Biogenesis of Intestinal Lactase-Phlorizin Hydrolase in Adults with Lactose Intolerance

Evidence for Reduced Biosynthesis and Slowed-down Maturation in Enterocytes

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Abstract

Enzymatic activity, biosynthesis, and maturation of lactase-phlorizin hydrolase (LPH) were investigated in adult volunteers with suspected lactose intolerance. Mean LPH activity in jejunal biopsy homogenates of these individuals was 31% compared to LPH-persistent individuals, and was accompanied by a reduced level of LPH-protein. Mean sucrase activity in individuals with low LPH was increased to 162% and was accompanied by an increase in sucrase-isomaltase (SI)-protein. Biosynthesis of LPH, SI, and aminopeptidase N (APN) was studied in organ culture of small intestinal biopsy specimens. In individuals with LPH restriction, the rate of synthesis of LPH was drastically decreased, reaching just 6% of the LPH-persistent group after 20 h of culture, while the rate of synthesis of SI appeared to be increased. In addition, maturation of pro-LPH to mature LPH occurred at a slower rate in LPH-restricted tissue. Immunoelectron microscopy revealed an accumulation of immunoreactive LPH in the Golgi region of enterocytes from LPH-restricted individuals and reduced labeling of microvillus membranes. Therefore, lactose intolerance in adults is mainly due to a decreased biosynthesis of LPH, either at the transcriptional or translational level. In addition, intracellular transport and maturation is retarded in some of the LPH-restricted individuals, and this leads to an accumulation of newly synthesized LPH in the Golgi and a failure of LPH to reach the microvillus membrane. (*J. Clin. Invest.* 1990. 86:1329–1337.) Key words: lactase • human intestine • biosynthesis • microvillus membrane • proteolytic processing • degradation

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Introduction

Lactase-phlorizin hydrolase (LPH)[1] (EC 3.2.1.23–3.2.1.62) is a major glycoprotein of the microvillus membrane in human small intestinal mucosa. It consists of two enzymic activities: lactase (β-D-galactoside galactohydrolase [EC 3.2.1.23]), which is responsible for the hydrolysis of lactose, and phlorizin hydrolase (glycosyl-*N*-acylsphingosine glucohydrolase [EC 3.2.1.62]) (1–3). The enzyme complex is synthesized as a single-chain precursor (M_r = 215–245 kD) with subsequent proteolytic processing taking place intracellularly (4–7). There is still uncertainty as to whether this processing takes place before (7) or after (6, 8) complex glycosylation. The primary structure of LPH from rabbit and human small intestine has recently been deduced from cDNA sequences (9). From these data it was concluded that the LPH complex is composed of a single polypeptide chain with both catalytic sites for lactase and phlorizin hydrolase located on this single polypeptide. LPH was further found to be anchored in the membrane via a hydrophobic amino acid sequence located near the COOH terminus of the protein with a short hydrophilic amino acid segment at the COOH terminus itself. LPH, it was further deduced, is synthesized in a pre-pro form with a cleavable signal peptide sequence at the NH_2 terminus, followed by a large (847 amino acids) pro-piece. It appears that only the mature form of LPH (M_r = 160 kD) reaches the microvillus membrane.

The milk of most mammals contains lactose, and small intestinal lactase activity is high during the newborn and suckling period when milk is the sole nutrient. In the rat, for example, lactase activity is high during the latter part of gestation and reaches a maximum at or shortly after birth, but declines markedly after weaning to a low adult level (10). Although lactase activity is low in the majority of human adults on a worldwide scale, certain individuals, notably Europeans and their descendants, have persistent high levels of lactase activity comparable with that found in healthy infants. The clinical presentation of a restriction in LPH in human adults may thus be the consequence of this "natural" decline in enzyme activity (11, 12) and is different from congenital LPH deficiency which has been reported as a rare cause of neonatal diarrhoea (13, 14). In adult-type hypolactasia, gel electrophoretic analysis of proteins obtained from brush border fragments showed

1. *Abbreviations used in this paper:* APN, aminopeptidase N; LPH, lactase-phlorizin hydrolase; pro-LPH, high-mannose precursor of LPH; SI, sucrase-isomaltase.

that the band corresponding to LPH was decreased proportionally to enzymatic activity (15). As the residual activity in the congenital form of LPH deficiency is lower than in the adult-type, an investigation of the molecular defects in these two forms is appropriate. A comparative gel electrophoretic analysis of both types revealed the same gel patterns and it was suggested that the origin of the deficiency may be similar (16). A simultaneous decrease of enzyme activity and LPH-protein has also been demonstrated by specific immunoelectrophoretic techniques and it was concluded that in adult-type hypolactasia the low enzyme activity was the result of decreased synthesis and not a modified inactive enzyme (17); rates of biosynthesis, however, were not measured.

In the present paper we present immunolabeling and immunoelectronmicroscopic studies undertaken with biopsy specimens from adults with lactose intolerance. The data from these studies show that this condition is the consequence of LPH restriction which is predominantly due to a greatly decreased rate of synthesis of LPH in the enterocyte. We further present evidence that in this type of LPH restriction (*a*) the molecular forms of the residual mature LPH are indistinguishable from those found in tissue with persistent high lactase activity, (*b*) the intracellular transport and maturation of LPH is slower than in individuals with LPH persistence, and (*c*) that in some instances the newly synthesized LPH fails to reach the microvillus membrane.

Methods

Patients/volunteers

32 adult volunteers known or suspected of having lactose intolerance were recruited for this study. Of these volunteers 19 were Caucasian, 11 Black, and 2 Chinese in origin. The study was approved by the Committee on the Conduct of Human Research at Medical College of Virginia, Virginia Commonwealth University. All volunteers gave their signed consent to take part in the study and underwent a brief medical history and examination at the outset. Volunteers with a history of any other gastrointestinal disorders, previous gastrointestinal surgery, excess alcohol ingestion, regular drug usage, or major systemic illness were excluded. Volunteers who had no history of lactose intolerance and who had jejunal lactase activity within the normal range served as controls.

Clinical phase

Volunteers were screened for lactose intolerance by an oral challenge with 50 g lactose on an empty stomach, followed by a hydrogen breath test over 2 h (18). A rise of breath hydrogen of > 20 ppm was taken as indicative of lactose intolerance. Those volunteers with a positive test underwent laboratory tests (hemoglobin, platelet count, prothrombin time, partial thromboplastin time, serum vitamin B12, folate, stool for occult blood) and, if normal, were scheduled to attend fasting for a suction capsule jejunal biopsy (19).

Laboratory phase

Processing of intestinal tissue. The biopsy specimens were cut into several pieces for further processing. One portion was immediately fixed for immunoelectron microscopy, two portions were frozen at −80°C for enzyme activity assays and ^{125}I-labeling, respectively, and the remaining pieces were used for organ culture experiments in which proteins were metabolically labeled with ^{35}S-methionine.

