Involvement of Detergent-Insoluble Complexes in the Intracellular Transport of Intestinal Brush Border Enzymes†

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Received August 24, 1994; Revised Manuscript Received November 16, 1994®

ABSTRACT: A number of transmembrane digestive enzymes of the porcine small intestinal brush border membrane were found to be partially Triton X-100-insoluble at 0 °C and colocalized in gradient centrifugation experiments with the GPI-anchored alkaline phosphatase in low-density, detergent-insoluble complexes commonly known as glycolipid "rafts". Thus, aminopeptidase N (EC 3.4.11.2), aminopeptidase A (EC 3.4.11.7), dipeptidyl peptidase IV (EC 3.4.14.5), and sucrase-isomaltase (EC 3.2.1.48−10) were 34−48% detergent-insoluble. Maltase-glucoamylase (EC 3.2.1.20) was markedly less detergent-insoluble (20%), and lactase-phlorizin hydrolase (EC 3.2.1.23−62) was essentially fully soluble in detergent. In radioactively labeled, mucosal explants, the newly synthesized brush border enzymes began to associate with detergent-insoluble complexes while still in their transient, high mannose-glycosylated form, and their insolubility increased to that of the steady-state level soon after they achieved their mature, complex glycosylation, i.e., after passage through the Golgi complex. Detergent-insoluble complexes isolated by density gradient centrifugation were highly enriched in brush border enzymes, and the enrichment was apparent after only 1 h of labeling, where aminopeptidase N, sucrase-isomaltase, and alkaline phosphatase together comprised 25−30% of the total labeled, detergent-insoluble proteins, showing that sorting of newly made brush border membrane proteins into the glycolipid "rafts" does take place intracellularly. I therefore propose that, in the enterocyte, the brush border enzymes are targeted directly from the trans-Golgi network toward the apical cell surface.

A common feature of simple epithelial cells is their ability to generate and maintain distinct, specialized cell surface domains that enable them to carry out various vectorial transport functions and act as protective barriers against the environment (Rodriguez-Boulan & Nelson, 1989; Simons & Wandinger-Ness, 1990). This plasma membrane polarity is established by a specific delivery of both protein and lipid constituents to the apical and basolateral surfaces (Simons & van Meer, 1988), and a considerable amount of evidence has accumulated, proposing the existence of an apical targeting mechanism that specifically relies on an intimate relationship between some of the membrane components of this plasma membrane domain (van Meer, 1989; van Meer & Burger, 1992; Brown, 1992). Thus, probably by virtue of their ability to participate in hydrogen bond formation, glycolipids are able to self-associate and form microdomains in the lipid bilayer. In the trans-Golgi network, newly made glycosylphosphatidyl inositol (GPI)-anchored plasmamembrane proteins become part of these glycolipid "rafts" which subsequently pinch off into transport vesicles, destined for the apical cell surface and which are characterized by being detergent-insoluble at low temperature (Brown & Rose, 1992; Dupree et al., 1993; Sargiacomo et al., 1993). A 21-kDa transmembrane protein, caveolin, present in these rafts has been proposed to be responsible for the observed clustering of the GPI-anchored proteins and to be a part of the vesiculation machinery (Lisanti et al., 1993), but so far, only a few transmembrane proteins have been reported to be ferried as cargo by this mechanism. Skibbens et al. (1989) observed the apically targeted influenza virus hemagglutinin to become partially detergent-insoluble during intracellular transport in transfected MDCK cells, and a similar observation was made for sucrase-isomaltase in Caco-2 cells, where other transmembrane proteins studied, whether basolateral or apical, were well solubilized by detergent (Garcia et al., 1993). Studying a large number of brush border enzymes from pig kidney proximal tubule cells, Hooper and Turner (1988) reported that only the GPI-anchored enzymes alkaline phosphatase, 5′-nucleotidase, trehalase, and renal dipeptidase resisted solubilization at low temperature by a number of detergents having a low critical micellar concentration, whereas the transmembrane proteins endopeptidase 24.11, aminopeptidase N, aminopeptidase A, dipeptidyl peptidase IV, and alkaline phosphodiesterase were all well solubilized. The brush border membrane of the small intestinal enterocyte functions as a digestive/absorptive surface and is endowed with many of the ectoenzymes present in the brush border membrane of the kidney proximal tubule cell (Kenny & Maroux, 1982; Semenza, 1986; Norén et al., 1986; Louvard et al., 1992). The lipid composition of small intestinal microvillar vesicles from the pig has previously been determined (Christiansen & Carlsen, 1981), and it was found to be unusually rich (>30%) in glycolipids, in particular in dihexosylceramides containing galactose, and in pentohexosylceramides containing fucose, galactose, glucose, and

† The work was supported by grants from Novo-Nordisk's Fond and Aage Louis-Hansens Mindefond and was part of a program under the Biomembrane Research Center, Aarhus University.

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® Abstract published in *Advance ACS Abstracts,* January 1, 1995.

0006-2960/95/0434-1596$09.00/0

hexosamines. The enterocyte would thus seem well equipped for glycolipid "raft" formation, a potential that prompted me to look for detergent-insoluble complexes in small intestinal mucosa and to investigate their possible role as vehicles in the biosynthetic transport of brush border enzymes. Surprisingly, I found that in addition to the GPI-anchored alkaline phosphatase, a number of the transmembrane ectoenzymes, including aminopeptidase N, aminopeptidase A, dipeptidyl peptidase IV, and sucrase-isomaltase partitioned significantly (34−48%) in the detergent-insoluble fraction of microvillar vesicles at low temperature, and that this association with glycolipid complexes occurs at an early stage during their intracellular transport. Maltase-glucoamylase was less detergent-insoluble than the other major brush border enzymes, and lactase-phlorizin hydrolase remained essentially fully detergent-soluble both during its biosynthetic transport as well as at the apical cell surface.

EXPERIMENTAL PROCEDURES

Materials. Equipment for performing organ culture, including Trowell's T-8 medium, culture dishes with grids, and [^{35}S]methionine (specific radioactivity >1000 Ci/mmol) was obtained as previously described (Danielsen et al., 1982). Rabbit immunoglobulins to calf alkaline phosphatase and peroxidase-conjugated swine immunoglobulins to rabbit immunoglobulins were obtained from DAKO (Glostrup, Denmark), and phosphatidylinositol-specific phospholipase C (from *Bacillus cereus*) was a product of Sigma Chemical Co. (St. Louis, MO).

Pig small intestine, kidney, and liver was kindly given by the Department of Experimental Pathology, Rigshospitalet, Copenhagen.

Organ Culture of Mucosal Explants. Mucosal explants of about 100 mg were excised from the jejunum of pigs weighing 30−40 kg that had been fasted overnight. The explants were cultured at 37 °C for periods up to 20 h and labeled with [^{35}S]methionine, and after culture they were quickly frozen at −20 °C until further processing.

Tissue Fractionation. Labeled mucosal explants were extracted at 0 °C with 1 mL of 25 mM Hepes, 150 mM NaCl, pH 6.5, and 1% Triton X-100 for 15 min and centrifuged at 48000*g* for 1 h at 3 °C to obtain a detergent-soluble (supernatant) and a detergent-insoluble (pellet) fraction. The pellet was resuspended and extracted for 5 min at 37 °C with 1 mL of the above buffer and centrifuged again to obtain an extract of "detergent-insoluble" proteins. Intracellular (Mg^{2+}-precipitated) and microvillar membrane vesicles were prepared from 100−200 g of pig jejunum by the divalent cation precipitation method (Schmitz et al., 1973; Booth & Kenny, 1974), essentially as described by Sjöström et al. (1978). In some experiments, pieces (about 1 g) of freshly obtained kidney cortex and liver were suspended in 10 mL of 25 mM Hepes and 150 mM NaCl, pH 6.5, homogenized for 20 s in a polytron homogenizer, and extracted with 1% Triton X-100 at 0 °C.

