cognate cDNA, and it is preceded by Arg^{363}. This means that the entire region I of the four homologous regions in Estr/PLase (Boll et al., 1993) must have been removed (see Figure 2). Since the four protein bands have different sizes but identical N termini, they must be heterogeneous in their C-terminal regions.

The Estr/PLase protein is most likely anchored to the membrane by a hydrophobic sequence at the very C terminus

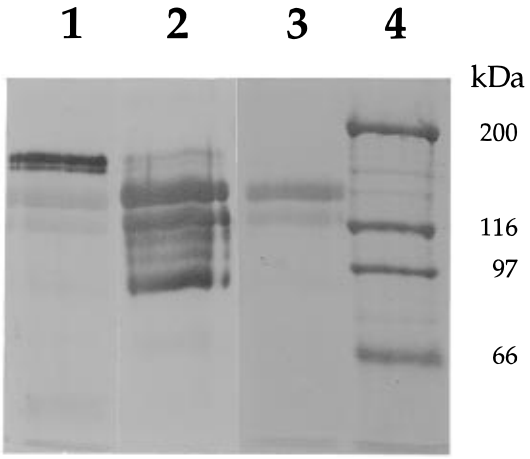

FIGURE 1: SDS−PAGE and immuno-overlay of Estr/PLase as isolated from the brush border membrane by immunochromatography. Estr/PLase was analyzed by SDS−PAGE using a sulfate/borate system (Neville, 1971). After transfer to a PVDF membrane, it was detected by Coomassie Blue staining (lane 3) or with antibodies directed against amino acids 39−178 (lane 1) and 703−1138 (lane 2). Molecular mass markers are in lane 4.

(see below); proteolytic processing by luminal proteases upstream of this region may thus change its amphipathic properties due to the loss of the hydrophobic stretch. This was studied using phase separation in Triton X-114. Estr/PLase predominately partitioned into the detergent phase (51 vs 30%, 10−15% being found in the interface) using either high- or low-ionic strength buffers. When the aqueous and detergent phases were analyzed by SDS−PAGE, no difference in band pattern was apparent, indicating that the heterogeneity at the C terminus did not significantly influence the amphipathic behavior of the protein. It is possible that Estr/PLase forms di- or oligomers in which only one (or few) of the polypeptide(s) has lost its C-terminal anchor; it is possible, also, that the cleaved C-terminal segment still remains associated noncovalently with the other, complete polypeptide(s) in the native state (i.e., during partitioning in Triton X-114) but dissociates in the subsequent SDS−PAGE analysis. Examples of partially nicked oligomers still remaining associated are known [see, e.g., Wacker et al. (1976)].

Estr/PLase Is Not Proteolytically Processed in Small-Intestinal Organ Cultures. We have used an established organ culture system [see, e.g., Lottaz et al. (1992) and Keller et al. (1992)] that was successfully used to study the in vitro biosynthesis of a number of small-intestine proteins, including LPH and SI. Figure 3 shows the results of such an experiment. Tissue pieces from the small intestine of a 6-month-old male rabbit were pulse labeled for 1 h and then chased for 1 or 5 h. Estr/PLase, LPH, and SI were immunoprecipitated from the solubilized tissue and analyzed by SDS−PAGE and autoradiography. LPH showed the usual processing pattern; i.e., 220 kDa pro-LPH was processed via a 180 kDa intermediate to mature 135 kDa LPH [see, e.g., Keller et al. (1992)]. SI was only in the form of pro-SI. Since pro-SI is known to be split by pancreatic proteases into heterodimeric SI [see, e.g., Hauri et al. (1979); reviewed by Semenza (1986)], the unprocessed pro-SI is a reliable control for the complete absence of pancreatic proteases [see also Keller et al. (1995)].

Estr/PLase immunoprecipitated with antibodies directed against the C-terminal region (amino acids 703 - 1138) gave a very closely spaced doublet of bands (approximately 175 and 185 kDa, respectively).[3] After the 1 h pulse, only the 175 kDa band was visible. The amount of the 185 kDa species increased with an increasing time of chase, whereas the amount of the 175 kDa species decreased, suggesting that this band is the high mannose form of Estr/PLase and that the 185 kDa species is the complex-glycosylated form (Figure 3, bottom right).[3] After a 20 h chase (data not shown), only the 185 kDa band was detectable. This corresponds fairly well to the calculated molecular mass [161 343 Da, from the cDNA-deduced amino acid sequence (Boll et al., 1993)], considering that the protein is glycosylated. The polyclonal antibody directed against the 39−178 sequence did not precipitate any Estr/PLase (Figure 3, bottom left); see more in the next section. No other bands were detectable at any time of this in vitro experiment, suggesting that the multiple Estr/PLase bands visible in SDS−PAGE of brush border membranes did not arise from intracellular processing, but rather from the action of luminal proteases.