Immunoelectronmicroscopy. Freshly obtained jejunal tissue was fixed in 3% (wt/vol) paraformaldehyde/0.1% (wt/vol) glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.4) for 2 h at room temperature and stored in 2% (wt/vol) paraformaldehyde in the same buffer until further processing. Low temperature embedding in Lowicryl K4M, and immunolabeling on ultrathin sections was as previously described (20). The monoclonal antibody used was HBB1/909 raised against LPH, the second antibody was a rabbit anti-mouse IgG and, finally, the protein A was complexed to 10-nm gold particles. The sections were stained with a saturated aqueous solution of uranyl acetate and lead citrate and observed in a Phillips 410 EM operating at 80 kV.

Enzyme and protein assays. Disaccharidase activities were measured according to Dahlqvist (21) using lactose, sucrose, and maltose as substrates. Aminopepsidase N (APN) was assayed as previously described (22). Protein was determined according to Peterson (23) using bovine serum albumin as standard.

Monoclonal antibodies. All monoclonal antibodies used have been described elsewhere (8) and included HBB 1/909 against LPH, HBB 2/614 against sucrase-isomaltase (SI), and HBB 3/153 against APN.

[^{35}S]Methionine labeling in organ culture. Intestinal biopsy explants were labeled with 150 μCi L-[^{35}S]methionine per 1 ml culture medium according to Naim et al. (24). Labeling was for 30 min, 4 h, and 20 h, respectively. At the end of the labeling period the tissue was chilled by washing three times with ice-cold homogenization buffer (25 mM Tris-HCl/50 mM NaCl, pH 8.1), and homogenized in 1 ml of the same buffer containing 1 mM phenylmethyl-sulfonyl fluoride, 1 μg/ml pepstatin, 5 μg/ml leupeptin, 17.4 μg/ml benzamidine, and 1 μg/ml aprotinin.

^{125}I-labeling. Biopsy samples were homogenized, Triton-solubilized and centrifuged as described (7). The resulting 105,000 *g* supernatant fraction was iodinated by the chloramine-T procedure of Hunter and Greenwood (25) using 1 mCi Na^{125}I per sample and free iodine was removed by gel filtration on Sephadex G25 columns.

Immunoprecipitation. [^{35}S]Methionine-labeled mucosal homogenates were solubilized with Nonidet P-40 and sodium deoxycholate, both at 0.5% (wt/vol) final concentration, by slowly stirring on ice for 30 min. The solubilized material was filtered through 0.45-μm Millex-HA filters, and the total protein determined in the filtrate. The filtrates were then diluted to contain 0.2 mg/ml of protein and 1 ml aliquots were used for immunoprecipitation. Samples were first precleared overnight using 80 μl protein A-Sepharose, followed by sequential immunoprecipitation with monoclonal antibodies against LPH, sucrase, and aminopeptidase N bound to (CnBr)-activated Sepharose. Typically, 35 μl of packed immunobeads were incubated at 4°C for 1.5 h. The immunoprecipitates were washed as previously described (7, 8) before analysis by SDS-PAGE.

Sodium dodecyl sulfate polyacrylamide-gel electrophoresis. Washed immunoprecipitates were solubilized with 50 μl of twofold concentrated electrophoresis sample buffer, 10 μl of 20% SDS, 10 μl of 0.1 M dithiothreitol, boiled for 4 min, and submitted to electrophoresis on 5% acrylamide gels according to Laemmli (26). The molecular weight standards were: myosin, 202 kD; β-galactosidase, 116 kD; phosphorylase b, 97 kD; bovine serum albumin, 66 kD; and ovalbumin, 45 kD. In some gels the position of actin is also indicated. The gels were stained with Coomassie blue, destained, and treated with Amplify, dried, and exposed on Kodak SO-282 films at −80°C. The films were developed in a 3M XP507 x-ray processor and scanned in a TLC scanner (Camag Ltd., Muttenz, Switzerland). Incorporation of radioisotope into specific molecular forms of proteins was expressed as integrated peak areas.

Statistical analysis. Student *t*-test statistical analyses were applied throughout.

Results

Assessment of lactose intolerance. Intolerance to lactose was established by challenge with 50 g lactose followed by the hydrogen breath test. Lactose intolerance was indicated by a rise in breath hydrogen of 20 ppm or more. Of the 32 volunteers, 18 were found to be lactose intolerant and 14 were tolerant. Of the individuals unable to tolerate lactose, seven were Cauca-

Table I. Disaccharidase activities in 14 patients with lactose intolerance and 5 control subjects

Group	Parameter	Specific activity (*IU/g protein*)			
		Lactase	Sucrase	Maltase	Isomaltase
Controls	Mean	27.4	46.1	191.0	67.9
(n = 5)	SD	7.9	4.2	24.4	7.5
Lactose intolerant	Mean	8.5	74.6	161.2	78.5
(n = 14)	SD	4.6	26.5	33.8	28.7
	P	<0.001	<0.05	<0.5	>0.5

Disaccharidase activities in homogenates of small intestinal mucosa, obtained by suction biopsy, in control subjects and individuals with clinical lactose intolerance. Enzyme activities were determined as described in Methods using lactose, sucrose, maltose, and isomaltose, respectively, as substrates. Activities are expressed as international units per gram mucosal protein.

sian, nine were Black, and two were Chinese. Of the 14 found lactose tolerant, 12 were Caucasian and two Black. All lactose-intolerant individuals gave a history of bloating, abdominal pain, or diarrhoea after the ingestion of milk. The onset of lactose intolerance varied in this population from childhood to young adulthood but the exact age of onset was difficult to assess.

14 of the 18 volunteers with lactose intolerance took part in the second stage of the study and underwent a jejunal biopsy. Five separate lactose-tolerant Caucasian young adults with no history of intestinal problems served as controls.

Disaccharidase activities. A decrease in lactase activity was found in all volunteers with a positive hydrogen breath test, with specific activity in mucosal homogenates ranging from 2.4 to 18 IU/g protein (mean, 8.5; SD, 4.6). Individuals within this range were considered to have LPH restriction. In contrast, the specific activity in control subjects ranged from 38 to 50 IU/g protein (mean, 27.4; SD, 7.9). The specific activity of lactase in the LPH-restricted group amounted to 31% of the control group (Table I). Lactase activity varied considerably in both groups, and all lactose-intolerant patients had some residual activity. Maltase and isomaltase activities were not significantly different in the two groups. Sucrase activity was increased to 162% in the lactose-intolerant group (mean, 74.6; SD, 26.5) compared to the control group (mean, 46.1; SD, 4.2).

