

Apical Secretion of Apolipoproteins from Enterocytes

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Abstract. Synthesis and secretion of apolipoproteins in pig small intestine was studied by pulse-chase labeling of jejunal segments, kept in organ culture. Apo A-1 and apo B-48 were the two major proteins released, constituting 25 and 10%, respectively, of the total amount of labeled protein in the mucosal-side medium where they appeared with a $t_{1/2}$ of 50–60 min. Using tissue from fasting animals, >85% of newly synthesized apo A-1 and about one third of apo B-48 was released to the mucosal-side medium. Newly synthesized apolipoprotein that remained associated with the intestinal segment accumulated in the soluble fraction, suggesting a basolateral secretion into the intercellular space, and both this accumulation and the release to the medium was prevented by culture at 20°C. The specific radioactivity of apo A-1 and apo B-48 released to the medium was significantly higher than that of the

corresponding apolipoproteins remaining associated with the intestinal tissue. Furthermore, during culture periods of up to 5 h, the enterocytes and their tight junctions largely remained intact as evidenced by the inaccessibility of the nonpermeable surface marker Ruthenium red. We therefore propose that enterocytes release most of their newly made free apo A-1 and a significant portion of apo B-48 by exocytosis via the brush border membrane into the intestinal lumen. Fat absorption reduced apolipoprotein secretion to the medium and induced the formation of chylomicrons, containing apo A-1 at their surface, as evidenced by immunogold electron microscopy. The chylomicrons were localized in the Golgi complex and near the basolateral plasma membrane, but not in the apical region of the enterocytes, indicating that only free apolipoproteins are secreted to the intestinal lumen.

To fulfill their role in the assimilation of dietary fat, small-intestinal enterocytes express a number of apolipoproteins, required for their synthesis of lipoproteins (Shiau, 1987). The major apolipoproteins synthesized by the gut include apolipoprotein A-1 (apo A-1)¹ and apolipoprotein B-48 (apo B-48), and during fat absorption, the concentration of these apolipoproteins inside enterocytes has been reported to increase considerably (Schonfeld et al., 1978; Glickman and Green, 1977; Glickman et al., 1978). In spite of this, the dietary status does not acutely affect the rate of biosynthesis of these apolipoproteins which remains high also in the fasting state (1–2% and 0.5–1% of total protein synthesis in the rat enterocyte for apo A-1 and apo B-48, respectively) (Davidson et al., 1987). That apolipoproteins are indeed major gene products of the enterocytes has also been established at the mRNA level of expression (Gordon et al., 1982a,b). After fat absorption, however, the pattern of apo A-1 and apo B-48 in lipoproteins of intestinal lymph does not mirror their distribution inside the enterocyte; thus, several investigators have observed that the major proportion of intracellular apo A-1 (~90%) is unassociated with lipoproteins and that apo B-48 is either unassociated or, if associated, then mainly with low density lipoprotein and high

density lipoprotein (Mougin-Schutz et al., 1983; Alpers et al., 1985; Magun et al., 1988).

It has been well documented that chylomicrons, after their assembly in the Golgi complex of enterocytes, are secreted by exocytosis from the basolateral plasma membrane into the intercellular space of the epithelium, and, after penetration of the basement membrane and diffusion through the lamina propria, reach the lymphatics (Sabesin and Frase, 1977; Christensen et al., 1983). However, since enterocytes do not have a large storage capacity for apolipoproteins (Alpers et al., 1982), the fate of the large intracellular amounts of free apolipoproteins is uncertain as these cannot be recovered from the lymph (Alpers et al., 1985). One possibility, to our knowledge not previously considered, would be that enterocytes secrete constitutively the major portion of newly synthesized, free apolipoproteins mainly by exocytosis via the apical brush border membrane into the lumen of the gut. In the present work, we tested this hypothesis by studying synthesis and secretion of apolipoproteins in organ-cultured segments of pig small intestine, a model system that has previously been useful for investigating biosynthesis, processing, and intracellular transport of brush border enzymes (Danielsen et al., 1987). Surprisingly, our results strongly argue that apo A-1 and apo B-48 are indeed secreted from the apical surface of the enterocyte and that this pathway, as shown for apo A-1, is the major route whereby the apolipoprotein exits the epithelial cells.

1. Abbreviations used in this paper: apo A-1, apolipoprotein A-1; apo B-48, apolipoprotein B-48.

Materials and Methods

Materials

Equipment for performing organ culture, including Trowell's T-8 medium, plastic dishes with grids, and [³⁵S]methionine (specific radioactivity >1,000 Ci/mmol) was obtained as previously described (Danielsen et al., 1982). Rabbit antibodies to human β -lipoprotein, rabbit antibodies to swine serum, and swine antibodies to rabbit immunoglobulins were purchased from Dako Corp. (Copenhagen, Denmark). Heparin (from porcine intestinal mucosa) and Ruthenium red were products of Sigma Chem. Co. (St. Louis, MO).

Pig small intestines were kindly given by the Department of Experimental Pathology, Rigshospitalet, Copenhagen.

Organ Culture of Intestinal Segments

Pigs (30–40 kg of weight) were fasted overnight except in one case where the animal, 3 h before surgery, was fed with 250 g of fat (butter). Segments of \sim 1 cm² of intact tissue were excised from the small intestine, taken \sim 2 m from the Pylorus, and cultured on grids placed in Falcon dishes essentially as earlier described for organ culture of mucosal explants (Danielsen et al., 1982). In culture for periods of up to 5 h, the intestinal segments remained viable, as judged by their morphology and their ability to synthesize, process, and transport secretory and membrane proteins. In other experiments, the intestinal segments were cultured in a type of Ussing chamber instead of a dish, allowing the mucosal and serosal sides of the tissue to be bathed by separate media. Otherwise, the culture conditions in the two types of experimental setup were identical. After culture, intestinal segments were rapidly frozen and kept at -20°C until further processing.

Fractionation of Labeled Intestinal Segments

In some experiments, the intestinal segments were extracted in 0.5 ml 25 mM Tris-HCl, 75 mM NaCl, 5% (wt/vol) Triton X-100, pH 7.3. The homogenates were centrifuged at 20,000 g, 5 min, to obtain supernatants of detergent-extracted proteins. In other experiments, the segments were gently homogenized (manually operated Potter-Elvehjem homogenizer) in 2 ml 50 mM Tris-HCl, 150 mM NaCl, pH 7.3 and the homogenates centrifuged at 48,000 g, 60 min, to obtain a soluble fraction (cytosol and intercellular fluid) and a pellet containing membrane-associated protein.

Immunoprecipitation

An antiserum to human apo A-1 (\sim 97% pure) was prepared and used in precipitation of the apolipoprotein from medium and tissue fractions as previously described (Danielsen, 1990). The same procedure was used for immunoprecipitation of serum proteins, using rabbit antibodies to swine serum. Aminopeptidase N (EC 3.4.11.2) and sucrase-isomaltase (EC 3.2.1.48, EC 3.2.1.10) were purified by a rabbit antiserum to pig intestinal brush borders which predominantly precipitates these two microvillar enzymes (Danielsen, 1989).

