Intestinal alkaline phosphatase: selective endocytosis from the enterocyte brush border during fat absorption

Gert H. Hansen, Lise-Lotte Niels-Christiansen, Liss Immerdal, Birthe T. Nystrøm, and E. Michael Danielsen

Department of Cellular and Molecular Medicine, and Department of Biomedicine, The Panum Institute, University of Copenhagen, Copenhagen, Denmark

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Hansen GH, Niels-Christiansen L-L, Immerdal L, Nystrøm BT, Danielsen EM. Intestinal alkaline phosphatase: selective endocytosis from the enterocyte brush border during fat absorption. Am J Physiol Gastrointest Liver Physiol 293: G1325–G1332, 2007. First published October 18, 2007; doi:10.1152/ajpgi.00379.2007.—Absorption of dietary fat in the small intestine is accompanied by a rise of intestinal alkaline phosphatase (IAP) in the serum and of secretion of IAP-containing surfactant-like particles from the enterocytes. In the present work, fat absorption was studied in organ cultured mouse intestinal explants. By immunofluorescence microscopy, fat absorption caused a translocation of IAP from the enterocyte brush border to the interior of the cell, whereas other brush-border enzymes were unaffected. By electron microscopy, the translocation occurred by a rapid (5 min) induction of endocytosis via clathrin-coated pits. By 60 min, IAP was seen in subapical endosomes and along membranes surrounding fat droplets. IAP is a well-known lipid raft-associated protein, and fat absorption was accompanied by a marked change in the density and morphology of the detergent-resistant membranes harboring IAP. A lipid analysis revealed that fat absorption caused a marked increase in the microvillar membrane contents of free fatty acids. In conclusion, fat absorption rapidly induces a transient clathrin-dependent endocytosis via coated pits from the enterocyte brush border. The process selectively internalizes IAP and may contribute to the appearance of the enzyme in serum and surfactant-like particles. Small intestine

Absorption of dietary fat in the small intestine of mammals involves a complex series of events. After luminal emulsion with bile and digestion with pancreatic lipase, fatty acids and monoacylglycerol are transported across the brush border and reassembled to triacylglycerol in the endoplasmic reticulum of the enterocyte before being packaged into chylomicrons and finally discharged by exocytosis from the basolateral cell surface (18, 28). It has long been recognized that fat absorption is accompanied by the appearance of a soluble form of intestinal alkaline phosphatase (IAP) in serum (29, 31, 32, 48). In the small intestine, IAP is a resident brush-border enzyme that hydrolyzes monophosphate esters (16, 27, 35). It is a glycoprotein linked to the membrane by a glycosylphosphatidylinositol anchor, and the mechanism underlying its release from the small intestine to the blood has been the subject of many studies over the past decades (11, 14, 38). Thus it has been established that, after fat absorption, IAP is incorporated into membranes surrounding intracellular lipid droplets and into basolaterally secreting surfactant-like particles (SLPs), so called because of their similarity to pulmonary surfactant (15). After secretion, these lamellar particles become distributed in layers on the luminal surface and in the lamina propria beneath the basal membrane of the mucosal cells (49). Interestingly, studies on IAP knockout mice have indicated that the enzyme participates in a regulatory rate-limiting step in fat absorption and that its absence may cause metabolic abnormalities leading to hepatic steatosis and visceral fat accumulation (37, 39).

In addition to their content of IAP, SLPs are enriched in dipalmitoylphosphatidylcholine and surfactant proteins, and these and other characteristics indicate that SLPs are not identical to fragments of the brush-border membrane (13). Accordingly, the IAP harbored in SLPs is thought to represent a newly synthesized enzyme of intracellular origin that assembles with the nascent lamellar particles (17).

In the present work, we studied the early events in the enterocytic uptake and accumulation of dietary fat. With the use of organ culture of small intestinal explants as a model system, a rapid appearance of coated pit formation at the apical surface was observed to accompany fat uptake across the brush border, indicative of an induced endocytosis. Only IAP out of several major brush-border enzymes studied was visibly translocated from the apical surface during the fat absorption process. Intracellularly, IAP accumulated in subapical endosomes and was seen in membranes surrounding the nascent lipid droplets. We therefore conclude that fat absorption is a physiological event that causes a rapid but transient endocytosis of IAP from the brush border.