Flotation of Detergent-Insoluble Complexes by Sucrose Gradient Centrifugation. This was performed essentially as described by Brown and Rose (1992) with the modification that a buffer of pH 6.5 was used, as recommended by Sargiacomo et al. (1993). Briefly, 2 mL of labeled mucosal extract, or, in some experiments, intestinal Mg^{2+}-precipitated or microvillar membranes or total homogenate extracts of kidney cortex or liver (in 25 mM Hepes, 150 mM NaCl, pH 6.5, 1% Triton X-100) was mixed in a centrifugation tube with an equal volume of 40% sucrose and made up in the same buffer, and 8 mL of a 5−30% linear sucrose gradient was layered on top of the extract which was then centrifuged in a Beckman SW40 Ti rotor (Beckman Instruments, Palo Alto, CA) at 35 000 rpm (g_{max} = 217 000) for 20−22 h at 3 °C. After centrifugation, the gradient was fractionated from the bottom into 15 fractions of 0.8 mL. In some experiments, the resulting low-density light-scattering band was carefully collected from the gradient by a pipette, mixed with 5 volumes of 25 mM Hepes and 150 mM NaCl, pH 6.5, and centrifuged at 48000*g* for 1 h to obtain a pellet of detergent-insoluble complexes.

Phospholipase Solubilization of Detergent-Insoluble Complexes. Samples (150 μL) of detergent-insoluble complexes made from a microvillar fraction (approximately 1 mg of protein/mL) in 10 mM Tris-HCl, pH 7.5, were incubated in the presence or absence of 1 unit of phosphatidylinositol-specific phospholipase C for 20 h at 37 °C. After incubation, the samples were centrifuged for 15 min at 20000*g*, and the supernatant and pellet fractions were collected and analyzed by SDS−PAGE.

Immunoprecipitation of Brush Border Enzymes. Aminopeptidase N and sucrase-isomaltase were immunoprecipitated from the detergent-soluble and detergent-insoluble fractions of labeled mucosal explants by addition of 25−100 μL of rabbit antisera raised to the respective pig brush border enzyme and incubation overnight at 4 °C. The immunoprecipitates were collected by centrifugation at 5000*g*, 5 min, and washed once in 0.5 mL of 50 mM Tris-HCl and 150 mM NaCl, pH 7.4.

Immunoelectrophoresis. Quantitative rocket immunoelectrophoresis in 1% agarose gels of sucrase-isomaltase, maltase-glucoamylase, and lactase-phlorizin hydrolase against the corresponding rabbit antisera was performed essentially as described by Weeke (1973).

SDS−PAGE. SDS−PAGE in 10% gels under reducing conditions was performed according to Laemmli (1970) and fluorography as described by Bonner and Laskey (1974). X-ray films of gel tracks were scanned in an Ultroscan XL densitometer (Pharmacia LKB, Bromma, Sweden). For Western blotting, proteins separated by SDS−PAGE were electrotransferred onto nitrocellulose paper (pore size 0.45 μm, from Schleicher and Schuell, Dassel, Germany) and visualized by the procedure of Bjerrum et al. (1983).

RESULTS

Intestinal Transmembrane Brush Border Enzymes Are Partially Detergent-Insoluble at 0 °C. Fractions of vesiculated microvilli were prepared from pig small intestinal mucosa by the divalent cation precipitation method and solubilized by 1% Triton X-100 or 60 mM *n*-octylglucoside at 0 °C. As shown in Table 1, the GPI-anchored enzyme alkaline phosphatase was predominantly Triton X-100-insoluble at this temperature. In addition, four of the transmembrane-anchored peptidases and glycosidases assayed partitioned substantially in the Triton X-100-insoluble fraction, in particular aminopeptidase N and sucrase-isomaltase which were almost 50% insoluble, but also aminopeptidase A and dipeptidyl peptidase IV which were 35−40% insoluble. This partial insolubility was also

Table 1: Detergent-Insolubility at 0 °C of Intestinal Brush Border Enzymes[a]

enzyme	Triton X-100-insoluble (%)	*n*-octylglucoside-insoluble (%)
alkaline phosphatase	88 (±3)	26 (±4)
aminopeptidase N	48 (±1)	12 (±1)
aminopeptidase A	41 (±1)	14 (±3)
dipeptidyl peptidase IV	34 (±1)	12 (±1)
sucrase-isomaltase	46	not determined
maltase-glucoamylase	20	not determined
lactase-phlorizin hydrolase	6	not determined

[a] An intestinal microvillar fraction was prepared by the divalent cation precipitation method and resuspended (at a protein concentration of 1.4 mg/mL) in 1 mL of 25 mM HEPES and 150 mM NaCl, pH 6.5, and solubilized at 0 °C by addition of Triton X-100 or *n*-octylglucoside (final concentration 1% and 60 mM, respectively). After 10 min with frequent vortexing, the suspension was centrifuged at 48000*g* for 30 min. The resulting supernatant (the detergent-soluble fraction) was collected and the pellet (the detergent-insoluble fraction) resuspended and solubilized for 10 min at 37 °C in 1 mL of the above buffer, containing 1% Triton X-100 or 60 mM *n*-octylglucoside. Alkaline phosphatase and peptidase activities were determined spectrophotometrically (Sjöström et al., 1978) in both the detergent-soluble and the detergent-insoluble fractions. The values listed are the mean (±SD) of six experiments. The relative amounts of sucrase-isomaltase, maltase-glucoamylase, and lactase-phlorizin hydrolase in the two fractions after solubilization with Triton X-100 was determined by quantitative rocket immunoelectrophoresis against the respective specific antisera, and the values listed are the mean of two determinations.

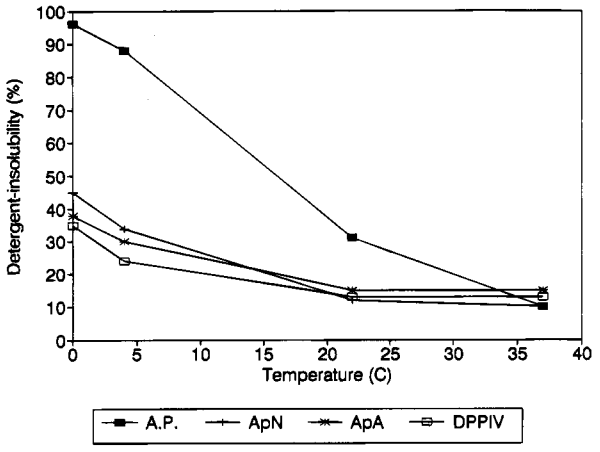


FIGURE 1: Temperature-sensitivity of detergent-insolubility of intestinal brush border enzymes. An intestinal microvillar fraction was prepared and solubilized at 0, 4, 22, and 37 °C as described in the legend to Table 1. The relative enzyme activities of the detergent-soluble and -insoluble fractions were determined, and each of the values given are the mean of two determinations. AP, alkaline phosphatase; ApN, aminopeptidase N; ApA, aminopeptidase A; DPPIV, dipeptidyl peptidase IV.