Precipitation with Heparin-Mn²⁺

Precipitation of apo B-containing lipoproteins from media and soluble fractions of intestinal segments with heparin-Mn²⁺ was performed according to the method of Warnick and Albers (1978) as follows: 0.5 ml of culture medium or soluble fraction was mixed with 50 μ l heparin (14 mg/ml) dissolved in 0.92 M MnCl₂, and incubated on ice for 30 min. The precipitate was collected by centrifugation at 10,000 g, 2 min, washed in 50 mM Tris-HCl, 150 mM NaCl, pH 7.3, containing 0.1 vol of heparin-Mn²⁺-solution, and pelleted by centrifugation as described above.

Ultracentrifugation Analysis

For ultracentrifugation analysis, 50- μ l samples of culture medium were adjusted with KBr to a density of 1.063 g/ml or 1.21 g/ml and centrifuged for 25–30 h at 100,000 g (30 psi) in an airfuge (at 4°C) (Beckman Instrs. Inc., Fullerton, CA). After centrifugation, the supernatants were collected and analyzed by SDS-PAGE.

Electrophoresis

SDS-PAGE in 10% gels under reducing conditions was performed accord-

ing to Laemmli (1970), and fluorography as described by Bonner and Laskey (1974). X-ray films of gel tracks were scanned in an Ultrosan XL densitometer (Pharmacia LKB, Bromma, Sweden).

Rocket immunoelectrophoresis in 1% agarose gels was performed as described by Weeke (1973).

Immunofluorescence Microscopy

Small segments of freshly obtained pig jejunum were frozen for 1 min in precooled hexane at -70°C . For immunofluorescence, 5- μ m sections were cut in a cryostat (Carl Zeiss, Inc., Thornwood, NY) operated at -25°C and incubated overnight with rabbit apo A-1 antiserum, diluted 600-fold. The sections were next incubated for 20 min with swine anti-rabbit IgG conjugated to FITC and examined in a Zeiss epifluorescence microscope equipped with interference filters for FITC (Optisk Laboratorium, Lundtofte, Denmark). After microscopic inspection, the sections were fixed in paraformaldehyde for 30 min and stained with Hematoxylin and Eosin.

Electron Microscopy

For ultrastructural studies, segments of jejunum obtained from a fat-fed animal were fixed for 2 h at 4°C in 2.5% glutaraldehyde in 0.1 M sodium phosphate, pH 7.2 (PB). After a rinse in PB, the tissue was postfixed in 1% osmium tetroxide in PB, dehydrated in ethanol, and finally embedded in Epon.

For staining with Ruthenium red, mucosal segments cultured for 5 h were fixed for 20 h at 4°C in a mixture of 3% glutaraldehyde and 2% paraformaldehyde in PB containing 0.2% Ruthenium red. After a rinse in PB, the tissue was postfixed for 2 h at 20°C in 1% osmium tetroxide in PB containing 0.3% Ruthenium red, treated for 1 h at 20°C with 1% uranyl acetate in water, dehydrated in ethanol, and finally embedded in Epon.

Ultrathin sections were cut in an Ultratome III (Pharmacia LKB), using a diatome diamond knife. The sections were collected on formvar- and carbon-coated slot grids, and examined in an EM 900 electron microscope (Zeiss, FRG). In the experiment, shown in Fig. 7 A, the sections were stained in 1% uranyl acetate and lead citrate before examination.

For immunoelectron microscopy (immunogold labeling), freshly obtained mucosal segments were fixed in 4% formaldehyde (freshly prepared from paraformaldehyde powder) in PB. The tissue was then placed in 2.3 M sucrose for 30 min at 20°C, mounted on top of a metal pin, and frozen in liquid nitrogen. Ultracryosections of \sim 85–100 nm were cut in an RMC MT 6000-XL ultracryomicrotome, using a glass knife, collected with a sucrose droplet and attached to formvar- and carbon-coated 75-mesh nickel grids.

Gold particles of \sim 10 nm were prepared according to Slot and Geuze (1985) and conjugated to sheep anti-rabbit IgG as described by Hansen et al. (1992). The immunogold labeling was performed essentially as described by Balslev and Hansen (1989).

Results

Release of Apolipoproteins into the Mucosal-Side Medium

Fig. 1 A shows the appearance of labeled proteins in the mucosal-side medium from a small intestinal segment cultured in an Ussing chamber. After a 20-min pulse with [³⁵S]methionine, the most abundant component (constituting \sim 25% of labeled protein in the medium after 3 h of chase) had a molecular mass of 27 kD and was previously identified as apo A-1 (Danielsen et al., 1991), but another distinct polypeptide with a molecular mass of 220 kD was visible as well. The latter, constituting \sim 10% of total labeled protein in the medium, represents apo B-48 as demonstrated by its recognition by an antibody to human β -lipoprotein and by its precipitation with heparin-Mn²⁺, a routine method for isolation of apo B-containing lipoproteins (Burstein et al., 1970; Warnick and Albers, 1978) (Fig. 2). (In the absence of detergent, apo A-1 was coprecipitated partially by the heparin/Mn²⁺ treatment, showing that the two lipoproteins released to the mucosal-side medium are associated.) Furthermore, apo B-48 was effectively immunoprecipitated