MATERIALS AND METHODS

Materials. Rabbit and sheep antibodies to IAP were from AbD Serotec (http://www.biogenesis.co.uk/), and Abcam (http://www.abcam.com/), respectively, and goat antibodies to annexin A2 (annexin II) were from Santa Cruz Biotechnology (http://www.scbt.com/). Monoclonal mouse antibodies to clathrin heavy chain were from Meridian Life Science (http://www.meridianlifescience.com/) and from Boehringer Mannheim (Mannheim, Germany). The following rabbit antibodies to other brush-border enzymes were also used in the present work and have been previously described elsewhere: aminopeptidase N (25), aminopeptidase A (9), meprin (neutral endopeptidase-2) (3), lactase (41), and maltase glucoamylase (42). Secondary Alexa Fluor 488-conjugated antibodies for immunofluorescence microscopy were obtained from Invitrogen (www.probes.invitrogen.com). Secondary horseradish peroxidase-coupled antibodies for immunoblotting, secondary antibodies for immunogold electron microscopy, and antifade mounting medium were from Dako.
Bovine bile and pancreatin from porcine pancreas (containing a mixture of digestive enzymes) and gold-conjugated protein G for immunogold electron microscopy were from Sigma-Aldrich (http://www.sigma-aldrich.com/).

Fasted mouse small intestines were kindly provided by the Department of Experimental Medicine, the Panum Institute (Copenhagen, Denmark).

Organ culture of small intestinal explants. The small intestines of adult mice (obtained from licensed staff at the Department of Experimental Medicine, University of Copenhagen) were rinsed in MEM medium, and explants were excised from the proximal part of the intestine and cultured in MEM medium essentially as described previously (10). In experiments with fat absorption, a solution of corn oil (10%), bile (1%), and pancreatin (1%) was made up in MEM medium, vortexed, and preincubated overnight at 37°C before it was diluted 1:1 with fresh MEM medium and added to intestinal explants.

Fluorescence microscopy. Immediately after culture, explants were fixed in 4% paraformaldehyde in 0.1 M sodium phosphate, pH 7.2

Fig. 1. Accumulation of lipid droplets in enterocytes. Electron micrographs of Epon sections showing the apical region of enterocytes after 20 min (A) and 60 min (B) of fat absorption during organ culture of small intestinal explants. Small- and medium-sized lipid droplets (arrows) were visible after 20 min, and full-size (>1 μm) droplets were formed by 60 min. No lipid droplets were seen in explants cultured in parallel for 60 min in the absence of fat (C). Notice the progressive reduction in the length of microvilli during fat absorption, compared with control samples (C). Bars = 1 μm.

Fig. 2. Fat absorption selectively changes localization of intestinal alkaline phosphatase (IAP). Localization of brush-border enzymes by immunofluorescence microscopy is shown for control explants and for explants exposed to the fat emulsion for 60 min. Mep, meprin; ApA, aminopeptidase A; Mal, maltase-glucoamylase. The images shown are representative results obtained from 3 separate experiments. Bar = 10 μm.
(PB), for 2 h at 4°C. After a rinse in PB, the explants were frozen in precooled 2-methylbutane and mounted on a precooled cryostat table. Sections were cut in a Leica CM1850 cryostat at −20°C, collected on glass slides, and labeled with primary antibodies to IAP, aminopeptidase N, aminopeptidase A, meprin, lactase, and maltase-glucosamylase, followed by Alexa Fluor 488-conjugated secondary antibodies. Control experiments were routinely performed in parallel by omission of the primary antibodies. Sections were mounted in antifade mounting medium and finally examined in a Leica DM 4000 B microscope equipped with a Leica DC 300 FX digital camera.

**Electron microscopy.** For Epon sectioning, cultured explants were fixed in a 3% glutaraldehyde-2% paraformaldehyde mixture in PB for 20 min at 4°C, and pellets of detergent-resistant membranes (DRMs) were fixed in 2.5% glutaraldehyde in PB for 2 h at 4°C. After a rinse in PB, the specimens were postfixed in 1% osmium tetroxide in PB for 1 h at 4°C, dehydrated in acetone, and finally embedded in Epon. Ultrathin sections were cut in a LKB Ultrrotome III, and some sections were incubated with sheep anti-IAP antibodies, followed by labeling with gold-conjugated protein G (2).

