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Evidence for the Transit of Aminopeptidase N through the Basolateral Membrane before It Reaches the Brush Border of Enterocytes

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Summary. *In vivo* pulse-chase labeling of rabbit jejunum loops was used in conjunction with subcellular fractionation and quantitative immunoprecipitation to determine whether or not the newly synthesized aminopeptidase N transits through the basolateral membrane before it reaches the apical brush border, its final localization. The kinetics of the arrival of the newly synthesized enzyme in the Golgi complex, basolateral and brush border membrane fractions strongly suggest that on leaving the Golgi aminopeptidase N is transiently integrated into the basolateral domain before reaching the brush border.

Key Words aminopeptidase · brush border · cell polarity · intracellular transport · membrane biogenesis

Introduction

The highly polarized intestinal absorbing cells provide a useful model for studying the biogenesis of various plasma membrane domains in epithelial cells. In these cells, the apical and basolateral domains are particularly well characterized by their morphology and their unique protein composition, which is well adapted to their specific function. The apical domain forms the microvilli of the luminal brush border and contains a large number of digestive hydrolases (Kenny & Maroux, 1982). Among these, aminopeptidase N constitutes 3% of the brush border membrane proteins in the rabbit (Feracci & Maroux, 1980). The basolateral domain is characterized by other specific markers such as the Na^+K^+-ATPase (Fujita et al., 1972; Gorvel et al., 1983) and the histocompatibility antigens (Kirby & Parr, 1979; Gorvel et al., 1984).

Immunofluorescence labeling of ultrathin frozen sections of jejunum has indicated the absence of aminopeptidase N in the basolateral domain (Feracci et al., 1982). However, histochemistry on the same material has revealed active enzyme in the apical portion of the lateral membrane (Feracci et al., 1982), and subcellular fractionation has pointed to the presence of small amounts of aminopeptidase N and other brush border hydrolases in the basolateral membrane (Colas & Maroux, 1980). After studying the intracellular pathway of total brush border glycoproteins and sucrase-isomaltase by pulse-chase labeling in conjunction with subcellular fractionation, Quaroni, Kirsch and Weiser (1979*a*,*b*) and Hauri, Quaroni and Isselbacher (1979) have proposed that these newly synthesized molecules might transit through the basolateral membrane. Because of the absence of immunolabeling *in situ* (Fransen et al., 1985) and the risk of cross-contamination between various membrane vesicles during the subcellular fractionation procedure used, these results have never been definitely accepted (Matlin & Simons, 1984; Misek, Bard & Rodriguez-Boulan, 1984).

Recently, we devised a new specific technique for the immunoisolation of basolateral membrane that results in a satisfactory separation of this from other membranes. Simultaneously, brush border membrane vesicles and a fraction highly enriched in membranes from Golgi apparatus plus smooth endoplasmic reticulum were obtained. The application of the flow microfluorometry technique to these membrane vesicles has made it possible to definitely demonstrate the presence of aminopeptidase N in the basolateral membrane, where its specific activity is about 4.5% of that in the brush border membrane vesicles (Moktari et al., 1986).

Here we used this subcellular fractionation procedure after *in vivo* pulse chase labeling of jejunum loops to study the routing of the newly synthesized aminopeptidase N, paying particular attention to a possible passage through the basolateral membrane. Our results indicate that the newly synthesized enzyme could be integrated into the basolateral membrane before reaching the brush border.

Materials and Methods

The rabbit typing (Feracci et al., 1982) and pulse-chase labeling (Feracci, Rigal & Maroux, 1985) procedures have been described previously.

Subcellular Fractionation

All subcellular fractions were concomitantly prepared from the same sample of intestinal mucosa of A^+ rabbits as described in detail elsewhere (Moktari et al., 1986). Briefly, mucosal scrapings were homogenized in 8 times their weight in a 20-mM TrisHCl buffer (pH 7.3) containing 0.25 M sucrose, 10 mM KCl, 1 mM phenylmethylsulfonylfluoride, 0.1 mg/ml of trypsin inhibitor and 2 μg/ml of leupeptin by 20 and 30 vertical strokes with the A and B pestles, respectively, in a Dounce homogenizer.

The resulting homogenate was centrifuged at 750 × g for 10 min and then at 9500 × g for 10 min.

The microsomal fraction was obtained by centrifugation at 105,000 × g of the 9,500 × g supernatant for 30 min.

By means of centrifugation of the 9,500 × g supernatant through a sucrose step gradient, the high density membranes (H Mbr) containing brush border (BB) and rough endoplasmic reticulum (RER) membranes were separated from the low density membranes (L Mbr) containing membranes from smooth endoplasmic reticulum (SER), Golgi complex (G) and basolateral plasma membrane domain (BL).