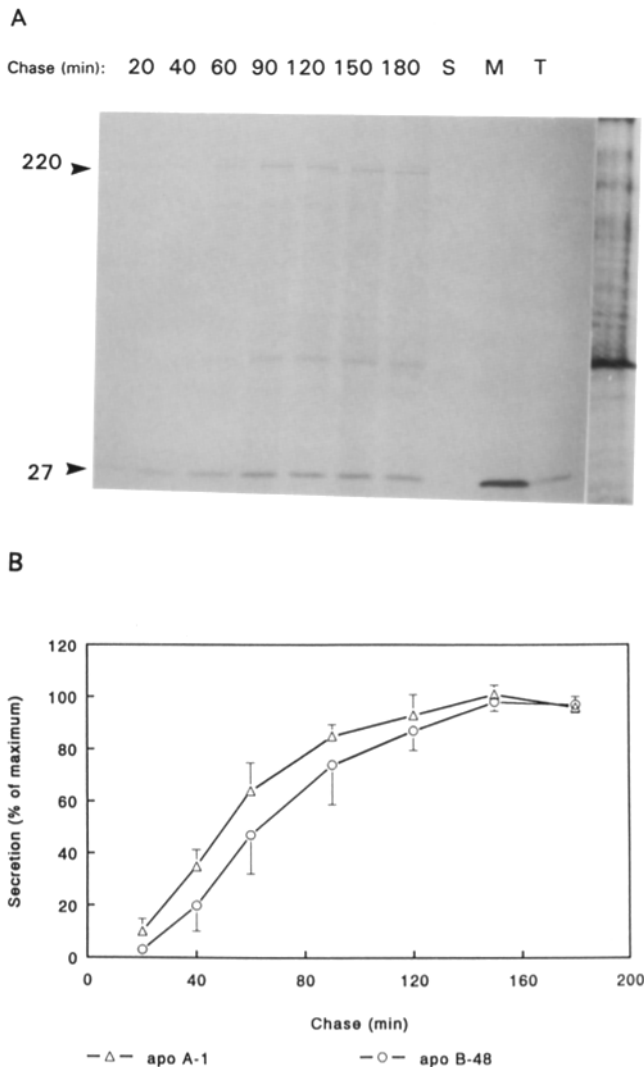


Figure 1. Release of proteins from cultured intestinal segments. (A) A small intestinal segment was mounted in a type of Ussing chamber with a circular aperture of 1 cm in diameter, and its mucosal and serosal sides were each bathed in 1 ml of medium. The segment was then labeled (from the mucosal side) for 20 min with 200 $\mu\text{Ci/ml}$ [^{35}S]methionine, followed by a chase with 1 mM nonradioactive methionine for 3 h. At the indicated time points (min), 100- μl samples of mucosal-side medium were collected for SDS-PAGE analysis, and the mucosal chamber was replenished with a similar volume of fresh medium. Lane S shows 100 μl of serosal-side medium collected after a 3-h chase. After culture, apo A-1 was immunopurified from the mucosal-side (M) medium (after 50 μl 10% [wt/vol] Triton X-100 had been added) and from a total detergent extract of the intestinal tissue (T). Molecular mass values of apo B-48 (220 kD) and apo A-1 (27 kD) are indicated by arrowheads. (B) The gel tracks of four experiments of the type shown in Fig. 1 A were densitometrically scanned and the relative amounts of apo A-1 (27-kD band) and apo B-48 (220-kD band) determined. The mean value at each time point is expressed as percent of maximal secretion to the medium (i.e., relative to the time point with the highest secretion of the respective apolipoprotein), and corrected for the "dilution" by replenishment with fresh medium after collection of samples at previous time points (bars indicate SD). The gel track furthest to the right shows a labeled total detergent extract of the intestinal tissue.

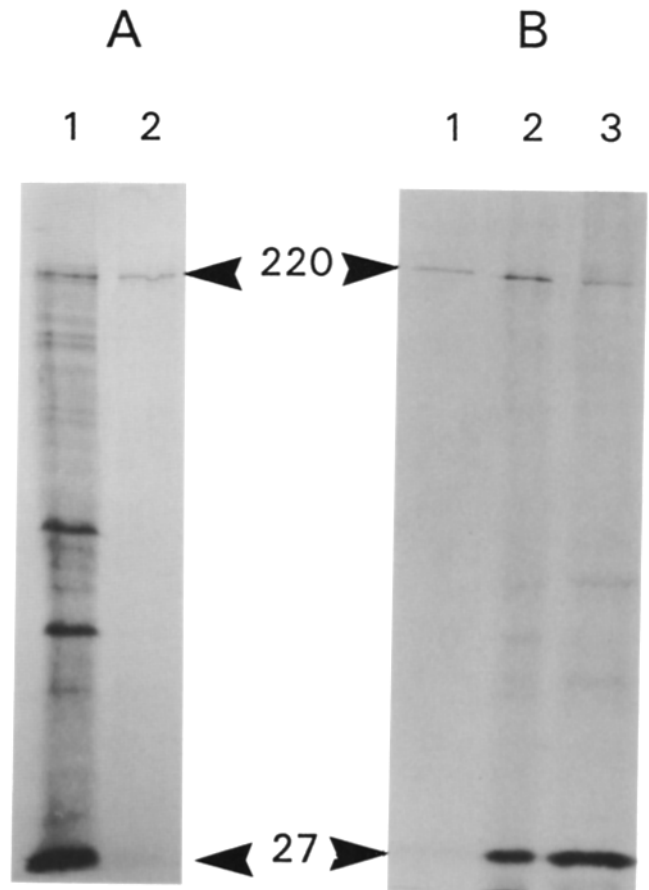


Figure 2. Identification of the 200-kD protein as apo B-48. (A) 0.5 ml of medium of an intestinal segment, labeled continuously for 3 h with 100 $\mu\text{Ci/ml}$ [^{35}S]methionine, mixed with Triton X-100 to 0.5% (wt/vol) and 25 μl of rabbit anti-human β -lipoprotein. After incubation for 6 h at 4°C, 50 μl swine anti-rabbit Ig was added and incubation continued for 20 h. The immunoprecipitate was collected by centrifugation at 5,000 g, 5 min and washed in 1 ml 50 mM Tris-HCl, 150 mM NaCl, pH 7.3 before SDS-PAGE (lane 2; lane 1 shows total medium). (B) 0.5 ml of medium of an intestinal segment, labeled as described above, was mixed with 50 μl of a heparin-MnCl₂ solution and apo B-48 isolated in the presence (lane 1) or absence (lane 2) of 1% Triton X-100 (lane 3 shows total medium). Molecular mass values of apo B-48 and apo A-1 are indicated.

by rabbit antibodies to swing serum and was by far the predominant radioactive serum protein thus isolated, both from the medium and the intestinal segment (see Figs. 3 and 5). Apart from apo B-48, immunoglobulins (most likely synthesized by intestinal lymphocytes) were the only radioactively labeled serum proteins that could be detected (results not shown).

Fig. 1 B shows the kinetics of appearance in the mucosal-side medium of pulse-labeled apo A-1 and apo B-48; maximal amounts of both proteins were released within 90–120 min of chase. The slight difference in the rate of appearance of the two apolipoproteins was consistent, with apo A-1 being released with a $t_{1/2}$ of ~ 50 min and apo B-48 with a $t_{1/2}$ of ~ 60 min. Small amounts of actin (42 kD, the most abundant protein synthesized by the intestinal segments) appeared in the medium progressively with time. This may well be due to a "budding off" of single microvilli, but to some extent it

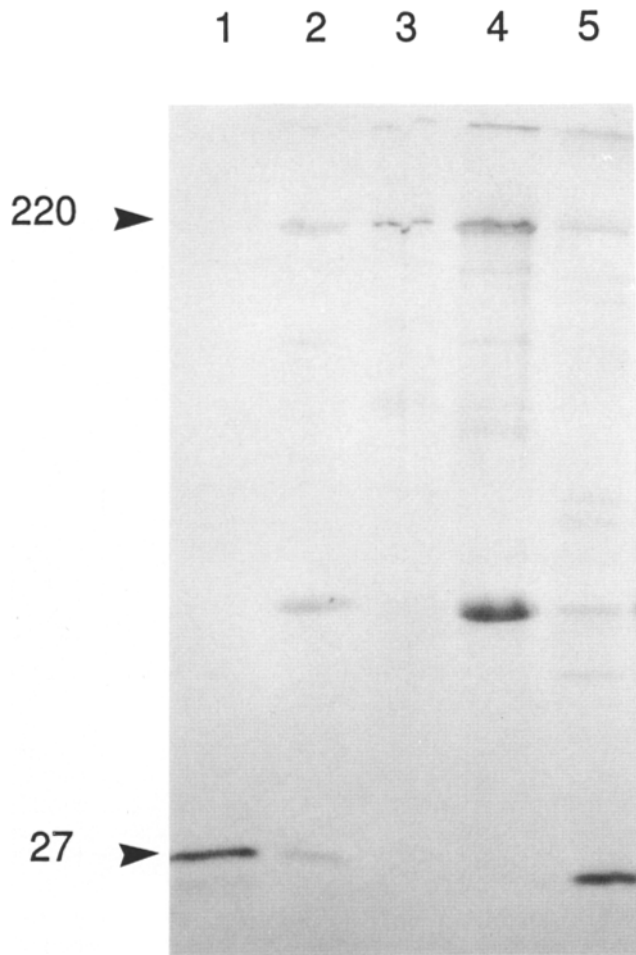


Figure 3. Apo A-1 and apo B-48 are differentially released to the medium. A small intestinal segment was pulse-chase labeled as described in the legend to Fig. 1 *A*. After culture, the medium was collected, 50 μ l 10% Triton X-100 was added, and a total detergent extract of the intestinal tissue was prepared. Both medium and tissue extract were divided in two samples of equal size, and apo A-1 and apo B-48 were immunoprecipitated by anti-apo A-1 and anti-swine serum, respectively. (Lane 1) Apo A-1 from medium; (lane 2) apo A-1 tissue; (lane 3) apo B-48 from medium; (lane 4) apo B-48 from tissue; and (lane 5) total medium. Molecular mass values of the two apolipoproteins are indicated. (Actin [42 kD] invariably appeared in the immunoprecipitates from the tissue extracts.)