Estr/PLase Is Not Proteolytically Processed in Transfected COS 7 Cells. As mentioned above, the antibodies directed against the N-terminal region of Estr/PLase precipitated little or no enzyme, whereas antibodies directed against a more C-terminal region precipitated it very efficiently. There are two possible explanations for this observation, i.e., (i) that the very N terminus of Estr/PLase has been proteolytically removed (this is however unlikely because the N-terminal antibodies do not even recognize the band synthesized during the continuous 1 h pulse) or (ii) that the N-terminal antibodies do not recognize the *native* conformation of Estr/PLase.

In order to distinguish between these two possibilities, we have compared immunoprecipitated ^{35}S-labeled Estr/PLase and immunoblots from transfected COS 7 cells using antibodies directed against the N- and C-terminal regions. On immunoblots, both antibodies recognized a band of approximately 175 kDa with the same efficiency, whereas only the C-terminal antibody immunoprecipitated Estr/PLase efficiently (data not shown).

Thus, the N-terminal antibodies did not immunoprecipitate Estr/PLase but did recognize it on immunoblots. It is therefore likely that the N terminus is present in the 175 kDa Estr/PLase form synthesized in vitro by intestinal transplants and by COS 7 cells.

Estr/PLase Carries One Single Active Site That Is Inactivated by DFP. Estr/PLase purified by immunoaffinity chromatography from the intestinal brush border membrane, like the protein A-Sepharose immunocomplex investigated previously (Boll et al., 1993), has both arylesterase and phospholipase A/lysophosholipase activities. The substrate specificity was now investigated further using 4-nitrophenyl esters with different chain lengths; nitrophenyl laurate was found to be the best substrate (Table 1).

Likewise, the effects of a variety of inhibitors tested were very much the same as in the immunocomplex.[4]

The interaction of Estr/PLase with DFP was studied more closely. Identical inactivation kinetics (approaching pseudo-

[3] Unfortunately, the two bands in the doublet are too close to one another to be visible on the reproduction.

[4] Stimulation by dithiothreitol, as obtained with the protein A−antibody complex, was not observed. This stimulation had been originally suggested (Boll et al., 1993) to be possibly due to a release of the antigen from the antibody by dithiothreitol.

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I       39 ESLKTFPFPCDPKTLAESVPSESVHSLRPSDIKFVAAIGN-VETAPDSGADDLEEQDGTE
II     362 VRe-GTeIRCPDKdPSdSVPT-SVHRLKPADIKVIGAMGDSLTAGNGAGSQPGNILDVLT
III    708 VQGFGTWLPCRDRSPSASPPT-SVHALRPADIQVVAALGDSLTAGIGIGSKPNDLSDGTT
IV    1064 VENWGSDFLCTAWNASRGVPN-SVHELQPGDIKVVAALGDSLT--LAMGARPSNSSDPPM
               .  . *   . . . *. *** *.*.**. ..*.*. ..   . *.   .  *

I       98 -------KRPEQACMGVVTVLSDIIGRFSPSALMPLCPET-----------RLVPRGGAE
II     420 QYRGLSWSVGGDQNISTVTTLANILReFNPS-LQGFSVGTGReTTSQAFFNQAVAGARAd
III    767 QYRGLSYSSGGDGSLDNVTTLPNILRQFNSN-LMGFAVGTGDASGTNAFFNQAVPGAKAR
IV    1121 FWRGLSWSIGGDGALETHTTLPNILKKFNPS-ILGFSTGTLEGTMG---LNVAVQGARAQ
                 .  ..  ..  *.*..*.  *... .  .  .*           *. . *

I      140 DLWMQATELVRSMRENPQLDFEHDWKLINVFFSNTSQCFPCPSAQQKGLVLGGMDKLTRT
II     479 GLIPQAQRLVALMKNDTRINFQEDWKIITVFIGGNDLCDFCNDPVRYSPQ-NFTDNIGTA
III    826 DLMSQVQTLVQRMKDDHRVNFQEDWKVITVQIGASDLCDYCTDSNLYSAA-NFYDHLRDA
IV    1177 DMPAQARDLVERMRNSPEIDLEKDWKLVTLFVGGNDLCHFCENPEGSSEG-EYVQHIQQA
           ..  *.  **  *...  .....***....  .... *  * ..   .   .  ...  .

I      200 LDYLQQEVPKAFVNLVDLSELAAFSRWRQG-----AQLSPAAEPCRCL----RETSQLTK
II     538 LDILHAEIPRAFVNLVKVLEISKLReLYQeTKVSCPRMILR-SLCPCVLKFddNSTEIAS
III    885 LDALHREVPRALVNLVDFMNPSVTRQVFLGNPDKCPVQQAS-ALCNCVLSPRENSYELAR
IV    1236 LDVLYEELPRTFVNVVEVMELAGLHQ---DQGGRCATLLAAQSHCTC-FKYSQSSVEMQE
           ** *  *.*...**.*.  . .       .     .      . * *      .. ..