Identification of LPH after iodination. Quantitative immunoisolation of iodinated enzymes using monoclonal antibodies, followed by SDS-PAGE and autoradiography, allowed the identification of the steady-state forms of these enzymes. Scanning of the autoradiographs further allowed a comparison of LPH-protein to APN-protein, another microvillar enzyme which is unchanged in LPH-restricted tissue. In both LPH-persistent and in LPH-restricted tissue, both the pro-LPH (215 kD) and the mature LPH (160 kD) were identified, the latter being the predominant form (Fig. 1, lanes *1–6*). In addition, a polypeptide has repeatedly been found with apparent $M_r \geq 260$ kD. This polypeptide had the same NH_2-terminal amino acid sequence (a.a 1–5) as the mature enzyme and is probably a dimeric form of the mature enzyme (Sterchi, E. E., unpublished data). The amount of LPH in mucosa from volunteers with LPH restriction (Fig. 1, lanes *1–4*) varied markedly but was significantly decreased compared to controls with persistent high lactase activity (Fig. 1, lanes *5* and *6*). No differences were detected in the molecular forms of APN between the two groups (Fig. 1, lanes *7–11*). The mature LPH-to-APN ratio, as determined from the mean peak areas of the appropriate bands after scanning of the autoradiographs, was decreased to 15% in hypolactasia (Fig. 2).

Identification of LPH after labeling with [^{35}S]methionine in organ culture. Fig. 3 shows the gel electrophoretic analysis of LPH in comparison to SI and APN immunoisolated from the mucosal biopsy of a control subject with persistent lactase activity after continuous labeling in organ culture with [^{35}S]me-

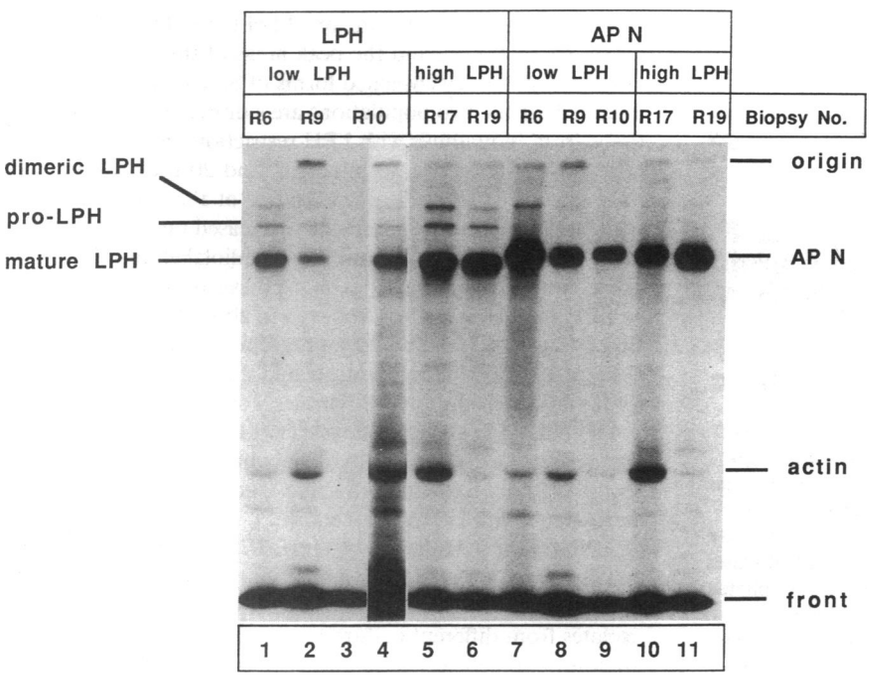

Figure 1. ^{125}I-Labeled LPH and APN from three volunteers with lactose intolerance and two control subjects. Triton X-100-solubilized mucosal homogenates were labeled with Na^{125}I by the chloramin T procedure and the brush border hydrolases immunoprecipitated with monoclonal antibodies against LPH (lanes *1–6*) or APN (lanes *7–11*). The immunoprecipitates were separated by SDS-PAGE (7.5% gel) followed by visualization through autoradiography. All lanes were derived from the same gel. Exposure times for lanes *1–3* and *5–11* were identical. Lane *4* represents a longer exposure of lane *3* to demonstrate that some residual LPH is in fact present. Pro-LPH, precursor of mature LPH hydrolase; L, mature LPH; dLPH, dimeric mature LPH; APN, position of mature aminopeptidase N.

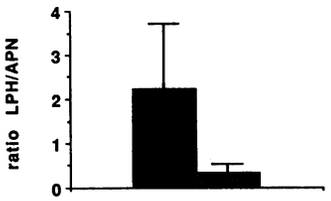

Figure 2. Ratio of mature LPH-protein to mature APN-protein in 10 volunteers with lactose intolerance and four control subjects. Iodination, immunoprecipitation, and separation on SDS-PAGE, and autoradiography were as described for Fig. 1. Autoradiographs were scanned, and the peak area of mature LPH and mature APN determined. The peak ratios LPH/APN were then calculated from the mean of the peak areas in patients with lactose intolerance (n = 10) and control subjects (n = 4). Black column, control subjects; shaded column, individuals with lactose intolerance. Bars = standard deviation. $P \leq 0.01$.

thionine for 30 min, 4 h, and 20 h, respectively. After 30 min of labeling single bands, representing high mannose precursors, were obtained for LPH and SI (Fig. 3, LPH and SI lanes *a*); for APN, some processing to the complex glycosylated form was evident (Fig. 3, APN, lane *a*). After 4 h and 20 h of labeling, the pro-LPH and the mature LPH could both be identified (Fig. 3, LPH, lanes *b* and *c*). As reported previously (7) no complex glycosylated pro-LPH was detected. As with the gel electrophoretic analysis of iodinated LPH the larger (M_r ≥ 260 kD) polypeptide species was often seen (Fig. 3, LPH lane *c*). The additional band below the mature LPH found after 20 h of culture (Fig. 3, LPH lane *c*) was not identified. For SI and APN both high mannose and complex glycosylated forms were identified (Fig. 3, SI and APN, lane *c*). The quantitative immunoisolation of the individual enzymes from equal amounts of solubilized mucosal protein allowed a direct comparison of the intensity of the different enzyme bands on the gels. The amount of radiolabeled LPH present after 4 h and 20 h of culture was thus comparable to APN. In comparison, the SI bands obtained were somewhat weaker.

Fig. 4 shows a gel electrophoretic comparison of LPH, SI, and APN in mucosa from an individual with LPH restriction. The molecular forms of all three microvillar enzymes investi-

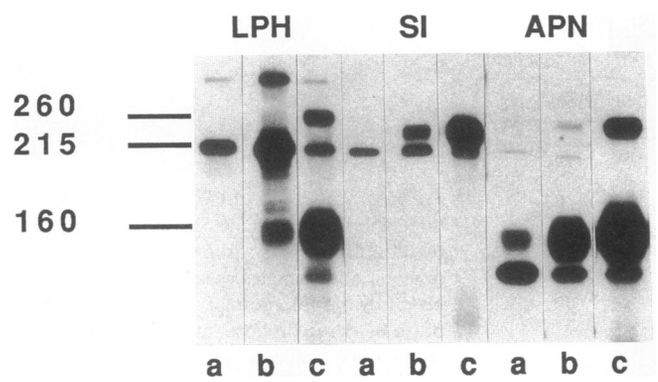

Figure 3. ^{35}S-labeled LPH, SI, and APN in control subject (LPH-persistent). Small intestinal biopsy specimens were cultured in organ culture dishes for 2 h in complete RPMI-1640 medium without methionine, then for 0.5, 4, and 20 h in the presence of 150 μCi of [^{35}S]methionine (continuous labeling). After homogenization in the presence of protease inhibitors, brush border membrane hydrolases were solubilized, and LPH, SI, and APN were immunoprecipitated and analyzed by SDS-PAGE and fluorography (see Methods). Exposure time was 2 d. (*a*) 0.5 h; (*b*) 4 h; (*c*) 20 h. 260 kD, dimeric form of mature LPH; 215 kD, LPH-precursor; 160 kD, mature LPH.