BB were completely separated from RER by Ca^{2+} precipitation of RER (Schmitz et al., 1973). Specific immunoprecipitation of BL by anti-human blood group A antibodies gave satisfactory separation from G + SER membranes.

Solubilization and Immunoprecipitation of Aminopeptidase N

Aminopeptidase present in membranes was solubilized by adding Triton X 100 and SDS to obtain final concentrations of 2 and 0.1%, respectively, followed by incubation overnight at 4°C. It was quantitatively immunoprecipitated by specific anti-AP (Feracci et al., 1985) coupled to Ultrogel ACA 22 (Ternynck & Avrameas, 1972). The quantity of immunogel required for quantitative immunoprecipitation of AP was determined by adding increasing amounts of beads to a constant amount of AP and testing the enzymatic activity remaining in the supernatant. The immunogel was incubated under vigorous agitation for 1 hr at 4°C in 10 mM phosphate buffer (pH 7.3), 0.15 M NaCl containing 1% bovine serum albumin for saturation of unspecific binding sites, then 3 hr at 4°C with the antigen solution. The gel was centrifuged for 30 sec in Eppendorf centrifuge 5414, washed and prepared for gel electrophoresis as previously described for direct immunoprecipitates (Feracci et al., 1985).

Quantification

AP was evaluated from enzymatic activity (Feracci & Maroux, 1980). The proportion of the transient intermediate of glycosylation (T form) and the mature form (M form) respectively accumulated in the SER and G membranes of the (G + SER) fractions (Gorvel et al., 1986; Moktari et al., 1986) was determined by quantitative rocket immunoelectrophoresis through two successive gels containing anti-human blood group A antibodies (anti-HBG-A) and anti-AP, respectively, as previously described (Feracci et al., 1985). The amount of T form in other fractions was taken to be negligible.

For radioactivity determination, 0.3 μg of immunoprecipitated AP from BL and (G + SER) and 3 μg of immunoprecipitated AP from total microsomes and BB were loaded in one and two 8-mm wide wells of 0.75 mm thick SDS-polyacrylamide gels, respectively, subjected to electrophoresis and quantitatively transferred to nitrocellulose as previously described (Feracci et al., 1985).

Staining by Ponceau S (Coudrier, Reggio & Louvard, 1983) revealed the M form of AP from microsomes and BB. Immunoblotting, which reveals the two forms from all fractions (Feracci et al., 1985) (*see also* Fig. 2), was not used to locate the bands before excision because peroxydase staining disturbs counting.

In the first series of measurements, autoradiography of nitrocellulose sheet was used as template for cutting out the T form and M form bands that were solubilized in 5 ml of PCS (Amersham) and counted. A background value measured from the nitrocellulose slice where no band was detected was subtracted from every determination. In the second series, T and M forms were counted together and their relative amounts determined by densitometer scanning of autoradiograms in a Vernon densitometer. Only the second method permitted the estimation of the very low labeling of M form after 0 min of chase. The two series of measurements were in good agreement.

Theoretical Kinetics

All calculations were performed on Plessy 6321 computer systems provided with a Hewlet Packard 7475 A plotter. Fitting of experimental data to theoretical equations was performed using Newton-Gauss algorithms as in Soulié et al. (1985).

Results

Characterization of Subcellular Fractions and their AP Content

Since the interpretation of the present study on the passage of aminopeptidase N through the basolateral membrane was dependent on the purity of the basolateral membrane vesicle preparation, it was essential to check carefully for any cross-contaminations by other types of membranes vesicles.

Notwithstanding the high sensitivity of the flow microfluorometric technique used, no brush border membrane vesicles (BB) were detected in the low density membrane fraction (L Mbr) from which basolateral membrane (BL) and membranes from Golgi complex and smooth endoplasmic reticulum (G + SER) were prepared (Moktari et al., 1986). Flow fluorometry showed that in L Mbr, 30% of membrane vesicles bore simultaneously on their external surface the class I histocompatibility antigen

Table. Enzyme content of BL and (G + SER) fractions

	Aminopeptidase					
	M form (units)	T form (units)	Na^+K^+-ATPase (units)	Gal. transferase (units)	Aryl sulf. C (units)	Proteins (ng)
BL	1	~0.1	1	0.6	0.006	18
(G + SER)[a]	1	1	0.5	4	0.060	36

The figures are the average of five independent assays.

[a] T and M forms of AP present in this fraction are located in the SER and G membranes, respectively (Gorvel et al., 1986; Moktari et al., 1986). Na^+K^+-ATPase, galactosyltransferase and aryl sulfatase C were the respective markers of BL, G and SER membranes. Their enzyme activities were determined as described elsewhere (Moktari et al., 1986). The relative values of these markers were normalized to M form of aminopeptidase N.