I      251 VLT-QWSYLEAWDSLLASSKYNTQESFAVVFQPFFYESSLS---------ALLAEPPLQ-
II     597 LIeTIKeYQeRTQQLIdSGRYDTRdDFTVVLQPFFeKVNMPKTQDGLPDNSFFAPDCFHF
III    944 LEALAQAYQSSLRELVESGRYDTREDFSVVLQPFFHSIQLPVLQDGRLDTSFFAPDCVHP
IV    1292 LKKVNWNLQSGLSRLSYSHQYVQREDFAVVVQPFFQNTLVPLNGRGDTDLTFFSDDCFHF
           .     .  .    *  * .*  ...*.** **** .  ..         ....    .

I      300 ---DPTTLALSLWNRMMEPIGRKEEPFS-EKERKPLRCPTQESPYLFTYRNS     347
II     657 SSKAHAhAASALWNNMLEPVGQKTThNDFe-GAVNITCPNQVWPFLSTYKNS     707
III   1004 NQKFHSQLSRALWRNMLEPLGGKTDALDLT-AAITLTCPTQNEPFLRTFRNS    1054
IV    1352 SERGHAEMAIALWNNMLEPVGHKTTSNNFTYSRTKLKCPSPDSPYLYTLRNS    1403
             ..  . .**..*.**.* *..  . . .   . **..  *.* * .**
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FIGURE 2: Internal homologies within the Estr/PLase amino acid sequence. Regions I (residues 39−347), II (362−707), III (708−1054), and IV (1064−1403) were aligned with the program Clustal. Amino acids common to all four repeats are indicated with asterisks and conservative substitutions with periods [from Boll et al. (1993)]. The N-terminal amino acid sequence found in Estr/PLase as isolated from the brush border membrane is underlined. Ser^{400} which was labeled with [^{14}C]DFP is in bold. His and Asp/Glu residues which are present in repeat II but not in repeats III and IV are lowercase.

first-order) were observed using three different substrates (2-nitrophenyl butyrate, 4-nitrophenyl palmitate, and phosphatidylcholine), which strongly indicated that these substrates are split by a single or by identical active sites (data not shown).

When the enzyme was incubated with [^{14}C]DFP for 20 min, 55% of the enzyme activity was lost, and a [^{14}C]DFP to protein molar ratio of 0.51 (moles:moles) was obtained. After overnight incubation, more than 99% of the enzyme activity was lost and a [^{14}C]DFP to protein ratio of 1.2 was measured. The molar ratio between incorporated [^{14}C]DFP and protein did not change significantly with an enzyme isolated in the presence of PMSF.

SDS−PAGE and PhosphorImager (Molecular Dynamics, Sunnyvale, CA) analysis of the labeled protein showed that essentially all the radioactivity (97%) was found in the 140−90 kDa bands; 52% of this radioactivity was in the 140 kDa band alone (Figure 4). The radioactivity profile exactly matched the protein mass distribution as determined by staining with Coomassie Blue; the intensity of staining roughly correlated with the height of the radioactivity peaks.

About 1% of the total radioactivity was detected in the range of 170−180 kDa. This again shows that a small amount of unsplit Estr/PLase protein is present in our preparation which is not detected by Coomassie Blue staining but can be seen only after affinity labeling or on immunoblots (see also Figure 1).

DFP Reacts with Ser^{400}. Having established an approximately 1:1 stoichiometry between DFP incorporated and the inactivation of the enzyme, we proceeded to identify the amino acid residue which had selectively reacted with DFP. The usual, obvious approach is to label this amino acid with radioactive DFP, to split the polypeptide chain, and to localize the radioactivity in the known sequence (Boll et al., 1993). However, in the case of Estr/PLase which has extensive 4-fold repeats (Figure 2), most procedures of selective degradation would yield sets of four or at best three peptides of nearly identical size and composition. Splitting with acid (Landon, 1977), BNPS-skatole (Fontana, 1972), or CNBr (Gross & Witkop, 1961) did show promise.

Degradation in 75% formic acid (splitting Asp−Pro bonds) should yield six peptides: one with a molecular mass of 1262 Da (the N-terminal region); 15 642, 65 998, and 33 259 Da molecular mass peptides; a 3782 Da molecular mass peptide comprising the presumed membrane anchor; and a 894 Da molecular mass segment at the C terminus (all molecular masses calculated disregarding post-translational modifications).