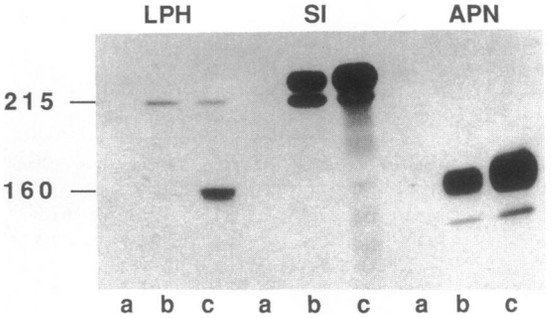

Figure 4. ^{35}S-labeled LPH, SI, and APN in subject with lactose-intolerance (LPH-restricted). Small intestinal biopsy specimens were cultured in organ culture dishes for 2 h in complete RPMI-1640 medium without methionine, then for 0.5, 4, and 20 h in the presence of 150 μCi of [^{35}S]methionine (continuous labeling). After homogenization in the presence of protease inhibitors, brush border membrane hydrolases were solubilized, LPH, SI, and APN immunoprecipitated and analyzed by SDS-PAGE and fluorography (see Methods). Exposure time was 2 d. (*a*) 0.5 h; (*b*) 4 h; (*c*) 20 h; 215 kD, LPH-precursor; 160 kD, mature LPH.

gated were the same as in controls (see Fig. 3). The intensity of the LPH bands was decreased considerably compared to the controls, indicating a decreased rate of biosynthesis of this enzyme. The SI bands displayed varying labeling intensities that correlated with variations in the enzymic activities of S and I. No significant difference in intensity of the APN bands was observed. Significantly, after 4 h of culture a higher proportion of the LPH present was still in the form of the high-molecular mass precursor in lactase-restricted individuals (Fig. 4, LPH, lane *b*).

Rate of incorporation of [^{35}S]methionine into LPH, SI, and APN. Densitometric scannings obtained from fluorographs of four controls and 13 individuals with LPH restriction were used to assess the incorporation of [^{35}S]methionine into LPH, SI, and APN protein. For this purpose, time of exposure (2 d) and scanner settings were calibrated for all fluorographs. The total incorporation of radiolabel was calculated by adding the peak areas obtained from the pro-LPH (215 kD) and the mature LPH (160 kD), and the peak areas of the high-mannose and the complex-glycosylated forms of SI and APN. The data obtained for the two populations are summarized in Fig. 5. In tissue from individuals with LPH restriction, incorporation of label into LPH during 30 min, 4 h, and 20 h of culture was only 0.6, 6.7, and 6.1%, respectively, of that obtained with control tissue, indicating a greatly decreased rate of biosynthesis of LPH-protein. Incorporation of radiolabel into SI, on the other hand, suggests that the biosynthesis of SI was increased in LPH-restricted tissue. Expressed in absolute terms the ratio SI/LPH after 0.5, 4, and 20 h of culture were 0.24, 0.21, and 0.45 in LPH-persistent controls and 12.7, 14.5, and 16.6 in LPH-restricted subjects. Biosynthesis of APN was not significantly altered in tissue with restricted LPH. These data are in line with the enzyme activity data, which showed diminished lactase activity and elevated sucrase activity in mucosa from individuals with lactose intolerance.

Proteolytic conversion of pro-LPH to mature LPH. The results of the scanned gels were employed to assess the distribution of the different molecular species of LPH in immunoisolates from different culture time points (Fig. 6). After 0.5 h of culture, practically all the LPH present was still in the pre-

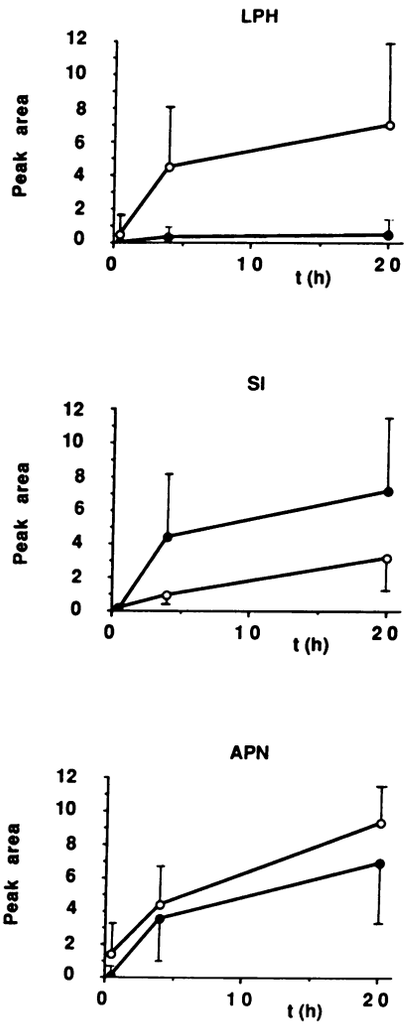

Figure 5. Rate of incorporation of [^{35}S]methionine into LPH, SI, and APN in organ culture of small intestinal biopsies. Biopsies from LPH-restricted patients and LPH-persistent subjects (controls) were all processed as described in the legend to Fig. 3 and in Methods. Fluorographs were scanned and peak areas of pro-LPH, mature LPH, SI, and APN determined. Incorporation into LPH-protein was determined by addition of the areas of the 215-kD and the 160-kD peaks; incorporation into SI and APN was determined by addition of the peaks corresponding to the high-mannose and the complex glycosylated (mature) forms. (*Open circles*) LPH-persistent control subjects (n = 4); (*closed circles*) LPH-restricted volunteers (n = 13). Bars = standard deviation of the data. Determination of significance was by Student t-test analysis. LPH: 0.5 h, $P \leq 0.1$; 4 h, $P \leq 0.002$; 20 h, $P \leq 0.001$. APN: 0.5 h, $P \leq 0.05$; 4 h, $P \leq 1$; 20 h, $P \leq 0.5$. SI: 0.5 h, $P \leq 1$; 4 h, $P \leq 0.2$; 20 h, $P \leq 0.2$.

cursor form in both LPH-persistent control tissue and in LPH-restricted tissue. After 4 h of culture, control tissue had processed more than half of the precursor to the mature enzyme, leaving 43% in the precursor form. LPH-restricted tissue had processed little of the precursor, as 85% of the total LPH was still present in this form. After 20 h of culture, 19% of LPH was present in the precursor form in LPH-persistent control tissue vs. 28% in LPH-restricted tissue.