may also reflect cell damage/turnover occurring during culture. However, the modest amount of actin generally observed in the medium of segments maintained for periods of up to 5 h demonstrates that the epithelium generally remains intact, and, consequently, that the mucosal-side release of apolipoproteins thus occurs by bona fide secretion. However, this release of apolipoproteins into the medium was never complete; as shown in Fig. 1 *A*, lane *T*, a minor portion of apo A-1 remained associated with the intestinal segment after 3 h of chase and could not be released by gently flushing the segment with medium. In a series of five experiments, $86.8 \pm 6.0\%$ (SD) of the total amount of labeled apo A-1 was recovered from the mucosal-side medium after a 3-h chase period. In contrast, the amount of apo A-1 released to the serosal-side medium was hardly detectable (Fig. 1 *A*, lane *S*).

For apo B-48, the relative amount secreted to the mucosal-side medium was much smaller. As shown in Fig. 3, the major proportion of apo B-48 (67%, as judged by densitometry) remained associated with the intestinal segment after 3 h of chase.

Ultracentrifugation analysis of apolipoproteins released to the culture medium showed that the major proportion of both apo A-1 and apo B-48 sedimented even at a density of 1.21 g/ml, indicating that the apolipoproteins thus released are mainly unassociated with lipid (results not shown).

Apolipoproteins Are Secreted to the Medium via the Apical Plasma Membrane

Of the two plasma membrane domains of the enterocyte, only the apical brush border membrane faces the (mucosal-side) culture medium directly. To appear in the medium, proteins secreted via the basolateral plasma membrane would have to pass through the tight junctions joining neighboring enterocytes or, alternatively, penetrate through leaky cells.

The general accessibility of the basolateral surface of enterocytes of cultured intestinal segments to the medium was tested by use of the nonpermeable surface marker Ruthenium red (Luft, 1971). This dye has previously been used to probe the tightness of MDCK cell monolayers (van Deurs et al., 1990). As shown in Fig. 4, *A* and *B*, the entire mucosal brush border surface along villi of intestinal segments cultured for 5 h was stained heavily by the dye whereas the basolateral cell surface and cytoplasm of the enterocytes both generally were negative. In only a few enterocytes was it possible to detect Ruthenium red intracellularly and at the basolateral plasma membrane (Fig. 4 *C*). Ruthenium red was observed occasionally in Goblet cells and in their surrounding intercellular space (Fig. 4, *D* and *E*). In the crypt regions, the staining pattern was similar to that seen along the villi (data not shown), but at the cell extrusion zone at the very tip of the villi, decaying enterocytes, leaky to the dye, could be seen (Fig. 4 *F*). The finding that Ruthenium red was able to penetrate into senescent enterocytes and Goblet cells verified the use of the dye as a sensitive marker for probing the tightness of the epithelial surface of the cultured intestinal segments. These experiments therefore strongly indicate that the tight junctions and apical plasma membranes of the enterocytes of cultured intestinal segments largely remain intact. Consequently, diffusion from the intercellular space between neighboring enterocytes into the culture medium by way of leaky junctions or enterocytes seems a very unlikely route for the rapid and massive release of apolipoproteins, shown in Fig. 1. In addition, the Ruthenium red labeling results show that diffusion through the submucosal and serosal layers likewise does not occur.

To test further the tightness of the epithelium, the relative specific radioactivity of apo A-1 and apo B-48 obtained from the intestinal segment and the culture medium, respectively, was compared by rocket immunoelectrophoresis. As shown in Fig. 5, independently of the amount of sample applied to the gel, the immunoprecipitates formed by both apolipoproteins from the medium were more intensively labeled than those formed by their counterparts extracted from the tissue. Such a difference in relative specific radioactivity can only arise because nonradioactive apolipoproteins, present in the intestinal segment, do not diffuse into the medium; as judged