Most of the radioactivity (50−60%) was found at about 25 kDa, and 15−20% at 16−18 kDa (Figure 5A). Partial N-terminal sequence analysis of the 25 kDa band yielded two sequences. One is PSDSVP, corresponding to positions 375−521 in the Estr/PLase sequence ("acid peptide" 2, calculated molecular mass of 15 642 Da; one potential

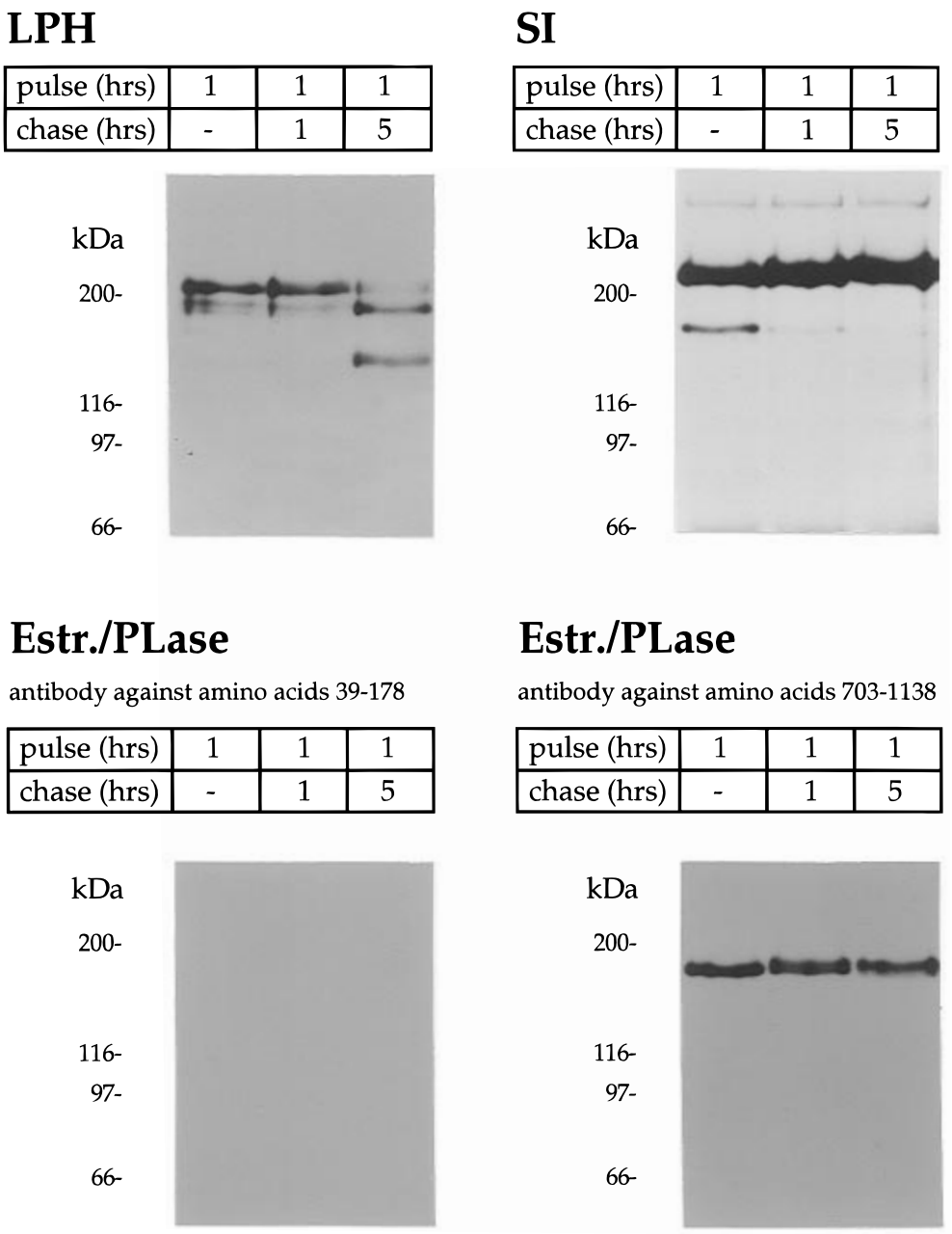

FIGURE 3: Estr/PLase is not proteolytically processed in organ cultures from rabbit small intestinal explants. The explants from a 6-month-old male rabbit were pulse labeled for 1 h in the presence of 150 μCi [^{35}S]methionine in 1 mL of medium and were then chased for 1 or 5 h in the presence of 2.5 mM unlabeled methionine. LPH (from $^{1}/_{10}$ of the solubilized tissue), SI ($^{1}/_{10}$), and Estr/PLase ($^{4}/_{10}$) were immunoprecipitated and analyzed by SDS−PAGE and autoradiography. Positions of molecular mass markers are indicated on the left. Note that the antibody against amino acids 39−178 does not precipitate Estr/PLase (bottom left), but it detects it well on overlays (see Figure 1, lane 1).