For ultracyrosectioning, cultured explants were fixed in a 4% paraformaldehyde in PB for 2 h at 4°C. After a rinse in PB, the specimens were immersed in 2.3 M sucrose, containing 1% paraformaldehyde overnight, mounted on top of a metal pin, and frozen in liquid nitrogen. Ultracyrosections were cut in an RMC MT 6000-XL ultramicrotome, collected with a sucrose droplet, and attached to formvar-coated nickel grids. Immunogold labeling using primary antibodies to IAP, meprin, and clathrin heavy chain, followed by secondary gold-conjugated antibodies, was performed as previously described (23).

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Finally, Epon and ultracyrosections were examined in a Zeiss EM 900 electron microscope equipped with a Mega View II digital camera system.

**Subcellular fractionation.** Cultured small intestinal explants were fractionated by the divalent cation precipitation method (4). Briefly, the explants were homogenized in 2 mM Tris-HCl and 50 mM mannitol, pH 7.1, containing 10 μg/ml aprotinin and leupeptin, by use of a manually operated Potter-Elvehjem homogenizer. The homogenate was cleared by centrifugation at 120 g for 5 min, and MgCl₂ was added to a final concentration of 10 mM. After 10-min incubation on ice, the preparation was centrifuged at 1,100 g for 10 min to sediment intracellular and basolateral membranes. Subsequent centrifugation of the supernatant at 48,000 g for 30 min yielded a pellet of microvillar membrane vesicles and a final supernatant of soluble proteins.

For preparation of DRMs, pellets of microvillar- and Mg²⁺-precipitated membranes were resuspended in 1 ml of 25 mM HEPES-HCl and 150 mM NaCl, pH 7.1, containing 10 μg/ml aprotinin and leupeptin, and extracted with 1% Triton X-100 for 10 min on ice. DRMs were isolated by sucrose gradient ultracentrifugation as previously described (5). Centrifugation was performed in a SW40 Ti rotor (Beckmann Instruments, Palo Alto, CA) for 20–22 h at 35,000 rpm (g_max = 217,000 g), as described previously (7). After centrifugation, the gradients were fractionated for subsequent analysis by SDS-PAGE and immunoblotting. Alternatively, the floating fractions were carefully collected by a pipette, diluted five times with 25 mM HEPES-HCl and 150 mM NaCl, pH 7.1, and centrifuged at 48,000 g for 1 h to obtain a pellet of DRMs for electron microscopy.

**SDS-PAGE and immunoblotting.** SDS-PAGE in 10% gels was performed as described previously (33). After electrophoresis and electrotransfer of proteins onto Immobilon polyvinylidene difluoride membranes, immunoblotting was performed with primary antibodies to IAP, aminopeptidase N, and annexin A2. The blots were developed using the electrochemiluminescence detection reagent according to the protocol supplied by the manufacturer (GE Healthcare, Little Chalfont, UK). After blotting was completed, total protein was visualized by staining with Coomassie brilliant blue.

**Lipid analysis.** For lipid analysis, Mg²⁺-precipitated microvillar membrane fractions were extracted with chloroform-methanol (6) as previously described (24). Aliquots of the lipid extracts were subjected to thin layer chromatography analysis together with appropriate lipid standards on 0.25-mm silica gel plates. After chromatography, the lipids were detected with a CuSO₄-H₃PO₄ reagent. Free fatty acids were determined after their chromatographic separation and excision of the silica gel spots (12).

**RESULTS**

**Rapid fat absorption by intestinal explants in culture.** In the present work, organ culture of mouse small intestinal explants...
was used as a model system for studying early events in the process of dietary fat absorption. To that effect, a pancreatin-predigested cornoil-bile emulsion was prepared to mimic the composition of a fatty meal after its passage into the jejunum where the fatty acids and monoacylglycerol are taken up by the enterocytes.

As shown in Fig. 1, culture of small intestinal explants in MEM medium containing the fat emulsion resulted in a rapid formation of lipid droplets in the apical cytoplasm of the enterocytes. Smaller droplets (up to 0.5 μm) were readily detectable by 20 min of culture (Fig. 1A); after 60 min, mature, micrometer-size droplets had formed (Fig. 1B). No lipid droplets were detected in control explants cultured in parallel in the absence of fat (Fig. 1C). Interestingly, fat absorption resulted in a shortening of the microvilli, suggesting an internalization of considerable amounts of apical membrane during the process. Otherwise, fat absorption was not observed to cause any morphological changes in other parts of the enterocyte. Thus the Golgi complex and the basolateral plasma membrane appeared unaffected by the process (data not shown). Unlike in vivo experiments, this model system allows well-defined short-time exposures to fat to be performed and is therefore well suited for studying early events during fat absorption in the gut.