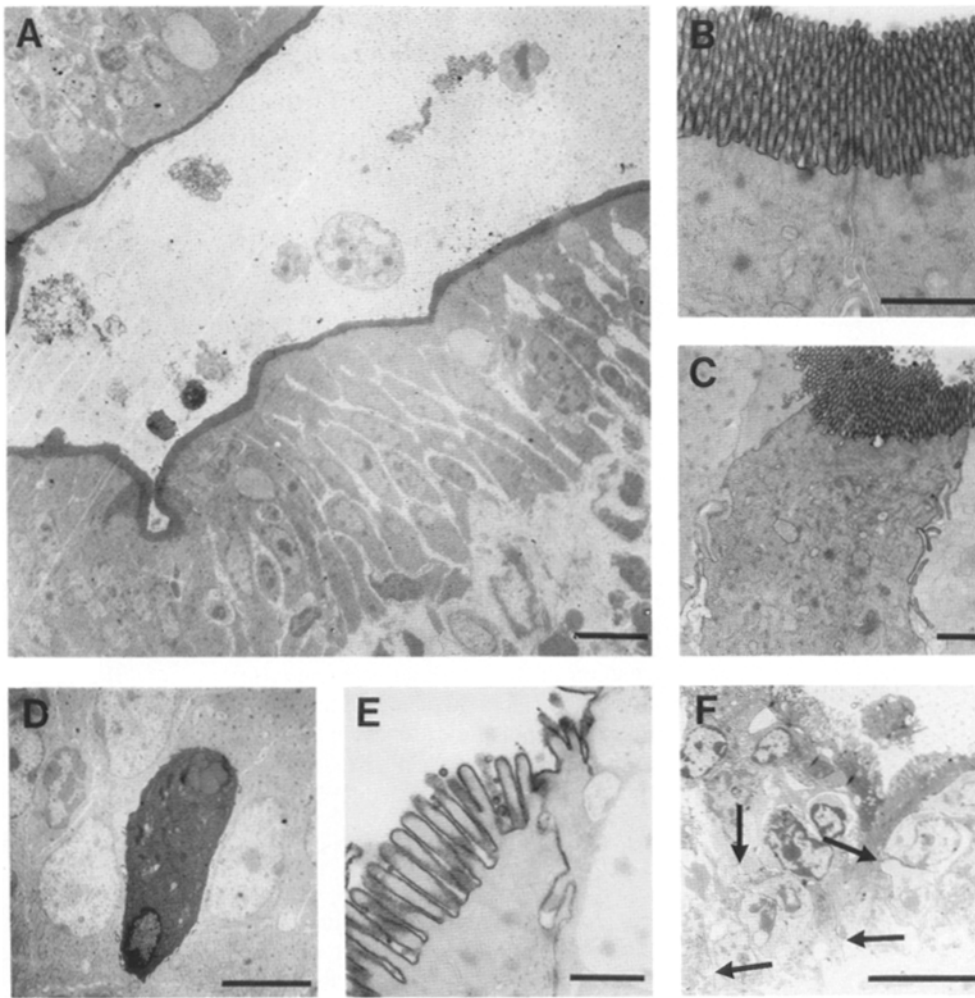


Figure 4. Staining with Ruthenium red. Electron micrographs of the villar region of intestinal segments cultured for 5 h before fixation in glutaraldehyde/formaldehyde fixative containing Ruthenium red. (A) Intense Ruthenium red staining is seen along the entire brush border surface, whereas the dye in only few cells can be detected at the basolateral surface and in the cytosol (C). (B) Tight junction between two neighboring enterocytes showing absence of penetration of Ruthenium red. (D and E) Staining in the cytosol of a Goblet cell and its surrounding intercellular space. (F) Cell extrusion zone at the tip of a villus with Ruthenium red staining in the cytosol and in the intercellular space (arrows). Bars: (A and F) 10 μm ; (B and C) 1 μm ; (D) 5 μm ; (E) 0.5 μm .

by densitometry, the difference in specific radioactivity was ~ 2.5 times for apo B-48. For apo A-1, this difference was even bigger, but it was difficult to determine because radioactive apo A-1 extracted from the tissue was barely detectable by this method. In conclusion, this experiment therefore strongly argues that neither of the two apolipoproteins appear in the culture medium by diffusion through leaks since this would have resulted in immunoprecipitates of comparable specific radioactivity.

Apolipoprotein Secretion via the Basolateral Plasma Membrane

Fig. 6 A shows the distribution of apo A-1 in the membrane and soluble fractions of labeled intestinal segments, obtained after a gentle homogenization of the tissue. Immediately after a 30-min pulse of [^{35}S]methionine, the major part ($\sim 85\%$) of apo A-1 was in the membrane fraction, but during a chase at 37°C , an increasing amount of the apolipoprotein appeared in the soluble fraction (and in the medium). As a control experiment, two brush border membrane proteins, aminopeptidase N and sucrase-isomaltase, were found to remain fully associated with the membrane fraction, indicating that all membranes of the secretory pathway partitioned in the pellet of the centrifugation step. Therefore, soluble apo A-1 (immunopurified in the absence of detergent and hence

not membrane confined) most likely represents apolipoprotein secreted through the basolateral plasma membrane of the enterocytes and subsequently trapped in the intercellular space or connective tissue of the intestinal segment. As shown in Fig. 6 B, this secretion occurred with a $t_{1/2}$ of ~ 30 min; i.e., somewhat more rapid than the rate of secretion into the medium. However, the relative amount of apo A-1 in the soluble fraction accumulated and remained high after 3 h of chase, indicating that the appearance of the apolipoprotein in this fraction is permanent rather than transient. Apo B-48 was coprecipitated by the apo A-1 antiserum from the soluble fraction (Fig. 6 A), indicating that the two apolipoproteins are also associated when exported from the enterocyte by this secretory pathway.

Culture at low temperature is a common way to interfere with the intracellular membrane traffic (Matlin and Simons, 1983). Posttranslational molecular processing as well as surface expression of newly synthesized brush border enzymes has previously been shown to be affected by culture at 20°C (Danielsen et al., 1989). In intestinal segments cultured at 20°C , both apo A-1's secretion to the medium and its appearance in the soluble fraction were essentially blocked (Fig. 6 A, lower panel; Fig. 6 B), thus confirming that both processes are indeed mediated by vesicular transport. As a control experiment, culture at this temperature was shown to arrest the posttranslational processing of the brush border

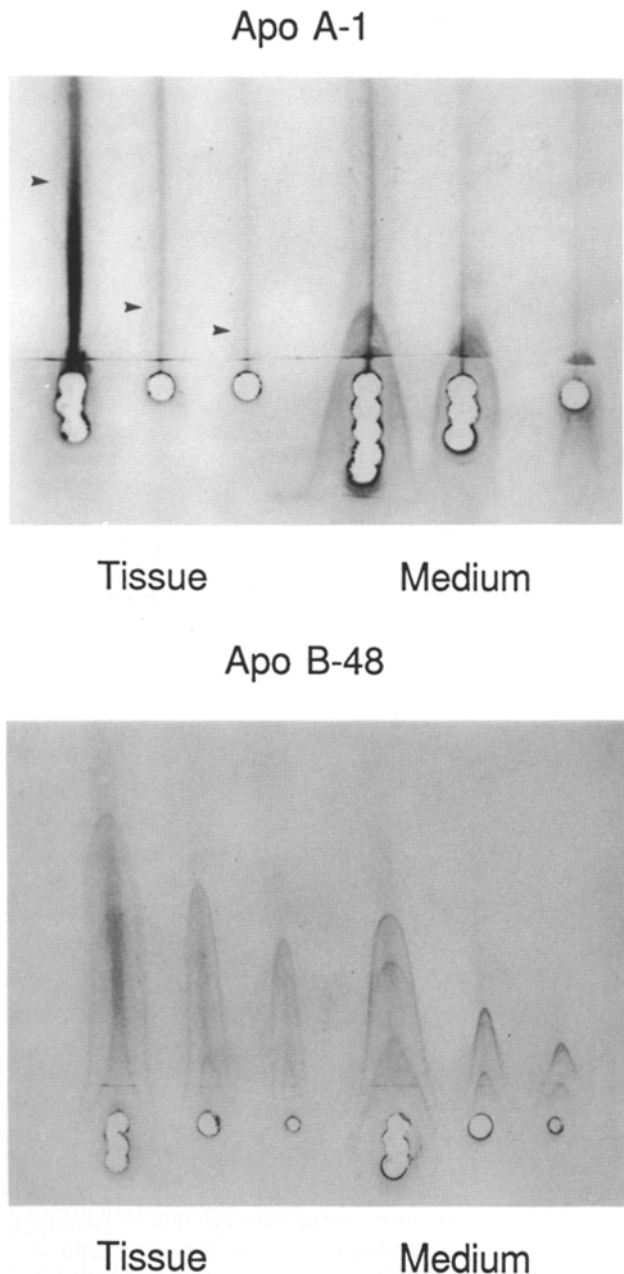


Figure 5. Relative specific radioactivity of apolipoproteins from tissue and medium. The medium and a detergent extract of an intestinal segment of a pulse-chase labeling experiment, performed as described in the legend to Fig. 1 *A*, were subjected to rocket immunoelectrophoresis against anti-apo A-1 (apo A-1) or anti-swine serum (apo B-48). (*Apo A-1*) 30, 10, or 5 μ l tissue extract and 50, 30, or 10 μ l medium was applied. (*Apo B-48*) 30, 10, or 5 μ l tissue extract and 30, 10, or 5 μ l medium was applied. Before electrophoresis, the medium samples were made 1% in Triton X-100 and concentrated three times by Speedvac centrifugation. After electrophoresis, the radioactive immunoprecipitates were visualized by autoradiography, and their relative intensity (a measure of the relative specific radioactivity of the apolipoproteins in the sample) was determined by densitometric scanning. The immunoprecipitates of apo A-1 from the tissue extract (marked by arrows) were barely detectable even after prolonged exposure of the film. The faint precipitates below those of apo B-48 represent immunoglobulins.

enzymes aminopeptidase N and sucrase-isomaltase from the transient, high mannose-glycosylated to the mature, complex-glycosylated form.