To amplify the LPH bands obtained, fluorographs were developed after an exposure time of 11 d. This time of exposure was only suitable for LPH from LPH-restricted tissue, as both SI and APN were overexposed and could not be scanned. The same was the case for all enzymes from LPH-persistent control mucosa. The labeling pattern for the three culture time points, i.e., the distribution of pro-LPH to mature LPH, varied considerably in hypolactasia tissue. Fig. 7 shows the immunoisolated species of LPH in three individuals with hypolactasia (R_5, R_6, R_{10}) after 30 min, 4 h, and 20 h of culture; they represent the whole spectrum of the pattern obtained. Visual analysis of these fluorographs showed that in tissue from subjects with hypolactasia residual LPH was synthesized. Both high-mannose pro-LPH and mature LPH isolated had the same relative molecular mass as those isolated from control tissue. A further observation was that in contrast to control tissue, additional peptide bands with a higher molecular mass than pro-LPH were detected in hypolactasia tissue (e.g., Fig. 7, R6, 20 h). Although this was not verified by analysis with endoglycosidases, one of these is likely to be a complex glycosylated form of pro-LPH. The extent to which the proteolytic conversion was retarded varied considerably among the hypolactasia patients, as demonstrated by the results shown in Fig. 7. In volunteer R_5 the distribution of pro-LPH/mature LPH was 68/32% (controls, 43/57%) after 4 h of culture and 23/77% (controls, 20/80%) after 20 h of culture; in volunteers R_6 and R_{10} the ratios were 90/10% after 4 h and 32/68% and 39/61%, respectively, after 20 h of culture. R_{10} represents a group of eight individuals with LPH restriction (out of a total of 13) where the amount of labeled LPH on the gel was diminished after 20 h of culture (Fig. 7). The remaining volunteers with LPH restriction showed an increase of labeled LPH over the 20-h culture period.

Immunolocalization of LPH in tissue from individuals with LPH restriction and from controls with persistent LPH. Immunolocalization of LPH was determined on ultrathin cryosections of freshly fixed mucosa with the protein A-gold method using the same monoclonal antibody to LPH as was

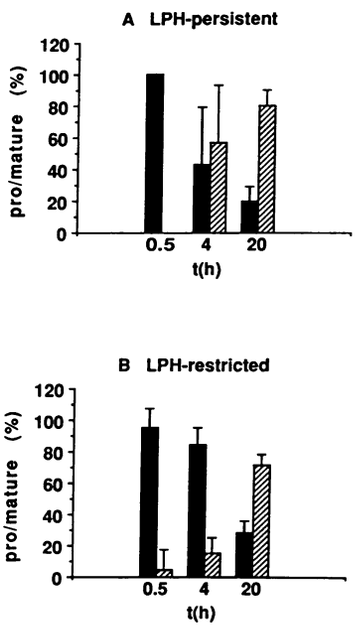

Figure 6. Ratio of pro-LPH to mature LPH after 0.5, 4, and 20 h of organ culture in the presence of [^{35}S]methionine. After culture, processing of the biopsies was as in the legend to Fig. 3. Integrated peak areas for pro-LPH and mature LPH were determined by scanning in a Camag TLC scanner and the distribution of the two forms calculated. For each time point pro-LPH + mature LPH = 100%. (*Solid columns*) pro-LPH; (*hatched columns*) mature LPH. Bars = standard deviation of the data. For 0.5 h timepoint of controls (n = 2) only pro-LPH was detectable, hence no SD bars are included. Determination of significance was by Student t-test analysis; 0.5 h, $P \leq 1$; 4 h, $P \leq 0.005$; 20 h, $P \leq 0.1$.

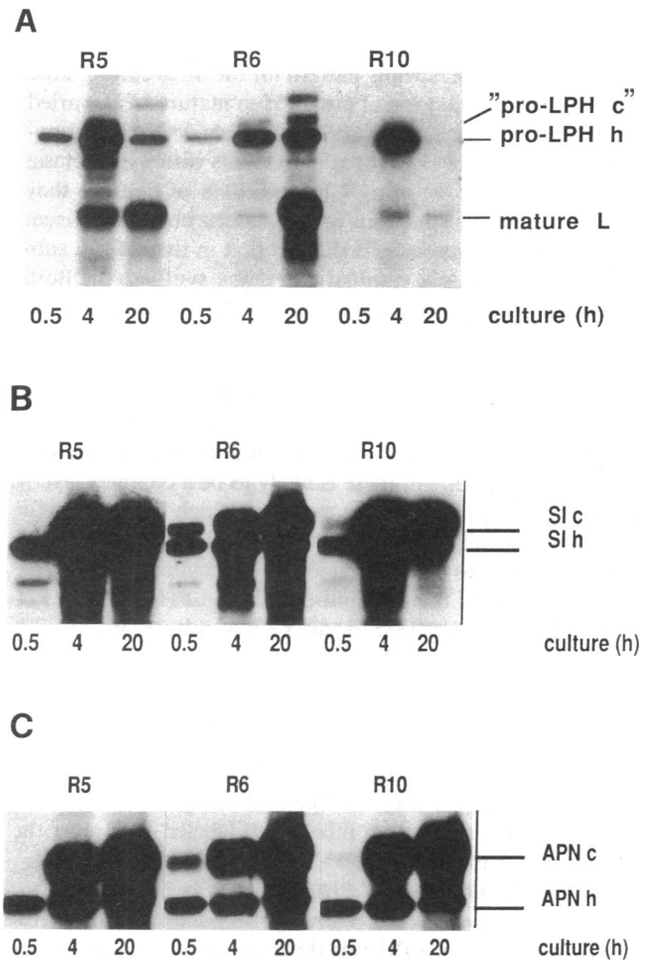

Figure 7. Identification of different forms of LPH in individuals with clinical lactose intolerance by SDS-PAGE after labeling with [^{35}S]methionine in organ culture. Small intestinal biopsies from three volunteers (R_5, R_6, R_{10}) with lactose intolerance were labeled in organ culture for 0.5, 4, and 20 h with 150 μCi of [^{35}S]methionine, LPH, SI, and APN immunoprecipitated and analyzed by SDS-PAGE and fluorography (see Methods). *A* = LPH, *B* = SI, *C* = APN. Exposure time was 11 d.

used for the immunoprecipitations. In control tissue with persistent LPH, labeling was practically limited to the microvillus membrane, i.e., little label above background was present intracellularly (Fig. 8, *a* and *b*). In contrast, tissue from individuals with LPH restriction showed different patterns of immunolabeling. First, labeling intensity was decreased in all tissue samples from these subjects. Second, the pattern of intracellular distribution varied, as is demonstrated by the EM pictures with tissue from patients R_5, R_6, and R_{10} (Fig. 9). Tissue from volunteer R_5 showed little to no labeling of the microvillus membrane or Golgi apparatus (Fig. 9, *a* and *b*). Tissue from volunteer R_6 showed labeling of the microvillus membrane which was reduced compared to LPH-persistent tissue and also showed positive labeling of the Golgi region (Fig. 9, *c* and *d*) and tissue from volunteer R_{10} exhibited very little labeling in the microvillus membrane but considerable accumulated immunoreactivity in the Golgi region (Fig. 9, *e* and *f*). These findings on the cellular distribution of LPH are consistent with the findings on the proteolytic conversion of pro-LPH to mature LPH, i.e., the more this conversion was delayed the more accumulation of immunoreactive LPH was found in the Golgi region of the enterocytes. In addition, a correlation was seen with the data from iodinated LPH, i.e., tissue from those individuals where processing was delayed and Golgi-accumulation was found, the amount of total ^{125}I-LPH-protein precipitated was decreased (e.g., R10).