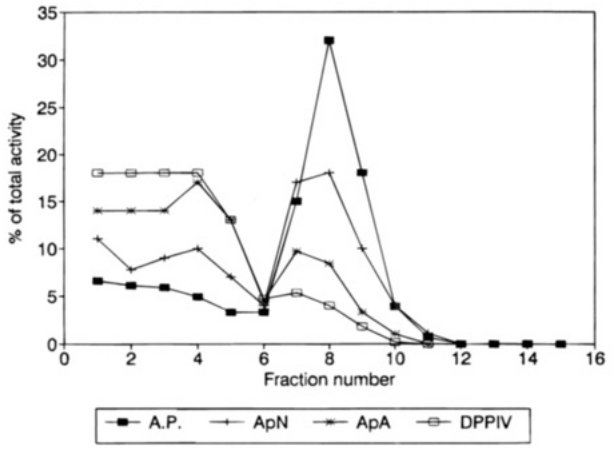
observed at Triton X-100 concentrations up to 3% (data not shown). Maltase-glucoamylase resisted detergent solubilization less well, and lactase-phlorizin hydrolase was essentially fully soluble under these conditions. The nonionic detergent *n*-octylglucoside which has a much higher critical micellar concentration than Triton X-100 (25 mM vs 0.24 mM), solubilized both alkaline phosphatase and the peptidases much more efficiently than Triton X-100 at 0 °C (Table 1).

As shown in Figure 1, the Triton X-100-insolubility of brush border enzymes decreased with increasing temperature. However, whereas alkaline phosphatase remained about 35% insoluble at 22 °C and only achieved maximal solubilization (approximately 90%) at 37 °C, the transmembrane peptidases were all maximally solubilized already at 22 °C, suggesting that the latter type of membrane proteins are less firmly associated with the detergent-insoluble complexes than the GPI-anchored protein.

The Brush Border Enzymes Are Present in Low-Density Complexes. To characterize further the partial detergent-insolubility of the brush border enzymes, a total homogenate of small intestinal mucosa was extracted in 1% Triton X-100 at 0 °C and subjected to sucrose gradient centrifugation, essentially as described by Brown and Rose, (1992). After centrifugation (which generated a light-scattering band of low density), the gradient was fractionated, and the distribution of various brush border enzymes was analyzed (Figure 2). The major portion of alkaline phosphatase activity was present in a sharp peak coinciding with the low density light-scattering band (Figure 2A). A prominent proportion of aminopeptidase N likewise flotated in this position of the gradient and, to a lesser extent, a fraction of aminopeptidase A and of dipeptidyl peptidase IV. The gradient distribution of the sucrase-isomaltase and lactase-phlorizin hydrolase from the same experiment was visualized by quantitative rocket immunoelectrophoresis, showing that only sucrase-isomaltase partitioned significantly in the low density fractions (Figure 2B). The flotation experiment confirms that the partial detergent-insolubility at low temperature of both alkaline phosphatase and the transmembrane brush border enzymes is due to an association with buoyant material and not solely to an association with the microvillar cytoskeleton. The partial detergent-insolubility of these brush border enzymes thus bears the hallmark of a presence in microdomains in the membrane enriched in glycosphingolipids and cholesterol (Brown & Rose, 1992; Sargiacomo et al., 1993), commonly referred to as "glycolipid rafts".

The brush border membrane of the kidney proximal tubule cell harbors many transmembrane and GPI-anchored ectoenzymes, including aminopeptidases N and A, dipeptidyl peptidase IV, and alkaline phosphatase. Studying their solubilization using a wide range of detergents, Hooper and Turner (1988) observed that only the GPI-anchored kidney brush border ectoenzymes resisted detergents with low critical micellar concentrations like Triton X-100 at low temperature, whereas the transmembrane ectoenzymes were well (>80%) solubilized. To examine this further, a pig kidney cortex homogenate was solubilized by 1% Triton X-100 at 0 °C and subjected to sucrose gradient centrifugation by the same protocol used in Figure 2 (Figure 3). As shown, the alkaline phosphatase activity flotated in a peak coinciding with the position of a light-scattering band (fraction 9), whereas the activities of kidney aminopeptidases N and A and dipeptidyl peptidase IV essentially remained at the bottom fractions (1−4) of the gradient. This experiment thus shows that kidney alkaline phosphatase owes its detergent-insolubility at low temperature to an association with low density complexes. Furthermore, it demonstrates that the transmembrane peptidases from the kidney, in contrast to their intestinal counterparts, are not associated with the low density complexes but on the contrary are fully detergent-soluble, as previously reported by Hooper and Turner (1988).

Both Mature and Transient Forms of Intestinal Brush Border Enzymes Are Present in Low Density, Detergent-Insoluble Complexes. The distribution in a density gradient of biosynthetic transient and mature molecular forms of



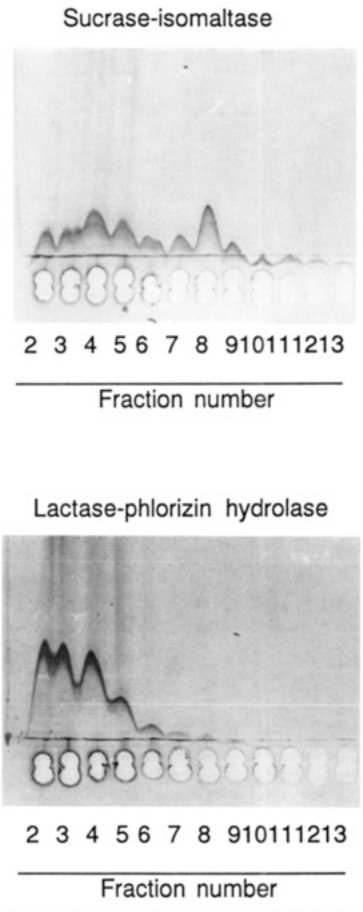


FIGURE 2: Flotation of detergent-insoluble intestinal brush border enzymes by sucrose gradient centrifugation. (A, top) A Triton X-100 extract of intestinal mucosa was subjected to sucrose gradient centrifugation as described under Experimental Procedures. After centrifugation, the gradient was fractionated from the bottom (fraction 1), and the relative amounts of activities of the brush border enzymes in each fraction were determined. AP, alkaline phosphatase; ApN, aminopeptidase N; ApA, aminopeptidase A; DPPIV, dipeptidyl peptidase IV. (B, bottom) Samples (20 μL) of the fractions from the gradient centrifugation were solubilized by 1% Triton X-100 at 37 °C and subjected to rocket immunoelectrophoresis against antisera to sucrase-isomaltase or lactase-phlorizin hydrolase. After electrophoresis, the immunoprecipitates were visualized by staining with Coomassie Brilliant Blue.

aminopeptidase N and sucrase-isomaltase was analyzed by SDS−PAGE and Western blotting of the gradient fractions. Figure 4A shows that the 166-, 125-, and 50-kDa bands of mature (and pancreatic proteinase-cleaved) microvillar aminopeptidase N [also termed "A", "B", and "C" subunits,

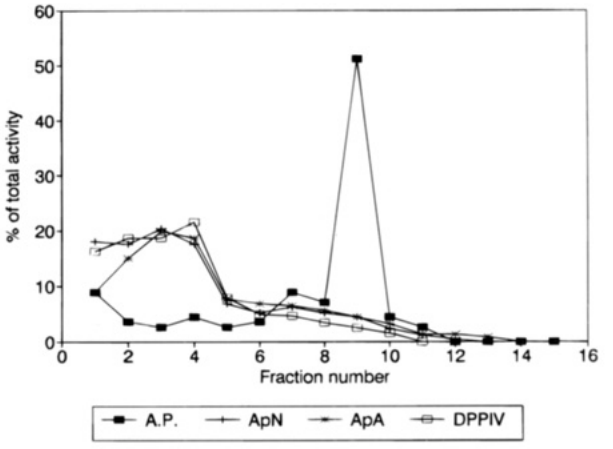


FIGURE 3: Flotation of detergent-insoluble kidney brush border enzymes. A Triton X-100 extract of a total kidney cortex homogenate was prepared and subjected to sucrose gradient centrifugation as described under Experimental Procedures. After centrifugation, the gradient was fractionated and enzyme activities determined as described in the legend to Figure 2A.