Table 1: Hydrolysis of Acyl *p*-Nitrophenyl Esters by Estr/PLase[a]

substrate	activity (u/mL)	relative activity (%)
p-nitrophenyl butyrate (C4)	0.3	2
p-nitrophenyl caprylate (C8)	3.8	23
p-nitrophenyl laurate (C12)	23.8	142
p-nitrophenyl myristate (C14)	17.2	102
p-nitrophenyl palmitate (C16)	16.8	100
p-nitrophenyl stearate (C18)	16.3	97

[a] Assay conditions: 0.5 mM substrate in 50 mM Hepes/NaOH at pH 8.0, 1% DOC, and 1% Nonidet P40. Activity using *p*-nitrophenyl palmitate is set to 100%.

glycosylation site). The other sequence, i.e., PVRYSPQ, corresponded to positions 522−1117 ("acid peptide" 3, calculated molecular mass weight of 65 998 Da, four potential glycosylation sites). We attribute the higher than expected mobility to an "unspecific" cleavage site in the peptide.

Following deglycosylation with *N*-glycosidase F (Figure 5B), the radioactive band of 25 kDa shifted to a molecular mass of 16−18 kDa; it yielded a single partial N-terminal sequence, PSDSVP.[5] No radioactivity was found in the first 20 cycles of Edman degradation of this [^{14}C]DFP-labeled peptide. This restricted the localization of the DFP-labeled amino acid to a residue between positions 395 and 521.

BNPS-skatole splits Trp−X bonds. After degradation of Estr/PLase with BNPS-skatole, radioactivity was found to

[5] The sequence PSDXXP was also found for the original peptide of 16−18 kDa, suggesting that it might have been generated from the 25 kDa peptide by chemical deglycosylation occurring during the acidic degradation reaction.

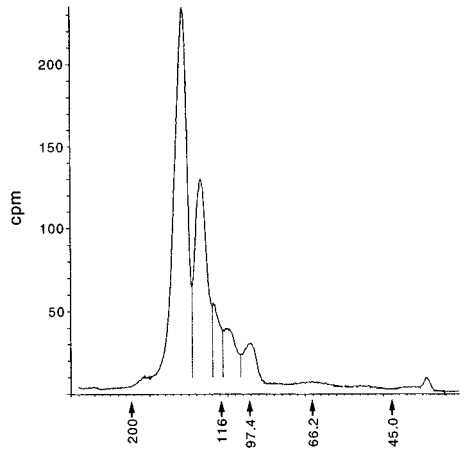

FIGURE 4: Inactivation of Estr/PLase with [^{14}C]DFP and incorporation of radioactivity into protein. After incubation for 20 min, non-protein-bound radioactivity was removed by gel filtration, and the protein was subjected to SDS−PAGE followed by transfer onto a PVDF membrane. Shown is the distribution of radioactivity as determined by scanning with the PhosphorImager. Migrations of molecular mass markers are indicated on the abscissa.

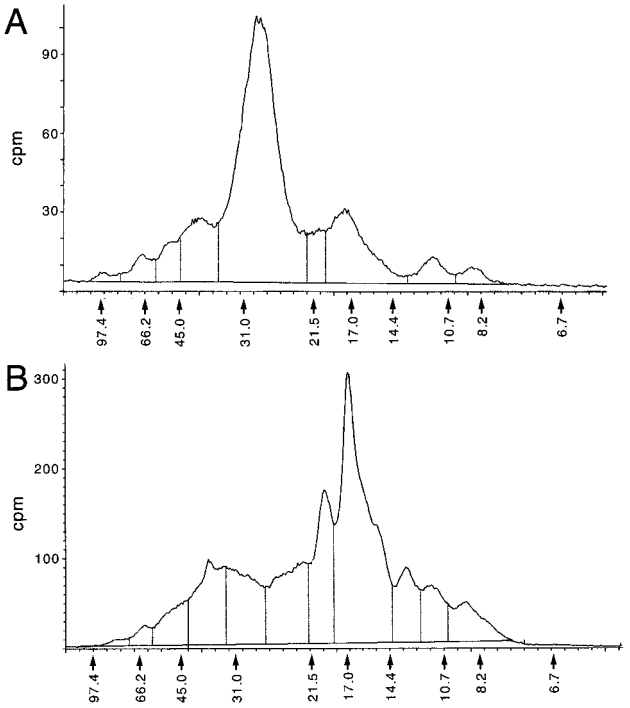

FIGURE 5: Distribution of radioactivity in peptides after cleavage of [^{14}C]DFP−Estr/PLase with formic acid. [^{14}C]DFP−Estr/PLase was treated with formic acid (see Materials and Methods) for 42 h, and the cleavage products were separated by SDS−PAGE using the Tricine system (Schägger & von Jagow, 1987) followed by electrotransfer onto a PVDF membrane. Shown is the distribution of radioactivity as determined by a PhosphorImager scan: (A) formic acid cleavage and (B) formic acid cleavage, followed by deglycosylation with *N*-glycosidase F. Migrations of molecular mass markers (in kilodaltons) are indicated on the abscissae (phosphorylase B, 97.4; bovine serum albumin, 66.2; ovalbumin, 45.0; carbonic anhydrase, 31.0; soybean trypsin inhibitor, 21.5; whale myoglobin, 17.0; lysozyme, 14.4; myoglobin, CNBr fragment (I + III), 10.7; CNBr fragment I, 8.2; CNBr fragment II, 6.2; CNBr fragment III, 2.5).