Molecular Processing of Newly Synthesized apo A-1

Apo A-1 is known to undergo a posttranslational proteolytic cleavage in the enterocyte, releasing a hexapeptide from its NH₂-terminal end (Gordon et al., 1982*b*). This is an essential event in the functional maturation of apo A-1 and defective processing believed to cause the abnormal plasma isoforms of apo A-1, associated with Tangier's disease (Gordon et al., 1983). As can be seen in Fig. 6 *A*, apo A-1 immunopurified from both the soluble fraction of the intestinal segment and from the medium after 1 h and 3 h of chase (at 37°C) migrated with a slightly higher mobility than the apolipoprotein from the membrane fraction, indicating a small decrease in molecular weight. The hexapeptide processing described above most likely accounts for this small but consistently observed electrophoretic mobility shift, and it must occur at a late stage in the secretory pathway (the Golgi complex or a post-Golgi compartment) since the mature, processed apo A-1 was not detectable in the membrane fraction of the intestinal segments. Interestingly, the small amounts of apo A-1 secreted at 20°C after 1 h and 3 h of chase were not of the processed form, indicating that the proteolytic cleavage of the propeptide does not take place at this temperature (Fig. 6 *A*).

Synthesis and Secretion of Apolipoproteins during Fat Absorption

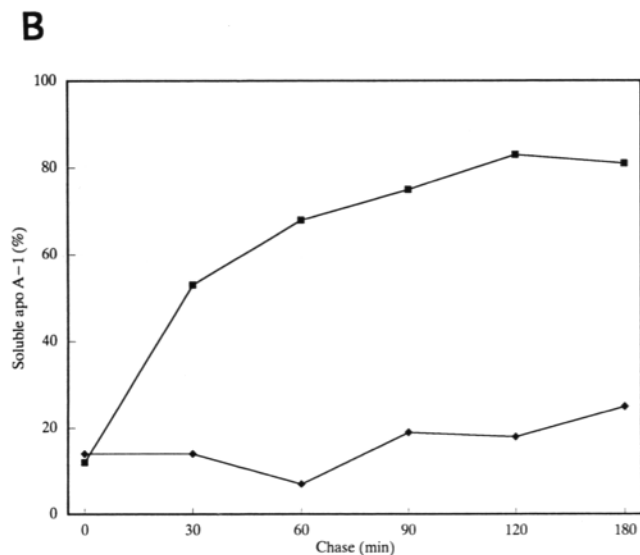
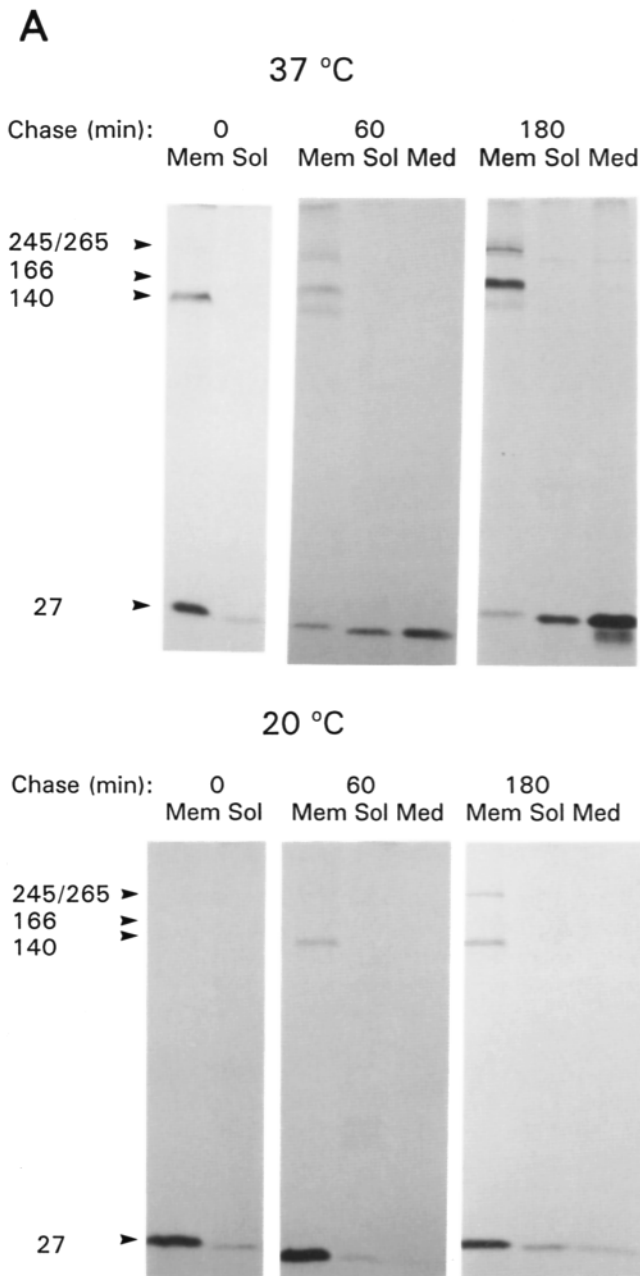
Fig. 7 *A* shows the ultrastructure of the jejunal epithelium 3 h after fat ingestion. Chylomicrons are present in the Golgi complex and in the vicinity of the lateral plasma membrane.

Fig. 7 *B* shows the synthesis and secretion of apo A-1 of an intestinal segment, obtained from an animal 3 h after fat ingestion. Both apo A-1 and apo B-48 were secreted with kinetics comparable to those of an intestinal segment of a fasting animal (data not shown), and after 3 h of chase, the major part of apo A-1 synthesized during a 20-min pulse was secreted to the medium. However, in comparison with the corresponding gel tracks of Fig. 1 *A*, it is apparent that the relative amount of apo A-1 associated with the intestinal tissue is increased. In two experiments, 66 and 72%, respectively, of the total amount was found in the medium. The considerable increase in immunogold labeling of enterocytes after fat ingestion indicates that the apolipoprotein is located predominantly intracellularly rather than intercellularly (Fig. 9; see below).

Immunolocalization of apo A-1 in Enterocytes

Fig. 8 shows the localization of apo A-1 in the jejunal epithelium of a fasting animal by immunofluorescence microscopy. Within the enterocytes, labeling was mainly seen in the apical region, but weaker labeling was apparent also in the lateral and basal areas of the cell. In addition, a weak band of immunofluorescence staining was visible centrally in the stroma of the villus, presumably representing apo A-1 in capillaries and lymphatics. Epithelial cells of the crypts only showed weak labeling (data not shown).