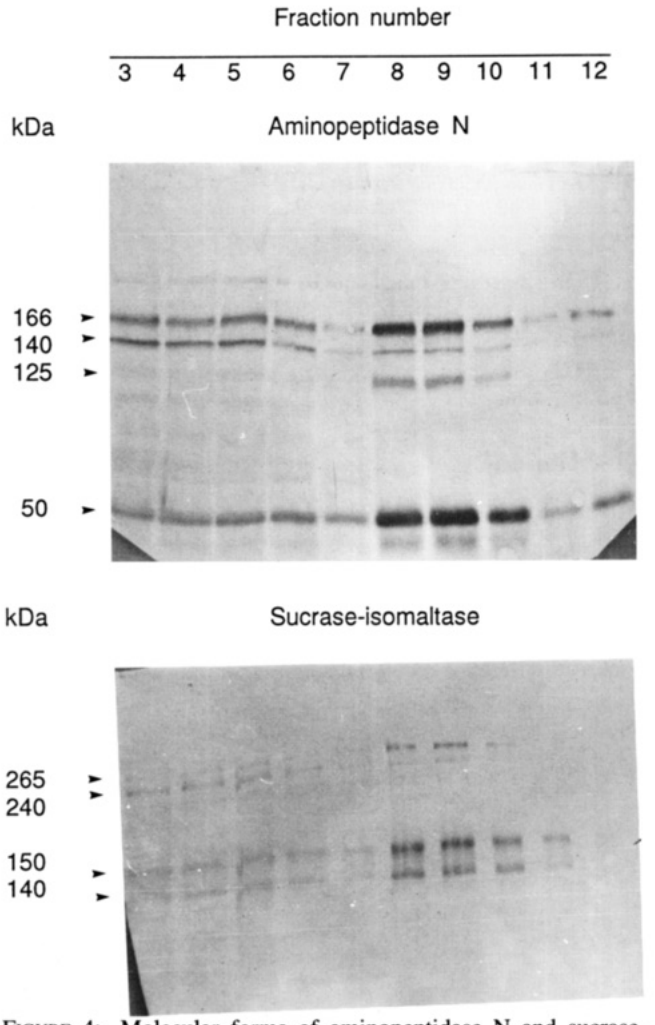


FIGURE 4: Molecular forms of aminopeptidase N and sucrase-isomaltase in detergent-insoluble complexes. Samples (50 μL) from the fractions of the sucrose gradient centrifugation experiment shown in Figure 2 were subjected to SDS−PAGE, followed by Western blotting using primary antibodies to aminopeptidase N or sucrase-isomaltase. Molecular mass values (kDa) are indicated.

respectively (Sjöström et al. 1978)] appeared predominantly in the low density fractions, whereas the transient, high mannose-glycosylated form of 140 kDa (Danielsen, 1982) was present mainly in the bottom fractions of soluble

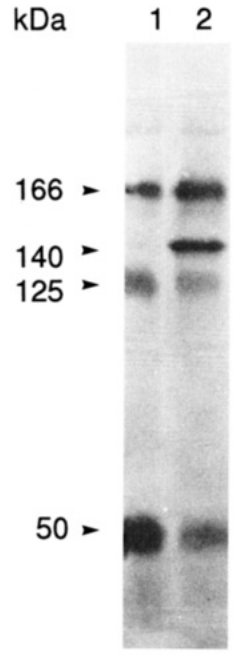


FIGURE 5: Transient form of aminopeptidase N in detergent-insoluble complexes prepared from intracellular membranes. Detergent-insoluble complexes were prepared from microvillar (1) and Mg^{2+}-precipitated (2) membrane fractions of mucosa by sucrose gradient centrifugation. After centrifugation, the light-scattering, low density band was collected by a pipette, diluted with 5 volumes of buffer, and pelleted by centrifugation as described iunder Experimental Procedures. The complexes were then re-extracted with 1% Triton X-100 for 5 min at 0 °C, pelleted by centrifugation again, dissolved and boiled in SDS−PAGE sample buffer, and analyzed by SDS−PAGE followed by immunoblotting, using a primary antibody to aminopeptidase N. Molecular mass values (kDa) are indicated.

proteins, albeit it was also clearly visible in the low density fractions. Similarly for sucrase-isomaltase, the 140- and 150-kDa sucrase and isomaltase subunits of the mature (pancreatic proteinase-cleaved) enzyme together with the complex-glycosylated precursor of 265 kDa were seen mainly in the low density fractions, whereas the high mannose-glycosylated precursor of 240 kDa (Danielsen, 1982) largely remained in the bottom fractions; however, as with aminopeptidase N, the latter molecular form was also visible in the low density fractions (Figure 4B). Together, these data indicate that it primarily is the mature forms of these two brush border enzymes that are detergent-insoluble. The presence in the low density fractions of small but significant amounts of the transient, high mannose-glycosylated forms of the brush border enzymes was a surprising finding that urged studying in more detail. Detergent-insoluble complexes were therefore prepared from intracellular (Mg^{2+}-precipitated) and microvillar membrane vesicles by gradient centrifugation, extracted once more with detergent at low temperature, and analyzed by SDS−PAGE and immunoblotting. As shown in Figure 5, the distinct band of the 140-kDa high mannose-glycosylated form of aminopeptidase N was present in the purified complexes, obtained from the intracellular, Mg^{2+}-precipitated fraction but not the microvillar fraction, demonstrating that the transient, pre-Golgi form of the brush border enzyme is indeed associated with purified glycolipid "rafts". (The presence of the 125- and 50-kDa bands indicates the presence in this fraction of some aminopeptidase N of microvillar origin.) Also in the case of alkaline phosphatase was seen a sharp, distinct band of higher mobility than the mature form in complexes obtained from the Mg^{2+}-precipitated but not the microvillar, fraction, indicating that the GPI-anchored enzyme behaves similarly to the transmembrane enzyme (data not shown).