be associated with bands of 7−8 kDa (20−25%; sequence EGTEIRXPD), 16−17 kDa (12−14%; sequence XGTEX and others), and 25−26 kDa (22−24%; sequence EGTEIRX-PD), the remaining radioactivity being distributed among peptides with higher molecular masses (Figure 6A). After

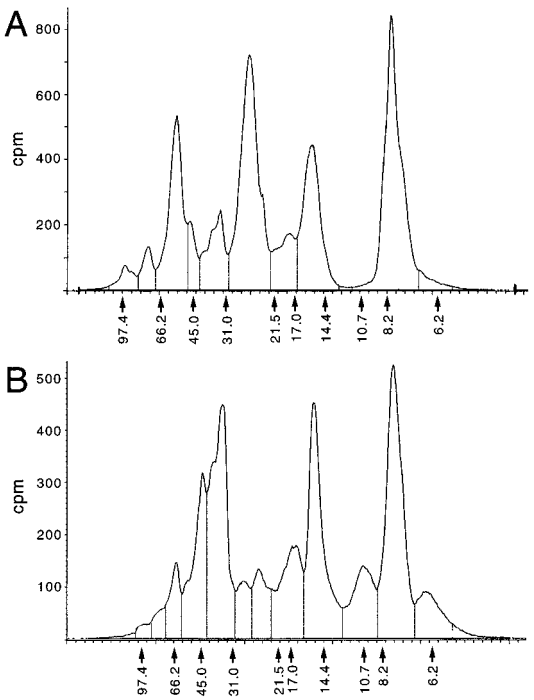

FIGURE 6: Distribution of radioactivity in peptides after cleavage of [^{14}C]DFP−Estr/PLase with BNPS-skatole. [^{14}C]DFP−Estr/PLase was treated with BNPS-skatole. The products were analyzed as in Figure 5: (A) BNPS-skatole cleavage and (B) BNPS-skatole cleavage, followed by deglycosylation with *N*-glycosidase F.

deglycosylation with *N*-glycosidase F (Figure 6B), the radioactive peptide of 25−26 kDa shifted to a molecular mass of 16−17 kDa (sequence EGTEIRX), while the 7−8 kDa peptide remained unchanged (sequence EGTEIRX). These sequences clearly corresponded to positions 364−426 (calculated molecular mass of the BNPS-skatole peptide 1 of 6593 Da). The 25 kDa peptide probably originated from an incomplete cleavage at Trp^{426} (calculated molecular mass of 14 936 Da; one potential glycosylation site), while the 16 kDa band again may have been generated by chemical deglycosylation under the acidic conditions of the BNPS-skatole reaction.

Summing up, from the two degradation reactions described above, it was clear that the active site serine residue labeled with [^{14}C]DFP must be located between Gly^{395} and Trp^{426}. To finally identify this serine residue, degradation of Estr/PLase with CNBr was used (note that Met^{397} is the only methionine residue within this short stretch). A radioactive band of approximately 13 kDa was generated in 66% yield; after incubation with *N*-glycosidase F, a band of approximately 8 kDa was obtained (Figure 7). Upon Edman degradation of the peptide, prior to or after deglycosylation, radioactivity emerged in cycle 3 (Figure 7, insets), thus identifying Ser^{400} as the amino acid residue labeled with [^{14}C]DFP [the radioactivity in the cycles immediately following cycle 3 can obviously be attributed to carryover from the previous cycle; also note that, with the possible exception of Thr^{402} (cycle 5), no other amino acid residue in the segment sequenced can be expected to react with DFP].