Selective translocation of IAP induced by fat absorption. Figure 2 shows the localization by immunofluorescence of four brush-border enzymes in control explants and in explants after 60 min of fat absorption. For IAP, the distinct and intense labeling of the brush border seen in the control situation was changed to a more diffuse staining pattern penetrating well into the apical cytoplasm of the enterocytes. In contrast, little or no
change in labeling pattern was seen for other prominent brush-border enzymes, such as the peptidases aminopeptidase A and meprin or the glycosidase maltase glucoamylase (Fig. 2), and for aminopeptidase N and lactase (data not shown). These results thus suggest that IAP rapidly and selectively translocates from the brush border to intracellular compartments during fat absorption.

Rapid induction of clathrin-coated pit formation and endocytosis by fat absorption. Figure 3, A and B, shows electron micrographs of the enterocyte brush border from control explants and after 5 min of fat absorption, respectively. In the control samples, pits or invaginations were rarely observed between adjacent microvilli of the enterocytes, in agreement with the fact that apical endocytosis seldom occurs in enterocytes from mature animals. A 5-min exposure to the fat emulsion, however, was sufficient to induce the formation of numerous pits along the apical cell surface. The pits were ~100 nm in diameter; at higher magnification, a coat was clearly seen surrounding the cytosolic face of the pit membrane (Fig. 3C). Small, coated vesicles, apparently detached from the cell surface, were also detected (Fig. 3D). Both pits and apical vesicles were labeled by an antibody to clathrin heavy chain (Fig. 3, E and F), indicating that the induced endocytosis is of the clathrin-dependent type.

Figure 4A shows an immunogold labeling for IAP in the apical region of an enterocyte from control explants. A dense labeling was seen along the microvilli, but only very few gold particles were present in the apical cytoplasm beneath the microvilli. In contrast, Fig. 4, B and C, shows that all the pits induced at the apical cell surface by 5 min of fat absorption were heavily labeled, indicating that the translocation of the enzyme to intracellular compartments occurs by this mechanism. Like for IAP, a strong labeling for meprin was seen over the microvilli; however, in contrast to IAP, meprin labeling was essentially absent from the pits seen between the microvilli by 5 min of fat absorption (Fig. 4, D and E).

At longer exposure times to the fat emulsion, fewer coated pits were generally observed at the apical cell surface; by 60 min, they could no longer be detected (results not shown). Together, this suggests that fat absorption induces a rapid but transient wave of clathrin-dependent endocytosis.

At 20 min of fat absorption, vesicles and endosomes present in the subapical cytoplasm beneath the terminal web region were heavily labeled for IAP (Fig. 5A); after 60 min, IAP labeling was seen at the membranes surrounding the numerous lipid droplets scattered in the apical cytoplasm (Fig. 5B).

In summary, the above results indicate that exposure to a “physiological” fat emulsion induces a rapid endocytosis of IAP from the enterocyte brush border. The internalization occurred via clathrin-coated pits, resulting in a significant redistribution of this brush-border enzyme to intracellular compartments. Because several other major brush-border enzymes studied were not similarly translocated, IAP appears to be selectively endocytosed during the process of fat absorption.