Newly Synthesized Intestinal Brush Border Enzymes become Detergent-Insoluble during Their Intracellular Transport. In cultured mucosal explants, newly synthesized aminopeptidase N and sucrase-isomaltase require 1−2 h to reach the apical cell surface, an event preceded by their conversion from a transient, high mannose-glycosylated form to a mature, complex-glycosylated form (Danielsen, 1982). The kinetics of acquisition of detergent-insolubility of aminopeptidase N and sucrase-isomaltase was studied by [^{35}S]methionine labeling of cultured intestinal mucosal explants. For both enzymes, the transient, high mannose-glycosylated molecular form was completely soluble immediately after 10 min of labeling, but after 20 min and at longer periods of labeling, a small but persistent fraction of this molecular form was seen in the detergent-insoluble fraction (Figure 6A). The mature, complex-glycosylated form of the brush border enzymes appeared after 40−60 min of labeling and was partially detergent-insoluble already at this stage; by 2 h, its detergent-insolubility had increased to a proportion roughly equivalent with the relative steady-state amounts of detergent-insoluble aminopeptidase N and sucrase-isomaltase in microvillar membrane vesicles (Figure 6B). It can therefore be concluded that they become part of the detergent-insoluble glycolipid complexes during their intracellular transport to the cell surface. For the major part, this takes place soon after complex glycosylation, most likely in the trans-Golgi network (Brown, 1992), but the small amounts of detergent-insoluble, radioactively labeled high mannose-glycosylated form of both aminopeptidase N and sucrase-isomaltase confirms the results of Figures 4 and 5 that the assembly of detergent-insoluble complexes begins at an earlier stage in the secretory pathway, possibly in the endoplasmic reticulum or in an intermediate compartment between the ER and the Golgi complex. Furthermore, once they have become part of the detergent-insoluble complexes during their intracellular transport, the brush border enzymes seem to remain stably associated with them.

Lactase-phlorizin hydrolase was the only brush border enzyme studied that seems to be virtually excluded from the detergent-insoluble complexes at steady-state (Table 1 and Figure2B). Since the work of Garcia et al. (1993) suggested that a part of alkaline phosphatase in Caco-2 cells disengage from the detergent-insoluble complexes after arrival at the cell surface, lactase-phlorizin hydrolase might exhibit a transient, biosynthetic association with detergent-insoluble complexes. Therefore, quantitative rocket immunoelectrophoresis was carried out to compare the relative amounts of labeled, detergent-insoluble lactase-phlorizin hydrolase after 1, 3, h and 10 h of labeling (Figure 7). As judged by the relative radioactivity of the precipitates of the detergent-insoluble forms, this enzyme was not associated with the detergent-insoluble complexes to a greater extent at 1 or 3 h than after 10 h of labeling, arguing against a transient, biosynthetic association any greater than that observed at steady state in the brush border membrane. A similar observation was made for maltase-glucoamylase which was markedly less detergent-insoluble than sucrase-isomaltase and the peptidases (data not shown).

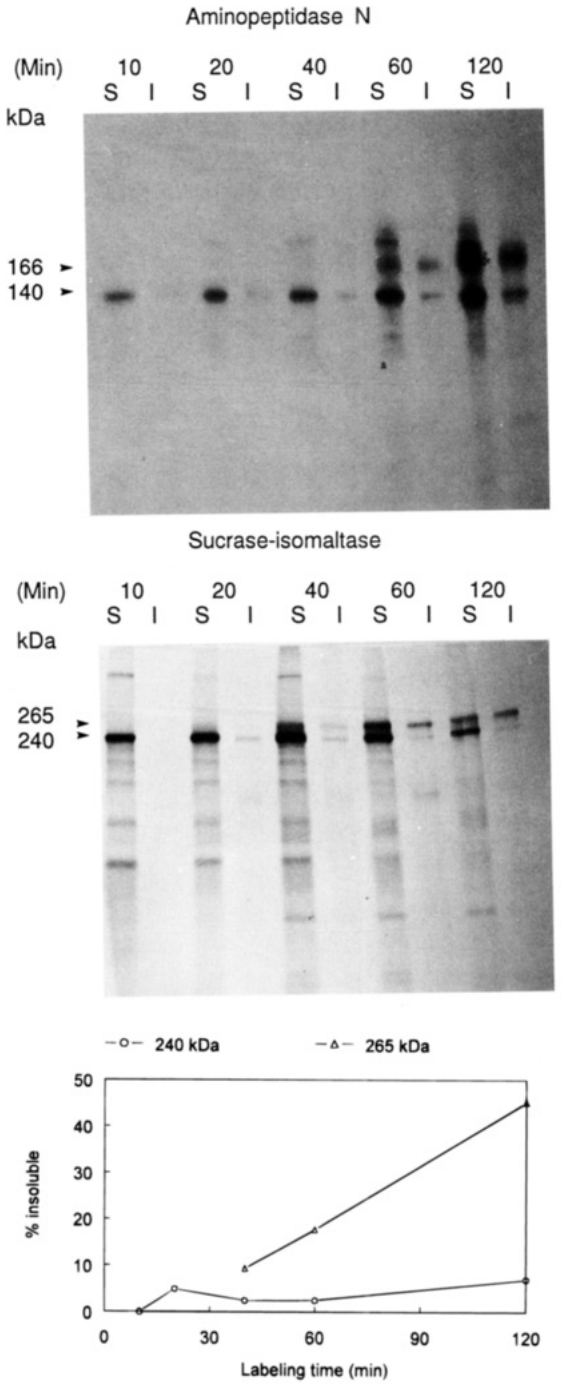


FIGURE 6: Aminopeptidase N and sucrase-isomaltase become detergent-insoluble during their intracellular transport. (A, top) Mucosal explants were labeled with [^{35}S]methionine (200 μCi/mL) for the indicated periods of time, and aminopeptidase N and sucrase-isomaltase were immunopurified from the detergent-soluble (S) and detergent-insoluble (I) fractions of each explant as described under Experimental Procedures and analyzed by SDS−PAGE followed by fluorography. Molecular mass values (kDa) are indicated. (B, bottom) The gel tracks of the sucrase-isomaltase experiment in panel A were densitometrically scanned and the insolubility (%) of the 240- and 265-kDa molecular forms calculated.

Detergent-Insoluble Complexes Are Highly Enriched in Intestinal Brush Border Enzymes. Detergent-insoluble complexes prepared from intracellular (Mg^{2+}-precipitated) and microvillar membrane vesicles by sucrose gradient centrifugation and analyzed by SDS−PAGE had a similar, but not identical, pattern of major polypeptide components (Figure 8). Most of the 9−10 major bands mainly represent molecular forms of aminopeptidase N, sucrase-isomaltase,

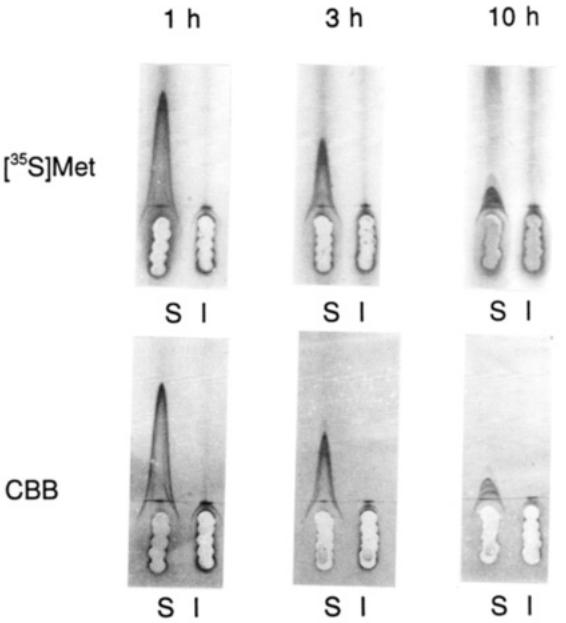


FIGURE 7: Lactase-phlorizin hydrolase remains soluble during biosynthesis and intracellular transport. Mucosal explants were labeled with [^{35}S]methionine (500 μCi/mL) for the indicated periods of time, and equal amounts (50 μL) of detergent-soluble (S) and detergent-insoluble (I) fractions were analyzed by rocket immunoelectrophoresis against an antiserum to lactase-phlorozin hydrolase. After electrophoresis, the immunoprecipitates were stained with Coomassie Brilliant Blue (CBB) and radioautographed ([^{35}S]Met). Note that the very small immunoprecipitate formed by the detergent-insoluble fraction was only weakly labeled both at 1, 3, and 10 h.