Ser^{400} is part of the sequence GDSLT which is reminiscent of the consensus sequence (Gly-X-Ser-X-Gly) for esterases and lipases (Derewenda & Sharp, 1993) and is conserved in homologous regions II−IV (see Figure 2). Interestingly, however, radioactivity from [^{14}C]DFP was not found to be associated with the corresponding Ser residues in the other

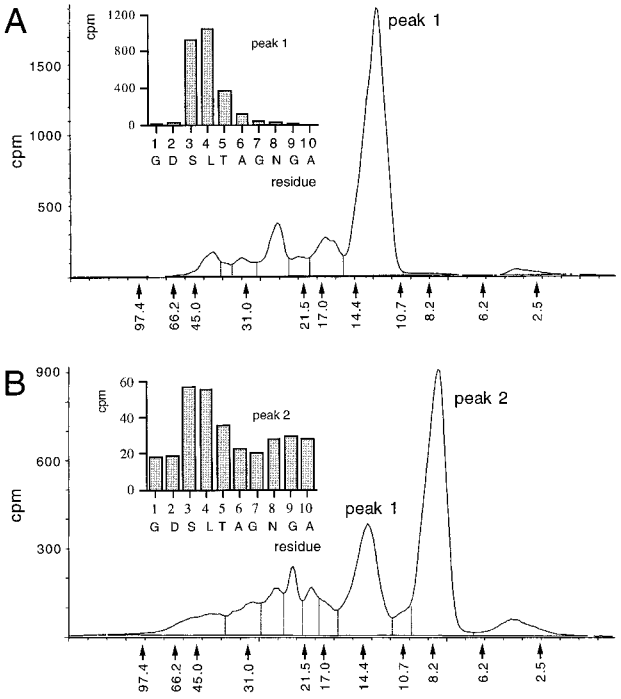

FIGURE 7: Radioactive sequences of [14C]DFP−Estr/PLase peptides after cleavage with CNBr. [^{14}C]DFP−Estr/PLase was treated with CNBr, and the cleavage products were worked up as described in Figure 5: (A) CNBr cleavage and (B) CNBr cleavage, followed by deglycosylation with *N*-glycosidase F. Radioactive peaks were cut from the membrane; and the radioactivity in each cycle of Edman degradation was determined (insets). Amino acid residues as expected from the cognate cDNA sequence (Boll et al., 1993) are indicated.

homologous regions, which agrees with the 1:1 stoichiometry of [^{14}C]DFP binding to the undegraded protein.

DISCUSSION

In addition to a non-membrane-associated enzyme (Négrel et al., 1978), at least one or two Ca^{2+}-independent esterase/phospholipases A which are associated with the brush border membrane have been described [in the guinea pig by Gassama et al. (1989) and in adult rabbit by us (Boll et al., 1993)]. It is not clear whether these two membrane-bound esterase/phospholipases are identical; they differ in the molecular masses reported, but this may be due to post-translational (pancreatic) proteolysis (see below).

In our previous paper (Boll et al., 1993), enzyme activities were tested using an enzyme−antibody−protein A complex. They differ only slightly from those reported in the present paper, where we have used the purified enzyme. This also holds for a variety of inhibitors tested.

The substrate specificity of the enzyme was studied using nitrophenyl esters of different chain lengths (Table 1). The hydrolysis of substrates was followed in the presence of 1% NP-40 and 1% DOC (essentially all substrates were sparely soluble in water); under these conditions, the C12 ester was found to be the best substrate.

The Estr/PLase Has a Single Catalytic Site. As we have reported previously (Boll et al., 1993), this protein has a peculiar structure, in the sense that it shows extensive, 4-fold repeats between the N-terminal signal sequence (presumably split after Gly^{19}) and the hydrophobic anchor which is located not far from the carboxyl-terminal region and is followed by a hydrophilic, presumably cytosolic C-terminal tail. We made a special effort to answer the question of how many catalytic sites are present in esterase/phospholipase A. Another enzyme with a similar general structure (pro-LPH) also has 4-fold repeats (Mantei et al., 1988), but only two of them have catalytic sites (Wacker et al., 1992); they are both located in that part of the polypeptide chain which reaches the brush border membrane (i.e., in LPH), and which accounts for only 60% of the original precursor (pro-LPH). In addition, other intrinsic membrane proteins of the brush border membrane have very large 2-fold repeats, and each repeat has its own catalytic site [sucrase-isomaltase (Kolínská & Semenza, 1967; Quaroni et al., 1974; Hunziker et al., 1986), maltase-glucoamylase (Messer & Kerry, 1967), and angiotensin-converting enzyme (Soubrier et al., 1988)].

The major tool in this investigation has been DFP, which we found to act as an affinity label. The degree of inactivation by DFP is the same, no matter which substrate is used to determine the residual enzymatic activity. This is strongly indicative that these substrates are split by a single catalytic site or, if they are split by different sites, that they have identical substrate specificities. Using [^{14}C]DFP, we inactivated the Estr/PLase to completion and found that the affinity label was incorporated with a stoichiometry of approximately 1:1. This, although in agreement with the other observations mentioned above, was rather surprising in view of the extensive 4-fold repeats in this protein (see Figure 2).