Using immunogold electron microscopy, significant, but



relatively weak apo A-1 labeling was seen only in the Golgi complex of the enterocytes of jejunal mucosa obtained from a fasting pig (Fig. 9, *A* and *C*), indicating that despite a high synthesis rate, the intracellular steady-state concentration of the apolipoprotein is relatively low. This result is consistent with the observed rapid and massive release of newly synthesized apo A-1 to the culture medium. After fat ingestion, the intracellular labeling intensity increased considerably and apo A-1 labeling was conspicuous on the surface of chylomicrons which were found in the Golgi complex and near the basolateral plasma membrane (Fig. 9, *B* and *D*). This increase in immunogold labeling after fat ingestion parallels the increase in immunofluorescence, observed previously by others (Glickman and Green, 1977; Schonfeld et al., 1978). With an unchanged apo A-1 synthesis rate, a slow assembly and release of chylomicrons, as compared with the rapid secretion of free apolipoprotein, probably accounts for the markedly increased labeling intensity. In contrast to chylomicrons, lipid droplet in the apical cytoplasm were devoid of any surface labeling (data not shown).

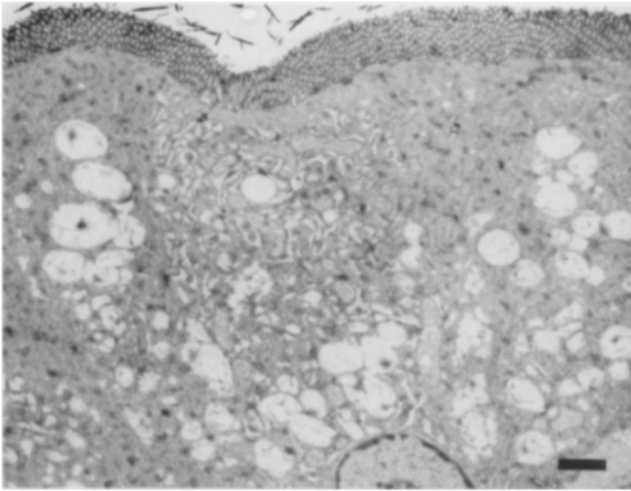
Discussion

The present work is in line with earlier reports that apo A-1 and apo B-48 are major proteins synthesized and secreted by the small intestine. These two apolipoproteins were the most abundant radioactive components rapidly released into the mucosal-side culture medium of pulse-chase labeled intestinal segments, and the kinetics and temperature dependence of their appearance proved this to represent a true secretion. On the basis of three lines of evidence, we propose that this secretion occurs via direct exocytosis from the apical brush border membrane of the enterocytes and not by basolateral secretion and subsequent diffusion through leaky cells/junctions: (1) Staining with the nonpermeable surface marker Ruthenium red showed that the epithelial cells and their junctions generally (>95%) remain tight. (2) After pulse-chase labeling, apolipoproteins secreted to the medium had a much higher relative specific radioactivity than those extracted from the intestinal segment. (3) Newly synthesized apo A-1 was predominantly (85%) secreted to the medium, whereas apo B-48 predominantly (about two thirds) remained associated with the tissue (despite their comparable kinetics of secretion to the medium).

The result of the present work thus strongly implies that a substantial portion of the apolipoproteins under normal

Figure 6. Basolateral secretion of apo A-1. (*A*) Small-intestinal segments were labeled for 30 min with 200 $\mu\text{Ci/ml}$ [^{35}S]methionine and chased with 1 mM methionine for the indicated periods of time at either 37°C (*upper panel*) or 20°C (*lower panel*). After culture, apo A-1, aminopeptidase N (transient, high mannose-glycosylated form of 140 kD and mature, complex-glycosylated form of 166 kD), and sucrase-isomaltase (transient form of 245 kD and mature form of 265 kD) were immunopurified from the membrane (intracellular) fraction (Mem), the soluble (intercellular) fraction (Sol), and the medium (Med). Molecular mass values are indicated. (*B*) The relative amounts of apo A-1 in the soluble and membrane fractions at each time point at 37°C (■) and 20°C (◆) were determined by densitometric scanning of the gel tracks, and the values express the fraction of apo A-1 in the soluble fraction as percent of the total, tissue-associated apo A-1 (soluble + membrane fraction) at that time point.

A



B

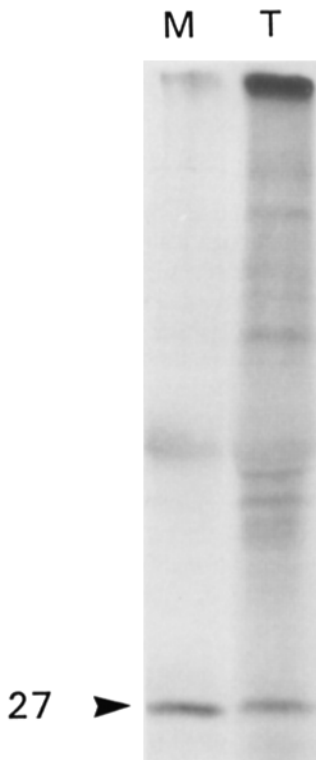


Figure 7. Apolipoprotein secretion during fat absorption. (A) The ultrastructure of enterocytes 3 h after fat ingestion, showing the presence of chylomicrons in the cytosol. Bar, 0.5 μm . (B) A pulse-chase labeling experiment identical to that described in Fig. 1 A, except that the cultured intestinal segment was obtained from an animal which 3 h before surgery had been given a fatty meal. After 3 h of chase, apo A-1 was immunopurified from the medium (M) and from a total extract of the intestinal tissue (T).

conditions *in vivo* is released into the lumen of the gut. To our knowledge, this has not previously been recognized although secretion of apolipoproteins into the medium of organ-cultured mucosal explants has been reported previously by other investigators (Zannis et al., 1980; Mougin-Schutz et al., 1983).

The human colon carcinoma cell line Caco-2 exhibits morphological and biochemical properties similar to enterocytes, including formation of an apical microvillar surface

A



B

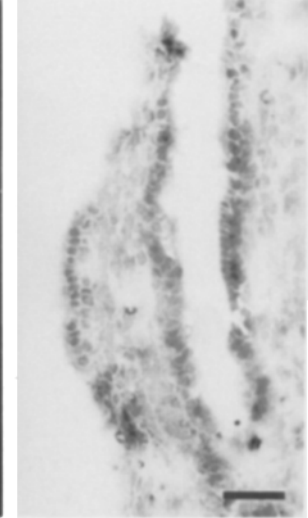


Figure 8. Apo A-1 localization by immunofluorescence microscopy. Immunofluorescence labeling of apo A-1 performed on frozen sections of jejunum from a fasting animal (A). The labeling is mainly confined to the apical region of the enterocytes, but weaker labeling is apparent also in the lateral and basal parts of the cells and in the stroma of the villus. The morphology of the same villus region was visualized by staining with Hematoxylin and Eosin. Bar, 50 μm .

separated from the basolateral plasma membrane by tight junctions (Pinto et al., 1983). Furthermore, it has been shown that confluent cultures of Caco-2 cells secrete apolipoproteins A-1, A-IV, B-100, B-48, C-III, and E (Hughes et al., 1986; Traber et al., 1987; Rindler and Traber, 1988). When grown as monolayers on porous filters, it was observed that 75-100% of the apolipoproteins, radioactively labeled for 4 h, were released into the basolateral medium. The two types of intestinal epithelial cells thus differ considerably in the polarity of their secretion of apolipoproteins. The Caco-2 cell line, which is widely used as a model intestinal epithelial cell in studies on cell polarity, is therefore not necessarily a true representative of the mature, fully differentiated enterocyte. Rather, the Caco-2 cell line resembles enterocytes from an early fetal stage of development. In support of this view, Traber et al. (1987) observed that apo B-100, which is not made by adult but only by fetal intestine, is more abundantly synthesized than apo B-48 in Caco-2 cells. Furthermore, the same investigators reported that Caco-2 cells secrete α -fetoprotein (likewise, preferentially from the basolateral side), a protein not synthesized by adult intestine (Rindler and Traber, 1988). One aspect of enterocytic differentiation during the embryonic development may therefore be the establishment of a major, direct apical secretory pathway. In line with this, it has recently been reported that \sim 50% of the endogenous plasma membrane protein dipeptidyl peptidase IV in a polarized thyroid cell line reaches the apical cell surface by a transcytotic pathway at an early stage of development, whereas a direct pathway from the TGN prevails at later stages (Zurzolo et al., 1992).