Discussion

In this study we have set out to investigate the expression and the posttranslational processing of LPH in individuals who, as adults, suffer from lactose intolerance. The findings are compared with those obtained for SI and APN, two other prominent hydrolases of the intestinal microvillus membrane.

All volunteers who proved lactose intolerant had decreased levels of enzymic lactase activity in their jejunal mucosa. This decrease in activity correlated with a specifically reduced level of LPH-protein in the intestinal mucosa from these individuals compared to controls (i.e., subjects with persistent lactase activity). All lactose-intolerant volunteers, however, had residual lactase activity and this, together with their medical his-

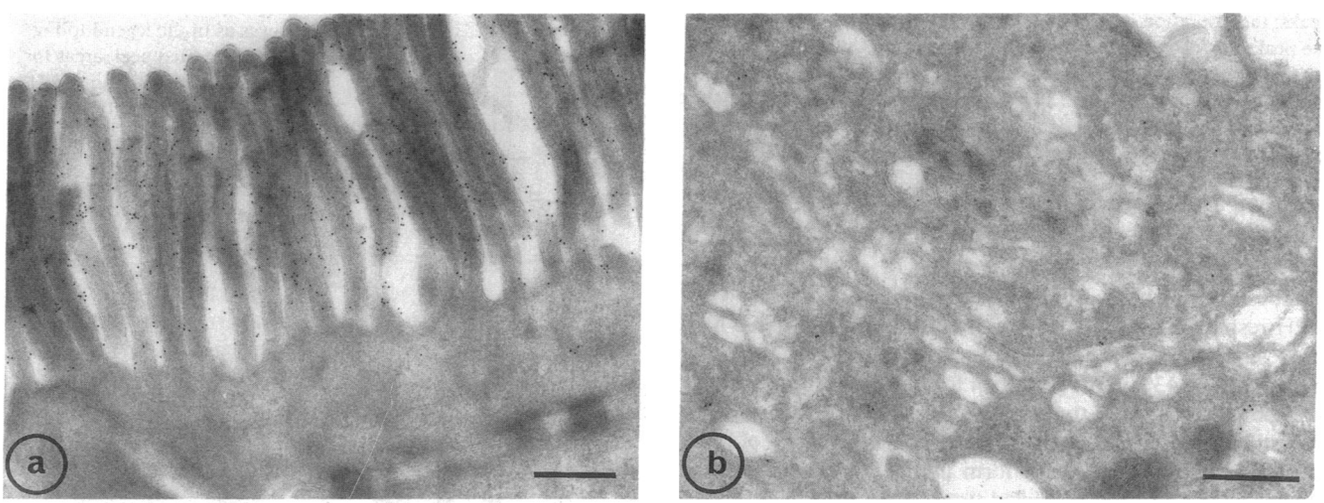

Figure 8. Immunolocalization of LPH in intestinal epithelial cells of adults with a high lactase activity. Ultrathin cryosection of a biopsy from a LPH-persistent control. Incubation with monoclonal antibody HBB1/909/34/74 against LPH shows a clear labeling of the microvillus membrane of intestinal epithelial cells (*a*). The Golgi apparatus is only weakly labeled (*b*). Bar = 0.5 μm.