and alkaline phosphatase, as identified by Western blotting (Figures 4 and 5): Bands 1, 2 (only present in complexes prepared from the Mg^{2+}-precipitated fraction), 4, and 5 reacted with antibodies to sucrase-isomaltase; bands 4, 6, and 8 reacted with anti-aminopeptidase N. [The less abundant aminopeptidase A (170 kDa) and dipeptidyl peptidase IV (137 kDa) would be minor constituents of bands 4 and 5, respectively (Danielsen et al., 1983), as would the subunits of maltase-glucoamylase (Sørensen et al., 1982).] Band 7, which reacted with antibodies to alkaline phosphatase, was the only major component that was significantly released from detergent-insoluble complexes from a microvillar fraction by an overnight incubation with a phosphatidylinositol-specific phospholipase C (data not shown). Band 9 represents actin (43 kDa), the most abundant microvillar protein, and band 3 may putatively be identified as myosin, since only these two bands were significantly released from the membrane complexes by extraction with a carbonate buffer at pH 11 (data not shown), and because myosin together with actin is a major component of the apical cytoskeleton of the enterocyte (Mooseker et al., 1978). The broad band of lower molecular weight (10) resolved into two major polypeptides of 37 and 35 kDa and a minor one of 32 kDa in SDS−PAGE in a 15% gel (data not shown) that cannot at the present time be identified. However, it can be concluded that most of the major bands of the detergent-insoluble complexes obtained from the two membrane fractions represent brush border enzymes, notably the two most abundant transmembrane enzymes aminopeptidase N and sucrase-isomaltase and the GPI-anchored alkaline phosphatase. In the enterocyte, the brush border membrane must therefore be the only major target organelle that contain detergent-insoluble complexes.

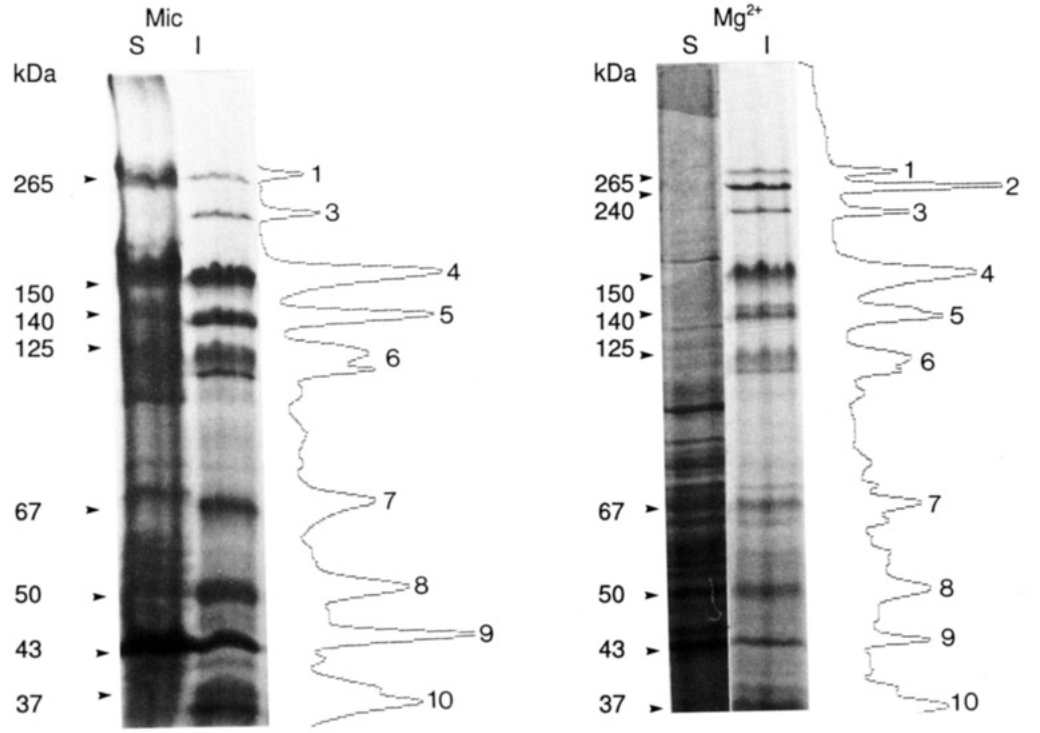


FIGURE 8: Polypeptide composition of detergent-insoluble complexes. Mg^{2+}-precipitated (Mg^{2+}) and microvillar (Mic) membranes were prepared from mucosa and solubilized by 1% Triton X-100 at 0 °C. The membrane extracts were subjected to sucrose gradient centrifugation to prepare a detergent-soluble fraction (S) and detergent-insoluble complexes (I) and analyzed by SDS−PAGE as described under Experimental Procedures. After electrophoresis, the gels were stained with Coomassie Brilliant Blue, and the gel tracks of the detergent-insoluble complexes were densitometrically scanned. Molecular mass values (kDa) are indicated.

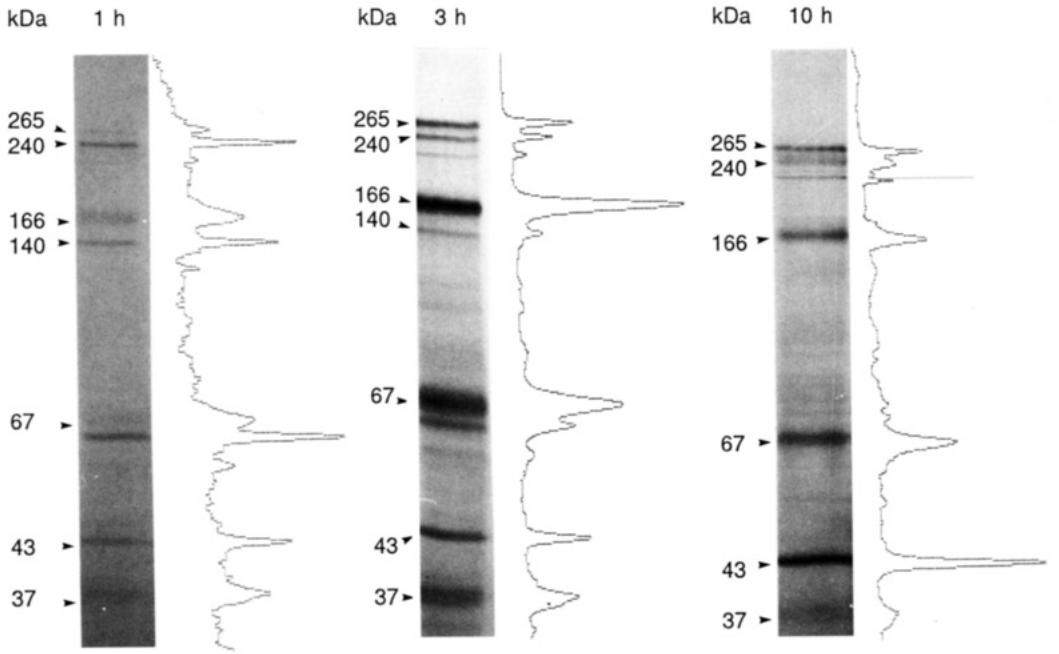


FIGURE 9: Intracellular detergent-insoluble complexes are highly enriched in newly synthesized brush border enzymes. Mucosal explants were labeled with [^{35}S]methionine (200−500 μCi/mL) for 1, 3, and 10 h, and detergent-insoluble complexes were prepared from total extracts of the explants and analyzed by SDS−PAGE followed by fluorography as described under Experimental Procedures. X-ray films of the gel tracks were densitometrically scanned. Molecular mass values (kDa) are indicated.