It is generally believed that specific sorting signals on

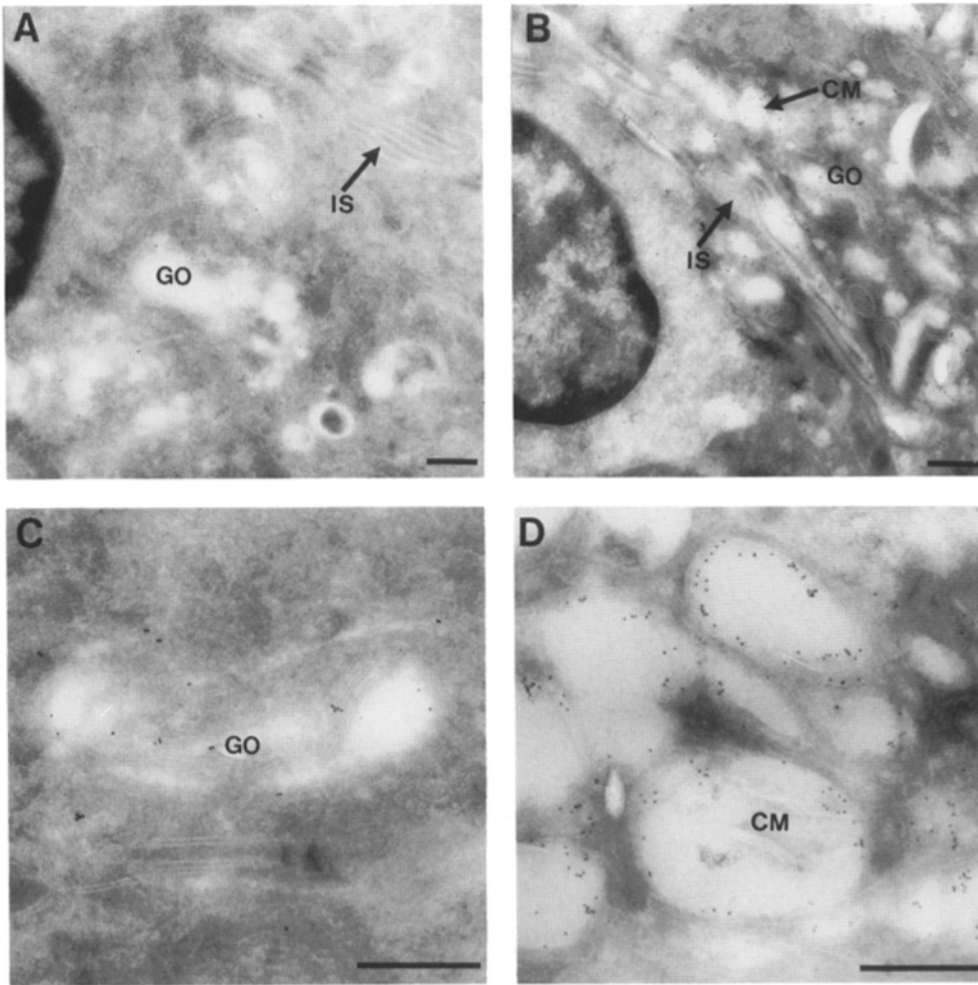


Figure 9. Apo A-1 localization by immunogold electron microscopy. Immunogold labeling of apo A-1 performed on ultracryosections of jejunal mucosa from a fasting (A and C) or fat-fed (B and D) animal. In the fasting state, a weak labeling is seen only in the Golgi complex (GO). After fat ingestion, the Golgi labeling is much more intense and apo A-1 lines the surface of chylomicrons (CM) both within the cell and in the intercellular space (IS). Bars, 0.5 μm .

newly synthesized plasma membrane and secretory proteins determine whether these will be transported to the basolateral or to the apical domain of the plasma membrane of polarized cells (Simons and Fuller, 1985; Rodriguez-Boulan and Nelson, 1989). It has also been proposed that the route to the basolateral surface represents the "default" pathway whereby proteins, lacking a sorting signal, are externalized (Simons and Wandinger-Ness, 1990). The finding that apo A-1 is secreted with opposite polarity from Caco-2 cells and enterocytes suggests that this apolipoprotein does not per se contain a sorting signal. This view is supported by the fact that when assembled into chylomicrons, apolipoproteins invariably exit the cell from the basolateral plasma membrane. If correct, this implies that the default pathway in mature, fully differentiated enterocytes is targeted predominantly towards the apical rather than towards the basolateral plasma membrane. A similar hypothesis has recently been proposed in case of the widely used MDCK cell line, based on observations that "tailless" molecular forms of a number of plasma membrane receptors with demonstrable basolateral sorting signals in this cell type are transported preferentially to the apical surface (Hopkins, 1991, 1992). In line with this, Mostov et al. (1992) have argued that evolutionary and developmental considerations favor the apical cell surface to be the more primitive domain and, consequently, the target for the default pathway.

Using MDCK cells, it was shown recently (Vogel et al., 1992b) that a secretory form of the brush border enzyme aminopeptidase N (lacking the cytoplasmic tail as well as the membrane anchor), like the native enzyme (Wessels et al., 1990), is secreted predominantly to the apical chamber, indicating that the putative apical sorting signal resides in the ectodomain of the molecule. A further study has indicated that this signal must be located in the catalytic head group and not in the "stalk" region of the enzyme (Vogel et al., 1992a). Contrary to apolipoproteins, it does seem fair to assume that this enzyme (and other apical ectoenzymes) possesses an apical sorting signal and that its apical expression, at least in hepatocytes and Caco-2 cells, is dependent upon it since these cell types (to a varying degree) use transcytosis from the basolateral cell surface as the last transport step in the apical targeting of the enzyme (Bartles et al., 1987; Matter et al., 1990; Le Bivic et al., 1990).

In contrast to the apical secretion of free apolipoproteins, chylomicrons were located exclusively in the vicinity of the basolateral plasma membrane and not seen near the brush border membrane. In view of the massive apical secretion of free apolipoproteins, it is unclear by which sorting mechanism chylomicrons are targeted to the basolateral plasma membrane. By acting as a barrier, the terminal web underlying the brush border membrane conceivably might prevent luminal secretion of large lipoproteins. Alternatively, the li-

poprotein assembly in the Golgi complex might itself be a sorting event that ensures a basolateral targeting. A further study to dissect this process might therefore provide more insight into some of the mechanisms that regulate intracellular membrane traffic.

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