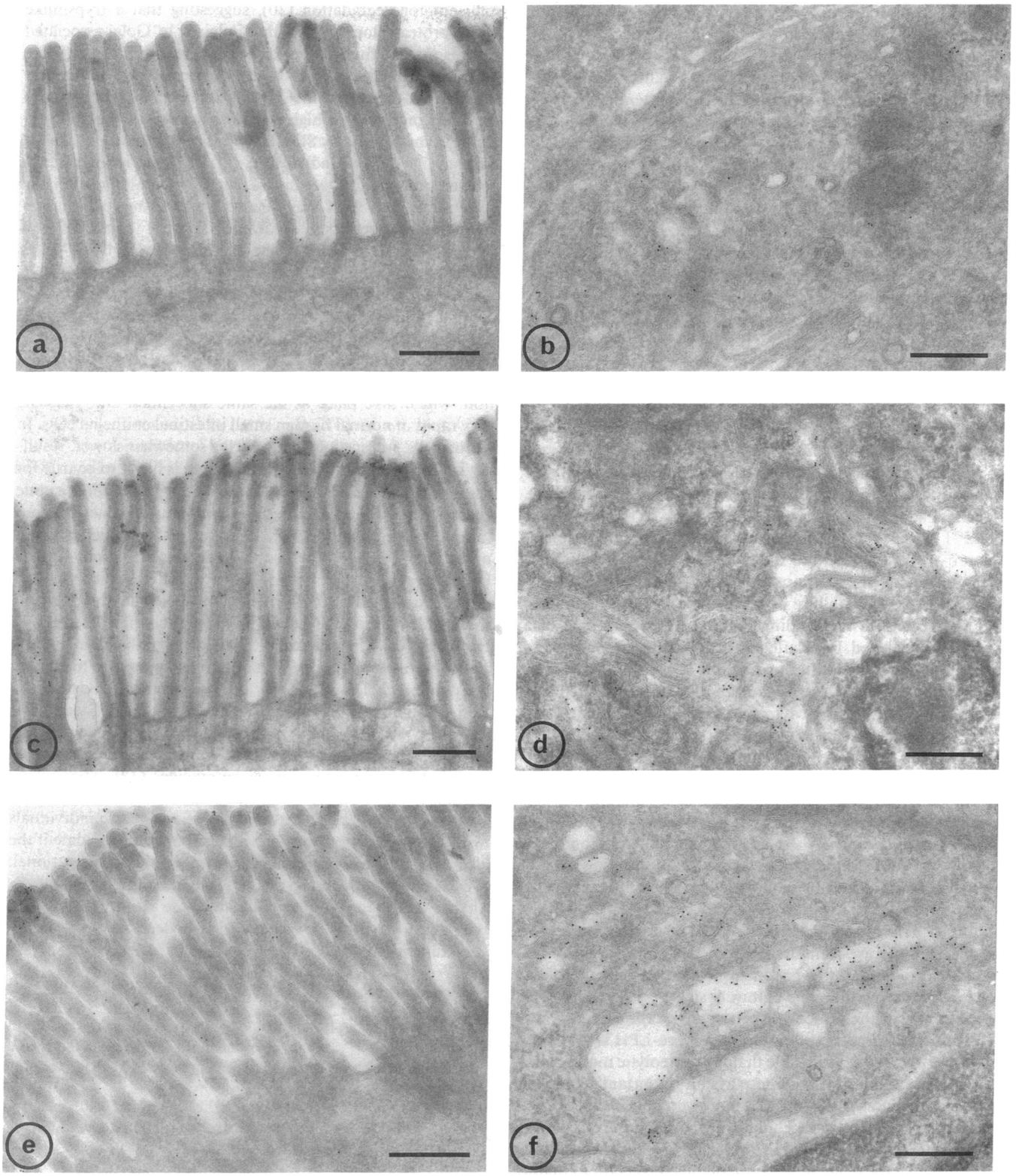

Figure 9. Immunolocalization of LPH in intestinal epithelial cells of adults with restricted LPH. Ultrathin cryosection of three individuals with LPH restriction incubated with monoclonal antibody HBB1/909/34/74. Volunteer R_5 shows little to no labeling of the microvillus membrane (*a*) and the Golgi apparatus (*b*). Volunteer R_6 shows labeling of the microvillus membrane (*c*) although less than observed in the controls. The Golgi region on the other hand shows more label (*d*) than LPH-persistent controls. Volunteer R_{10} shows a minor labeling of the microvillus membrane (*e*). However, the Golgi region in this individual (*f*) shows a very strong labeling as compared with control biopsies and volunteer R_6. Bar = 0.5 μm.

tory, is a strong indication for late onset LPH restriction and not congenital LPH deficiency.

Labeling studies with ^{125}I and [^{35}S]methionine showed the molecular forms of pro-LPH (215 kD) and mature LPH (160 kD) to be indistinguishable in both LPH-persistent and LPH-restricted individuals (see also reference 7). On the basis of NH_2-terminal amino acid sequencing it must be concluded that the additional high molecular mass LPH-polypeptide (260 kD) is a dimeric form of the mature LPH (Sterchi, E. E., unpublished data) (9). Evidence for dimers have been found by others (15, 27), although detailed gel electrophoretic analysis of the different molecular species was only carried out in one study (27). Earlier studies in our laboratory, using endoglycosidases, have yielded the same molecular forms of deglycosylated LPH (7). Therefore, it appears unlikely that there are differences in glycosylation pattern of LPH between individuals with persistent LPH and those with LPH restriction. On the basis of biosynthetic labeling of jejunal biopsy explants with [^{35}S]methionine in organ culture, it can be concluded that the decreased LPH activity in individuals suffering from lactose intolerance is the consequence of a greatly decreased rate of synthesis of LPH, although an increased cellular degradation cannot be excluded. Others have also reported the decline in lactase activity to be related to a decrease in the expression of LPH (16, 28). Furthermore, the proteolytic conversion of the pro-LPH to the mature LPH may be delayed, resulting in some instances in an intracellular degradation of newly synthesized LPH and a failure of this protein to reach the microvillus membrane. The finding in LPH-restricted tissue of a decrease in labeled LPH after 20 h of culture is difficult to explain due to the continuous labeling. It was not observed in LPH-persistent tissue (see Fig. 3) and is thus considered specific for the LPH-restricted group. It cannot be excluded that LPH synthesis (or degradation) is especially sensitive to prolonged organ culturing in LPH-restricted tissue. There is, however, no indication that the structure of LPH is altered in the restricted group, and other brush border membrane enzyme data indicate the system does not lose viability in a 20-h period. Another possibility is a conformational alteration of the synthesized LPH in enzyme-restricted tissue leading to a diminished affinity for our monoclonal antibody. This is, however, unlikely because the enzymatic activity parallels the amount of immunoprecipitated LPH. In earlier studies on congenital sucrase-isomaltase deficiency (24), we have observed degradation of SI-precursor protein in some molecular phenotypes using the same culture system.

The role of the proteolytic processing in the maturation of LPH is not clear. It is still not known if pro-LPH is enzymatically fully active. It is possible that the proteolytic modification leading to the mature enzyme plays an important role in the maturation and sorting of LPH. However, a more likely interpretation is that the intracellular transport and the maturation of LPH is slower in many individuals with LPH restriction. In this instance the delay in the proteolytic conversion to mature LPH would merely be an indication of a delayed arrival of newly synthesized LPH in the Golgi. Nothing is known about the enzyme or enzymes involved in the proteolytic conversion of pro-LPH to mature LPH. Most of the available information on posttranslational proteolytic processing has come from work on prohormones and neuropeptides (29). Proteolytic processing of some of these peptides requires pairs of dibasic amino acids, whereas for others a single dibasic amino acid is sufficient for degradation (30), suggesting that a trypsinlike serine protease localized in the Golgi or in Golgi-associated vesicles may be involved. Such a localization for the putative processing enzyme of LPH would be consistent with our earlier findings that in normal human intestine no complex glycosylated precursor or high-mannose mature LPH can be detected upon analysis by SDS-PAGE of biosynthetically labeled enzyme (7). In those individuals with LPH restriction where a delayed proteolytic processing occurs, some putative complex glycosylated pro-LPH was detected, suggesting that the delayed transport results in an extended stay of the pro-LPH in those cellular organelles where complex glycosylation takes place. The protease(s) involved are thus probably located in the Golgi itself. This localization could also be reconciled with the data obtained with the human colon adenocarcinoma cell-line Caco2 (8) and with pig small intestine (31), where complex-glycosylated pro-LPH is normally detected. It must be assumed that proteolytic processing and complex glycosylation, which take place at the same subcellular site, must be very rapid in normal human small intestinal epithelial cells. In Caco2 cells, intracellular transport is somewhat slower, resulting in these subtle differences. The same is likely to be true for tissue from subjects with LPH restriction. In addition, subtle changes in the microenvironment of the intestinal mucosa from some individuals with LPH restriction which are not morphologically or biochemically detectable, might further impede transport of LPH to the microvillus membrane. It is well documented that lactase activity is affected earlier and more severely than the activity of most microvillus membrane hydrolases by different pathological states of the gastrointestinal tract. In celiac disease, lactase activity is the first to decline after gluten challenge and the last to recover after reintroduction of a gluten-free diet (Ambühl, P. M., E. E. Sterchi, H. Gaze, H. Y. Naim, and M. J. Lentze, unpublished results). Also, low levels of treatment with colchicine has been shown to inhibit LPH expression in rat enterocytes (32). The experimental design of the present study has enabled us to analyze expressed protein. The next step will be to analyze individuals with LPH restriction with molecular probes to elucidate if the decrease in LPH expression is transcriptional or translational. This is particularly interesting in the light of findings by others, that in adult rabbits the decline in lactase activity during normal development is not paralleled by a decrease in the corresponding mRNA (9, 33).

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References

1. Schlegel-Haueter, S., P. Hore, K. R. Kerry, and G. Semenza. 1972. The preparation of LPH and glucoamylase of rat small intestine. *Biochim. Biophys. Acta.* 258:506–519.

2. Colombo, V., H. Lorenz-Meyer, and G. Semenza. 1973. Small intestinal phlorizin hydrolase: "the β-glycosidase complex." *Biochim. Biophys. Acta.* 317:412–424.