IAP redistribution to low-density DRMs after fat absorption. To study in closer detail the composition of the IAP-containing translocated membranes, Mg²⁺-precipitated membranes from control and fat-exposed explants were subjected to lipid raft analysis by detergent extraction followed by gradient centrifugation. In both situations, IAP was seen in immunoblotting as a single band of ~65 kDa, exclusively residing in DRMs, in agreement with its membrane anchorage by a glycosylphosphatidylinositol (GPI). However, fat absorption resulted in a marked change in the buoyant density of the IAP-containing DRMs. Thus, from IAP appearing in a broad range of fractions (fractions 6–11) in the control membranes, the enzyme became narrowly confined to fractions 11 and 12 after fat absorption (Fig. 6), indicating a change in its microdomain environment. Another lipid raft-associated protein, annexin A2 (26), believed to be involved in endocytic membrane trafficking (19), also shifted position in the gradient to the upper two fractions. The shift observed for annexin A2 was less distinct, indicating that this protein has a wider distribution in intracellular DRMs than IAP. The morphology of the isolated DRMs was studied by electron microscopy (Fig. 7). In Epon-embedded sections, microvillar DRMs appeared mainly as closed vesicular structures of ~100–150 nm in diameter, regardless of whether they were prepared from control or fat-exposed explants. In contrast, DRMs from Mg²⁺-precipitated membranes of control explants were more pleomorphic, consisting of rod-like membrane fragments and large (>0.5 μm in diameter) vesicle-like structures. However, after fat absorption, DRMs from this fraction mainly included the type of small vesicles, characteristic of microvillar DRMs. Immunogold labeling confirmed the presence of IAP on these membranes (Fig. 7).

In conclusion, the marked change in both density and morphology of IAP-containing DRMs isolated from intracellular membranes indicates a redistribution of the enzyme during fat absorption. This result agrees well with the induced apical endocytosis observed by immunomicroscopy and the microvillar shortening but might also, to some extent, reflect a redistribution of IAP between different intracellular compartments.

Changes in enterocyte membrane lipid composition during fat absorption. During dietary fat absorption, large amounts of free fatty acids and monoacylglycerol are transported across the brush border and assembled into triacylglycerol in the endoplasmic reticulum. To evaluate the effect of this flux of lipid molecules on the membranes of the enterocytes, the lipid composition of the microvillar- and Mg²⁺-precipitated fractions was analyzed (Fig. 8, Table 1). Fat absorption caused no overall changes in the relative contents of glycerolipids, glycerolipids, and cholesterol.
colipids, and cholesterol in either of the fractions, but the amounts of free fatty acids, particularly in the microvillar fraction, were markedly increased. This observation suggests that a fraction of the dietary free fatty acids transiently becomes incorporated into the enterocyte membranes, most notably the microvillar membrane. As should be expected, Mg²⁺-precipitated membranes contained relatively smaller amounts of glycolipids compared with microvillar membranes.

**DISCUSSION**

The rapid appearance and growth of intracellular lipid droplets in enterocytes of cultured explants seen in the present work testify to the extraordinary capacity of this cell type for import, resynthesis, and storage of fat. Our main objective was to study how the physiological process of fat absorption affects the brush-border membrane and in particular its contents of IAP. Of several major brush-border enzymes localized by immunofluorescence microscopy, only IAP appeared to be markedly affected, indicating the induction of a selective translocation of this enzyme by fat absorption. At the level of electron microscopy, the rapid (within 5 min) formation of numerous coated pits at the apical cell surface and subapical vesicles showed that endocytosis accounted for the translocation of IAP. Although lipid rafts are abundant in the brush border, the endocytosis occurred by a clathrin-dependent mechanism, as was previously observed for cholera toxin (20). A plausible explanation for this might be the low expression of caveolin and scarcity of caveolae in enterocytes (1, 44).

Inside the enterocyte, endosomes were the main initial destination of the internalized IAP; however, within 60 min, the membranes surrounding lipid droplets also acquired IAP. Like lipid droplets, these membranes, as previously demonstrated (11, 13), are generated in the endoplasmic reticulum and give rise to the SLPs subsequently secreted by exocytosis from the basolateral cell surface together with chylomicrons. In previous work, it has been argued that IAP in SLPs must represent enzyme protein synthesized de novo because both their protein and lipid compositions are markedly different from those of the brush border (11, 13). However, the results of the present work suggest that brush-border IAP may also in part be recruited for incorporation into SLPs following its apical endocytosis. The contribution of this pathway relative to that of the de novo synthesis of IAP is difficult to assess.