The Enrichment Occurs during Intracellular Transport. Detergent-insoluble complexes were prepared by density gradient centrifugation from whole extracts of mucosal explants, labeled for periods of 1, 3, and 10 h (Figure 9). By 1 h, the high mannose-glycosylated 240- and 140-kDa forms of sucrase-isomaltase and aminopeptidase N, respectively, were more abundant than the corresponding complex-glycosylated 265- and 166-kDa forms. Likewise, the distinct band of slighlty higher mobility than the 67-kDa mature alkaline phosphatase was the dominant molecular form of this enzyme. No other major components apart from actin and band 10 were present. After longer periods of labeling, the relative amounts of mature form relative to transient form of the three brush border enzymes gradually increased, but no other components, apart from band 3 (tentatively identified as myosin), appeared. It can therefore be concluded

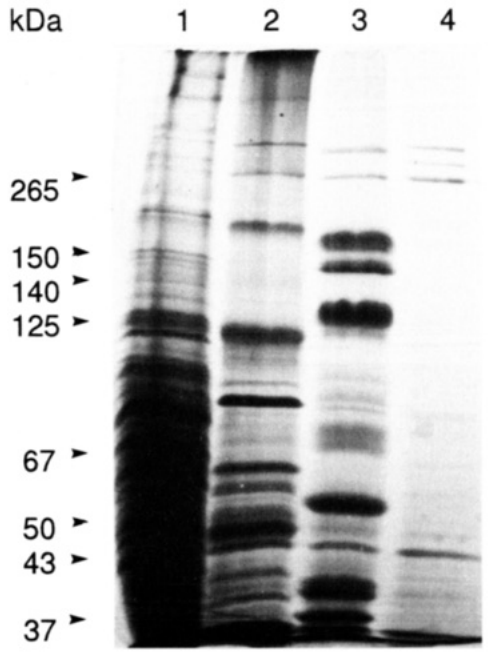


FIGURE 10: Detergent-insoluble complexes prepared from kidney and liver. Triton X-100 extracts of equal amounts of total homogenates of kidney cortex and liver were prepared and subjected to sucrose gradient centrifugation. After centrifugation, the low-density light-scattering bands were carefully collected with a pipette, re-extracted once more with 1% Triton X-100 at 0 °C, and analyzed by SDS−PAGE. After electrophoresis, the gel was stained with Coomassie Brilliant Blue. Molecular mass values (kDa) are indicated. (Lane 1) Kidney cortex homogenate; (lanes 2, 3, and 4) and detergent-insoluble complexes from kidney cortex, small intestine, and liver, respectively.

that the major protein composition of the detergent-insoluble complexes does not vary markedly with the labeling time and it is essentially similar to that revealed by protein-staining (taking into account that newly synthesized aminopeptidase N and sucrase-isomaltase are not cleaved by pancreatic proteases during culture of the mucosal explants). This confirms therefore that detergent-insoluble complexes formed during the biosynthetic transport to the brush border membrane remain as stable structures once expressed at the apical cell surface, at least with regard to their content of major proteins. The conspicuous bands of high mannose and complex-glycosylated aminopeptidase N and sucrase-isomaltase and of alkaline phosphatase were calculated to represent 25−30% of the total amount of labeled protein in the detergent-insoluble complexes after 1 h of labeling. This high enrichment, occurring well before the newly synthesized enzymes appear in the brush border membrane, strongly argues that their sorting from membrane proteins with other subcellular destinations in the enterocyte occurs during the intracellular transport. It probably begins in an intermediate compartment between the endoplasmic reticulum and the Golgi complex and proceeds during passage through the Golgi complex.

Detergent-Insoluble Complexes from Kidney and Liver. Figure 10 shows the polypeptide composition of detergent-insoluble complexes, prepared by sucrose gradient centrifugation of a kidney cortex homogenate. The profile of major bands of the preparation was different from the corresponding pattern of bands of intestinal detergent-insoluble complexes. Essentially the same profile of major bands was seen in detergent-insoluble complexes prepared from a kidney microvillar fraction (data not shown). This indicates that although transmembrane ectoenzymes are excluded from kidney detergent-insoluble complexes (Hooper & Turner, 1988; Figure 3), the brush border membrane of the proximal tubule cell is nevertheless the main source of detergent-insoluble complexes also in this organ. Using the same protocol and similar amounts of total homogenate as starting material, a preparation from liver resulted in a barely detectable low density, light-scattering band which, in comparison with kidney and intestine, was almost void of protein components.

DISCUSSION

The high content of glycosphingolipids of the small intestinal brush border membrane of the pig previously reported by Christiansen and Carlsen (1981) was suggestive that detergent-insoluble complexes might be abundant in this tissue and possibly have specific biological functions. Surprisingly, not only the GPI-anchored alkaline phosphatase but in addition most of the transmembrane brush border enzyme activities determined turned out to be partially Triton X-100-insoluble at low temperature whereas they were well solubilized by *n*-octylglucoside. This relatively high degree of Triton X-100-insolubility of transmembrane peptidases is at variance with their reported solubility from pig kidney microvillar vesicles (Hooper & Turner, 1988). Failure to solubilize a membrane protein can hypothetically be ascribed to use of insufficient amounts of detergent or to an association between the membrane protein and the underlying, insoluble cytoskeleton. However, the following evidence strongly indicates that the partial, but significant detergent-insolubility of the intestinal transmembrane brush border ectoenzymes is not artifactual but represents a *bona fide* association with detergent-insoluble complexes or glycolipid "rafts": (1) Extraction of membranes with detergent was carried out in excess (10 mg/1.4 mg of protein) of Triton X-100 as described by Hooper and Turner (1988), and detergent-insolubility was selective since one transmembrane enzyme, lactase-phlorizin hydrolase, was fully soluble. Furthermore, re-extraction with Triton X-100 at 0 °C of isolated detergent-insoluble complexes did not solubilize the insoluble transmembrane (or GPI-anchored) brush border enzymes. (2) Insolubility at low temperature was much reduced using *n*-octylglucoside instead of Triton X-100 as detergent, and the Triton X-100-insolubility of both transmembrane and GPI-anchored brush border enzymes was temperature-sensitive, as described for placental alkaline phosphatase by Brown and Rose (1992). (3) The high mannose-glycosylated forms of the aminopeptidase N and sucrase-isomaltase were much less detergent-insoluble than the corresponding mature forms, showing that this property depends upon the lipid microenvironment in the membrane. (4) In sucrose gradient centrifugation, detergent-insoluble transmembrane and GPI-anchored brush border enzymes both flotated in the position of a distinct low density, light-scattering band, showing that insolubility must include an association with buoyant lipid and cannot be ascribed solely to interactions with the microvillar cytoskeleton. (5) Using the same protocols for detergent extraction and sucrose density centrifugation, the transmembrane peptidases from kidney were fully soluble and absent from the detergent-insoluble complexes.