3. Skovbjerg, H., H. Sjöström, and O. Norén. 1981. Purification

and characterization of amphiphilic lactase-phlorizin hydrolase from human small intestine. *Eur. J. Biochem.* 114:653–661.

4. Skovbjerg, H., E. M. Danielsen, O. Norén, and H. Sjöström. 1984. Evidence for biosynthesis of lactase-phlorizin hydrolase as a single chain high molecular weight precursor. *Biochim. Biophys. Acta.* 798:247–251.

5. Büller, H. A., R. K. Montgomery, W. V. Sasak, and R. J. Grand. 1987. Biosynthesis, glycosylation, and intracellular transport of intestinal lactase-phlorizin hydrolase in rat. *J. Biol. Chem.* 262:17206–17211.

6. Danielsen, E. M., H. Skovbjerg, O. Norén, and H. Sjöström. 1984. Biosynthesis of intestinal microvillar proteins. Intracellular processing of lactase-phlorizin hydrolase. *Biochem. Biophys. Res. Commun.* 122:82–90.

7. Naim, H. Y., E. E. Sterchi, and M. J. Lentze. 1987. Biosynthesis and maturation of lactase-phlorizin hydrolase in the human small intestinal epithelial cells. *Biochem. J.* 241:427–433.

8. Hauri, H. P., E. E. Sterchi, D. Bienz, J. A. M. Fransen, and A. Marxer. 1985. Expression and intracellular transport of microvillus membrane hydrolases in human intestinal epithelial cells. *J. Cell Biol.* 101:838–851.

9. Mantei, N., M. Villa, T. Enzler, H. Wacker, W. Boll, P. James, W. Hunziker, and G. Semenza. 1988. Complete primary structure of human and rabbit lactase-phlorizin hydrolase: implications for biosynthesis, membrane anchoring and evolution of the enzyme. *EMBO (Eur. Mol. Biol. Organ.) J.* 7:2705–2713.

10. Doell, R. G., and N. Kretchmer. 1962. Studies of small intestine during development. I. Distribution and activity of β-galactosidases. *Biochim. Biophys. Acta.* 62:343–362.

11. Kretchmer, N. 1971. Memorial lecture: lactose and lactase—a historical perspective. *Gastroenterology.* 61:805–813.

12. Asp, N. G., and A. Dahlqvist. 1974. Intestinal β-galactosidases in adult low lactase activity and different small intestinal β-galactosidases in adults with and without lactase deficiency. *Scand. J. Gastroenterol.* 6:755–762.

13. Holzel, A., V. Schwarz, and K. W. Sutcliffe. 1959. Defective lactose absorption causing malnutrition in infancy. *Lancet.* i:1126–1128.

14. Launiala, K., P. Kuitunen, and J. K. Visakorpi. 1966. Disaccharidases and histology of duodenal mucosa in congenital lactose malabsorption. *Acta Paediatr. (Uppsala).* 55:257–263.

15. Crane, R. K., D. Menard, H. Preiser, and J. Cerda. 1977. The molecular basis of brush border membrane disease. *In* The International Conference on Biological Membranes. L. Bolis, editor. Elsevier Science Publishers B.V., Amsterdam.

16. Freiburghaus, A. U., J. Schmitz, M. Schindler, H. W. Rotthauwe, P. Kuitunen, K. Launiala, and B. Hadorn. 1976. Protein patterns of brush border fragments in congenital lactose malabsorption and in specific hypolactasia of the adult. *N. Engl. J. Med.* 294:1029–1032.

17. Skovbjerg, H., E. Gudmand-Høyer, and H. J. Fenger. 1980. Immunoelectrophoretic studies on human small intestinal brush border proteins: amount of lactase protein in adult-type hypolactasia. *Gut.* 21:360–364.

18. Newcomer, A. D., D. B. McGill, P. J. Thomas, and A. F. Hofmann. 1975. Prospective comparison of indirect methods for detecting lactase deficiency. *N. Engl. J. Med.* 293:1232–1236.

19. Crosby, W. H., and H. W. Kugler. 1957. Intraluminal biopsy of the small intestine. *Am. J. Digest. Dis.* 2:236–241.

20. Sterchi, E. E., H. Y. Naim, M. J. Lentze, H. P. Hauri, and J. A. M. Fransen. 1988. *N*-Benzoyl-*l*-tyrosyl-*p*-aminobenzoic acid hydrolase: a metalloendopeptidase of the human intestinal microvillus membrane which degrades biologically active peptides. *Arch. Biochem. Biophys.* 265:105–118.

21. Dahlqvist, A. 1968. Assay of intestinal disaccharidases. *Anal. Biochem.* 22:99–107.

22. Sterchi, E. E., and J. F. Woodley. 1980. Peptide hydrolases of the human small intestinal mucosa: distribution of activities between brush border membranes and cytosol. *Clin. Chim. Acta.* 102:49–56.

23. Peterson, G. 1983. Determination of total protein. *Methods Enzymol.* 91:95–119.

24. Naim, H. Y., J. Roth, E. E. Sterchi, M. J. Lentze, P. Milla, J. Schmitz, and H.-P. Hauri. 1988. Sucrase-isomaltase deficiency in humans: different mutations disrupt transport, processing, and function of an intestinal brush border enzyme. *J. Clin. Invest.* 82:667–679.

25. Hunter, W. M., and F. C. Greenwood. 1962. Preparation of iodine-130 labeled human growth hormone of high specific activity. *Nature (Lond.).* 194:495–496.

26. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.).* 227:680–685.

27. Potter, J., M.-W. Ho, H. Bolton, A. J. Furth, D. M. Swallow, and B. Griffiths. 1985. Human lactase and the molecular basis of lactase persistence. *Biochem. Genet.* 23:423–439.

28. Jonas, M. M., R. K. Montgomery, and R. J. Grand. 1985. Intestinal lactase synthesis during postnatal development in the rat. *Pediatr. Res.* 19:956–962.

29. Steiner, D. F., K. Docherty, and R. Carroll. 1984. Golgi/granule processing of peptide hormone and neuropeptide precursors: a minireview. *J. Cell. Biochem.* 24:121–130.

30. Rosen, A. M., and D. M. Geller. 1977. Chicken microsomal albumin: amino terminal sequence of chicken proalbumin. *Biochem. Biophys. Res. Commun.* 78:1060–1066.

31. Danielsen, E. M., H. Skovbjerg, O. Noren, and H. Sjöström. 1984. Biosynthesis of intestinal microvillar proteins. Intracellular processing of lactase-phlorizin hydrolase. *Biochem. Biophys. Res. Commun.* 122:82–90.

32. Hudson, D. A., and M. W. Smith. 1986. Colchicine selectively inhibits lactase expression by rat enterocytes. *Biochem. Pharmacol.* 35:3929–3934.

33. Kruse, T. A., L. Bolund, K.-H. Grezeschik, H. H. Ropers, H. Sjöström, O. Noren, N. Mantei, and G. Semenza. 1988. The human lactase-phlorizin hydrolase gene is located on chromosome 2. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 240:123–126.