**Table 1. Free fatty acids in enterocyte membranes**

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<thead>
<tr>
<th>Fraction</th>
<th>Control (%)</th>
<th>+Fat (%)</th>
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<tr>
<td>Mg²⁺-precipitated membranes</td>
<td>15.6%</td>
<td>26.6%</td>
</tr>
<tr>
<td>Microvillar membranes</td>
<td>2.6%</td>
<td>7.5%</td>
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Values are amounts of free fatty acids as percentage of the total lipid in the respective fractions and are the mean of 2 determinations. The lipids of the Mg²⁺-precipitated- and microvillar fractions of intestinal explants, cultured for 1 h in the absence (control) or presence of fat, were extracted and separated by thin layer chromatography as described in MATERIALS AND METHODS and Fig. 8. The amounts of free fatty acids in the gel spots excised from the thin layer plates were determined spectrophotometrically according to the method of Duncombe (12).
However, the rapidity and transient nature of the induced endocytosis suggest that this pathway might be quantitatively most important in the early phase of fat absorption. At later stages, the slower flow of newly synthesized enzyme may be the main provider of IAP for SLP formation and export. Accordingly, a retrograde pathway must subsequently transport the endocytosed IAP to the endoplasmic reticulum. The precise itinerary of this pathway must await further elucidation but is likely to include a subapical endosomal compartment (46), as evidenced by the IAP-positive endosomes seen after fat absorption. The existence of a retrograde pathway has been convincingly demonstrated by several works studying the route taken by bacterial toxins, such as cholera toxin, to accomplish their intoxication of the target cell (34, 40).

From studies on IAP knockout mice, it appears that the role of the enzyme is to decrease, rather than increase, the rate of transport of dietary lipid through the enterocyte, probably by affecting the lipid flow through the Golgi complex (37, 39). This organelle is a major hub in cellular membrane trafficking, and it is conceivable that the fat absorption-induced endocytosis of IAP from the brush border could affect the overall membrane dynamics of the enterocyte, leading to a slower basolateral secretion of chylomicrons and SLPs.

In addition to its absorptive functions, the small intestinal brush border must also act as a permeability barrier for luminal pathogens. Unlike other types of cell surfaces, the brush border is therefore normally considered nonpermissive for endocytic uptake by any mechanism (45). This may in part be explained by its unusually high amounts of glycolipids (6) that are organized in lipid raft microdomains stabilized by the divalent lectins galectin-4 (8, 21) and intelectin (47). Why and how a physiological process like fat absorption is capable of rapidly triggering an abundant apical coated pit formation and endocytosis are intriguing questions but at present unclear to us. Conceivably, the presence of bile and pancreatin in the culture medium of the mucosal explants might affect the epithelium differently from the situation in vivo. However, we consider it more likely that these harsh conditions genuinely mimic those existing in the gut lumen during digestion and absorption of nutrients and transiently compromises the barrier function of the brush border. A possible physiological role of this could be that the resulting membrane lipids thus internalized become available for assembly of chylomicrons and SLPs. As for a possible mechanism triggering the endocytosis, a clue might be the marked increase in the contents of free fatty acids in the microvillar membrane. Dietary free fatty acids may pass the brush border either by a saturable mechanism involving fatty acid translocase/CD36 and fatty acid transport proteins or through passive flip-flop across the lipid bilayer (43). Here, the latter pathway would be likely to give rise to a transient increase in microvillar free fatty acid content as the one observed. Conceivably, a flip-flop mechanism must involve a transient incorporation of the free fatty acid into the outer leaflet of the bilayer before translocation to the inner leaflet and subsequent delivery to a cytosolic fatty acid binding protein (43). How this process in turn may affect the physical properties of the microvillar bilayer is unknown, but one may speculate that a transient imbalance in the relative surface areas of the inner and outer leaflets somehow triggers membrane curvatures that stimulate coated pit formation and endocytosis.

Like other membrane proteins anchored by a glycosylphosphatidylinositol, IAP firmly resists extraction with cold Triton X-100 and is consequently fully DRM associated. By comparison, the transmembrane brush-border peptidases and glycosidases included in the present work reside either only partially in DRMs or are fully detergent soluble (7, 30). Whether this difference is the reason why IAP is selectively endocytosed from the brush border remains to be clarified. It should also be noted that the selectivity may not be absolute. Thus we have previously observed that the scavenger receptor SR-BI is found in lipid droplets after fat absorption (22), and cubilin, a large multiligand endocytic receptor of the LDL-receptor family, is secreted in SLPs together with IAP after fat feeding (36).

In conclusion, the observation that the absorption of dietary fat is accompanied by an endocytosis from the brush border adds yet another level of complexity to the overall process of fat assimilation. Clearly, more work is needed to obtain a full understanding of this important physiological process.

REFERENCES


GRANTS

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