We thus decided to localize the residue which had reacted with [^{14}C]DFP. As specified in the Results, a single residue was found to react with the affinity label, namely Ser^{400}. Inspection of Figure 2 shows that this serine residue belongs to repeat II, that no serine occurs in the corresponding region of repeat I, and that serine residues occur in the corresponding, homologous positions of repeats III and IV. The following question arises, therefore. Why did the two serines in repeats III and IV not react with DFP, and why were they thus apparently devoid of catalytic activity?

Ser^{400} is part of the sequence GDSLT which bears some resemblance to the pentapeptide motif GXSXG identified as the consensus sequence in other serine esterases [see Derewenda and Sharp (1993)]. Serine esterases (like serine proteases) operate via a catalytic mechanism involving the classical triad Ser-His-Asp/Glu. An appealing possibility to explain why repeats III and IV are not catalytically active is that this triad is incomplete. Assuming that the whole triad (when present) occurs within the same repeat, inspection of Figure 2 shows that His^{663} and His^{682} of repeat II do not have their pendants in repeats III and IV. Hence, if either one of these histidines were operative in the triad of repeat II, this would provide a simple explanation as to why repeats III and IV are not catalytically active. A similar reasoning can, of course, be made for Asp or Glu residues; however, the outcome of these considerations becomes even more ambiguous due to the richness in acidic amino acids. For example, there are not less than seven aspartate residues and eleven glutamate residues in repeat II which do not have their pendants in repeats III and IV. Clearly, more work is needed to identify the other members of the triad operating in this enzyme.

Membrane Anchor of Estr/PLase. From the cDNA-deduced amino acid sequence, we have indicated that this Estr/PLase most likely has a membrane-spanning domain between positions 1415 and 1440, which is a stretch of 24

highly hydrophobic amino acids (Boll et al., 1993). The apical plasma membrane of epithelia is rich in glycosylphosphatidylinositol (GPI)-anchored proteins, and GPI anchors are exchanged during biosynthesis for a C-terminal hydrophobic anchor peptide in the primary translation product [for a review, see Cross (1990)]. It is, however, unlikely that Estr/PLase is GPI-anchored, since with Triton X-100 at 0 °C it was solubilized from the brush border membrane to the same extent as LPH, SI, and aminopeptidase N, three proteins which are anchored via a transmembrane hydrophobic stretch. Contrary to this, under these conditions, trehalase (which is GPI-anchored; Takesue et al., 1986) was very poorly solubilized [results not shown; see also Hooper and Turner (1988)].

Biosynthesis of Estr/PLase. The enzyme, as isolated from adult rabbit brush border membranes, is composed of four bands of 140, 125, 100, and 90 kDa (see Figure 1). All these bands have an identical N-terminal sequence (EGTEIRXP) which in the cDNA-deduced amino acid sequence begins at position 364 and is preceded by an Arg residue.

In vitro biosynthetic studies using primary small-intestine cultures showed that Estr/PLase is synthesized as a single-polypeptide chain of approximately 180 kDa which corresponds fairly well to the calculated molecular mass (161 343 Da, from the cDNA-deduced amino acid sequence) considering that the protein is glycosylated. Most probably, the final biosynthetic product, once it has reached the brush border membrane, is subjected in vivo to the action of (pancreatic?) proteases, in particular of trypsin, leading to hydrolysis of the $Arg^{363}-Glu^{364}$ bond.[6] Furthermore, trypsin and/or other pancreatic proteases are likely to be responsible for the heterogeneity of the C-terminal region of Estr/PLase as isolated from small-intestine brush border membranes (see Figure 1 and Results). Note that the small-intestine explants which yielded the high-molecular mass Estr/PLase in biosynthetic experiments were totally devoid of pancreatic proteases, as shown by pro-SI not being split to the SI heterodimer (Figure 3) [pro-SI is an excellent internal control for this purpose; see, e.g., Keller et al. (1995)]. Expression of Estr/PLase in transiently transfected COS 7 cells corroborated the above results.

At What Age Is Estr/PLase Expressed? The cDNA clone of this enzyme had been originally isolated and identified in a differential cDNA library [adult intestinal minus baby rabbit cDNA (Boll et al., 1993)]. We nevertheless checked whether explants from the small intestine of rabbits aged 40 days synthesized Estr/PLase in vitro. As expected, no incorporation of [^{35}S]methionine was observed (data not shown). This enzyme seems therefore by all criteria to be confined to adulthood, at least in the rabbit. Presently, we have no data on the question of whether the appearance of this enzyme after weaning is due to a genetic program or whether it is perhaps "induced" by dietary components or by other factors.

[6] In spite of the use of protease inhibitors, we cannot completely rule out that some limited proteolysis may occur during the solubilization of Estr/PLase from the brush border membrane. Even so, however, this would not raise doubt about the conclusions of the present paper.

ACKNOWLEDGMENT

We are indebted to Dr. P. James for the amino acid sequences.

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BI962398A