It is not at present possible to explain why the transmembrane brush border enzymes are only partially detergent-insoluble and not fully insoluble like alkaline phosphatase. The observation that the association of the transmembrane enzymes with the detergent-insoluble complexes seemed to be more temperature-sensitive than that of alkaline phosphatase (Figure 1), may reflect a weaker association with these complexes. However, given that detergent extraction must be considered a harsh treatment to a lipid bilayer, the transmembrane enzymes might have sufficient affinity for the glycolipid "rafts" *in vivo* to be fully—rather than partially—associated with them. Alternatively, the transmembrane brush border enzymes may exist in an equilibrium between the "rafts" and the surrounding, mobile bilayer.

Small amounts of actin and (putatively) myosin were present in the isolated detergent-insoluble complexes, but whether their presence is significant or caused by unspecific association is uncertain. The presence of actin in caveolin-rich membranes from mouse lung endothelium, prepared essentially by the same protocol, was recently reported, suggesting that these membrane domains could represent sites for microfilament assembly (Lisanti et al., 1994).

It is intriguing that the transmembrane brush border peptidases are part of the intestinal, but not the kidney detergent-insoluble, complexes. Tissue-specific variations in posttranslational processing of the enzymes, for instance *O*-linked glycosylation of the juxtamembraneous stalk region of these lollipop-shaped molecules, might be the reason for this. It is also possible that association of transmembrane (but not GPI-anchored) proteins with the glycolipid "rafts" requires an unknown adaptor protein. Fiedler et al. (1994) have recently described a transmembrane protein, VIP36, present in the detergent-insoluble fraction of MDCK cells, the luminal domain of which shows sequence homology to leguminous plant lectins. In this context, it is interesting that the only major components of intestinal detergent-insoluble complexes that were not identified as brush border proteins had molecular masses in the range of 32−37 kDa (band 10 in Figure 8). This subset of proteins was either absent or present in much lower amounts in the detergent-insoluble complexes from kidney (Figure 10).

Relative to the intestine and kidney, the liver was observed to be a poor source of detergent-insoluble complexes. If these complexes in epithelial cells are generally found mainly at the apical cell surface, an explanation for this could be that the apical (bile canalicular) plasma membrane of the hepatocyte comparatively makes up only a small proportion of the cell surface area of this cell type.

Caveolae or plasmalemmal vesicles are small uniform pits at the cell surface involved in potocytosis, i.e., receptor-mediated import of small molecules such as folate (Anderson et al., 1992), and like detergent-insoluble complexes they are enriched in glycosphingolipids and cholesterol as well as GPI-anchored proteins. In MDCK cells, detergent-insoluble complexes at the cell surface were reported to resemble ultrastructurally caveolae and to make up less than 1% of the plasma membrane (Sargiacomo et al., 1993), and immunolocalization of caveolin, a transmembrane protein which contributes to the characteristic striated coat of caveolae, largely confined the labeling to non-clathrin-coated invaginations that made up only 3−4% of the plasma membrane surface area (Dupree et al., 1993). Caveolae are generally not seen along the densely packed microvilli of the enterocyte brush border membrane. Consequently, the large amounts of detergent-insoluble complexes, containing almost half the microvillar content of the two most abundant brush border enzymes aminopeptidase N and sucrase-isomaltase as well as a significant proportion of a number of the minor components, cannot be derived from caveolae. This notion is supported by the absence in the complexes of any major polypeptides of the size of caveolin (20−25 kDa). Hence it must be concluded that detergent-insoluble complexes cannot generally be considered synonymous with caveolae.

In polarized cells, newly synthesized proteins destined for the basolateral and apical surface domains are generally believed to be sorted into their appropriate transport vesicles in the trans-Golgi network (Simons & van Meer, 1988; Simons & Wandinger-Ness, 1990). In previous work, acquisition of detergent-insolubility of GPI-anchored proteins has been reported to occur only after the newly made proteins become resistant to endo H, i.e., at the earliest in the medial Golgi (Brown & Rose, 1992; Garcia et al., 1993; Lisanti et al., 1993). Nevertheless, our data consistently showed that transient (endo H-sensitive) forms of the brush border enzymes are indeed present in isolated and twice extracted complexes (Figure 5), albeit they are less insoluble than their corresponding mature forms (Figure 4). This, taken together with the time lag of the acquisition of detergent-insolubility in labeling experiments (Figure 6), implies that complexes begin to form either in an intermediate compartment between the endoplasmic reticulum and the Golgi complex or, at the latest, in the cis-Golgi prior to the modifications of the carbohydrate chains. [It should be noted that in the papers of Brown and Rose (1992) and Lisanti et al. (1993) transient forms of placental alkaline phosphatase and gD-1-DAF, respectively, are visibly present in the detergent-insoluble fractions.]

A surprising finding of the present work is that the enrichment of aminopeptidase N, sucrase-isomaltase, and alkaline phosphatase in detergent-insoluble complexes had occurred already by 1 h of labeling at a time when the enzymes were still predominantly in their transiently glycosylated form. This shows that protein components, destined for other compartments than the brush border membrane, are excluded from the glycolipid "rafts" already at this early stage of the intracellular transport and—in consequence hereof—that sorting and targeting of apically destined proteins in the enterocyte takes place intracellularly, as we have earlier argued (Danielsen & Cowell, 1985), most likely in the trans-Golgi network, as has been proposed for MDCK cells (Simons & Fuller, 1985). This contrasts with the routing in the enterocyte-like Caco-2 cell, where these enzymes have been reported to reach the apical surface, at least in part, by an indirect transcytotic pathway (Matter et al., 1990; Le Bivic et al., 1990). The comparatively limited partition in detergent-insoluble complexes of apically destined proteins in Caco-2 cells (Garcia et al., 1993) probably reflects the different targeting strategies used by the two types of epithelial cells. That enterocytes, in comparison with Caco-2 cells, rely more heavily on use of a direct apical targeting mechanism is also reflected in the polarity of protein secretion: Where Caco-2 cells secrete apolipoproteins from the basolateral surface (Traber et al., 1987), enterocytes predominantly release free apolipoprotein A-1 from the brush border membrane when they are not required for chylomicron

formation (Danielsen et al., 1993).

Lactase-phlorizin hydrolase was the only brush border enzyme studied that seemed to be efficiently excluded from the detergent-insoluble complexes both during its intracellular transport and at the cell surface. This may reflect the fact that this enzyme has a membrane topology different from the other enzymes in being a type 1—rather than a type 2—membrane protein (and in being synthesized with a large N-terminal propeptide) (Mantei et al., 1988). An inability to associate with detergent-insoluble complexes might be an explanation why the time required for transport to the apical cell surface of lactase-phlorizin hydrolase is considerably longer than for the other brush border enzymes, particularly in Caco-2 cells (Hauri et al., 1985), and it might also reflect that, in the small intestinal enterocyte of the pig, lactase-phlorizin hydrolase assembles into homodimers at a later stage (mainly a post-Golgi event) than aminopeptidase N and sucrase-isomaltase (Danielsen, 1990, 1994).

ACKNOWLEDGMENT

I thank Prof. Hans Sjöström, Prof. Ove Norén, and Prof. Bo van Deurs for valuable discussions of the manuscript.

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