(RLA), aminopeptidase N (AP) and human blood group A determinants (HBG-A) and that these vesicles could be very efficiently immunoprecipitated by anti-HBG-A antibodies. Judging from the distribution of specific markers, this immunoprecipitation seemed to be highly specific for BL. The SER and G were recovered in the nonprecipitable material (Moktari et al., 1986). During this subcellular fractionation, vesicles responsible for intracellular transport might be concentrated in L Mbr. In view of their membrane orientation, only a very improbable specific interaction with BL vesicles could lead to their copurification. It is more likely that they behave like G and SER vesicles, and they should therefore be found in the same fraction. The AP they contain will be included in the intracellular pool recovered in the G + SER fraction.

Little trapping of other membranes in the immunoprecipitate of BL occurred since, as shown in the Table as little as 10% of SER membrane, identified by means of its enzymatic marker, aryl sulfatase C, and from the presence of the only stable transient intermediate of glycosylation of AP (T form), was recovered in this fraction (Gorvel et al., 1986; Moktari et al., 1986). The Golgi marker (galactosyltransferase) reached higher levels. Some inside-out Golgi membrane vesicles may have been immunoprecipitated by anti-HBG-A antibodies since A antigenicity was acquired in the *trans* cisternae of the Golgi complex (Bernadac et al., 1984). However, the Table shows that no more than 15% of AP present in BL could arise from G or any related transporting vesicles. In the (G + SER) fraction 50% of the M form could arise from BL.

During subcellular fractionation, membranes from the different organelles characterized by their specific markers were obtained with about the same yield (Moktari et al., 1986). The microsomal fraction contained 35–45% of membranes from endoplasmic reticulum, Golgi complex, brush border and basolateral domain of the plasma membrane, which indicates that the same mixture of membranes was present in the homogenate. The loss of 65 to 55% of each type of membrane during preliminary low speed centrifugations (*see* Materials and Methods) were probably due to inefficient cell rupture (Moktari et al., 1986). In view of the fact that 8.5% of Na^+K^+-ATPase and 0.5% of AP were recovered in the BL fraction and 7% of galactosyltransferase and 0.6% of AP in the (G + SER) fraction (Moktari et al., 1986), it can be assumed that in the homogenate, these two fractions contained 6 and 8%, respectively, of the aminopeptidase N, 85% of which was in the BB.

The molecular forms of AP present in the SER and G membranes are different (Ahnen et al., 1983; Gorvel et al., 1986; Moktari et al., 1986): SER contains the only stable transient intermediate of glycosylation of AP (T form), whereas G contains the newly processed mature AP (M form). The (G + SER) fraction can be said to be the G fraction if only the M form it contains is taken into consideration. As shown in Fig. 1, with rocket immunoelectrophoresis as described by Feracci et al. (1985), it was possible to quantify the T form, which represented 40 to 60% of AP present in all (G + SER) fractions tested. A complete separation of T and M forms contained in a (G + SER) fraction prepared from mucosa scrapings from several A^+ rabbits was also performed by immunoaffinity chromatography on anti-HBG-A immunoabsorbent column (Feracci et al., 1985; Massey & Maroux, 1985); the unretarded T form of AP represented 48% of the total activity loaded on the column. An average value of 50% was used in the following studies. From results of subcellular fractionation discussed above, the

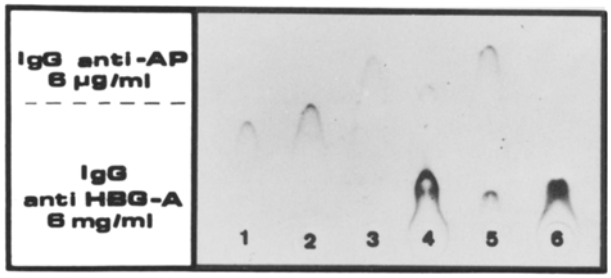

Fig. 1. Quantitative estimation of the transient form of aminopeptidase in G + SER fraction by rocket immunoelectrophoresis through agarose gels containing successively anti-human blood group A antibodies and anti-aminopeptidase. All mature molecules bearing human blood group antibodies were precipitated in the first gel, whereas the transient form devoid of these determinants was specifically precipitated by anti-aminopeptidase of the second gel. 0.5, 1, 1.5 units of AP from BB from A^- rabbits were loaded in 1, 2 and 3 to obtain a quantitative reference. The area of AP peak is proportional to the amount of immunoprecipitated enzyme. 3 units of AP from L Mbr, G + SER, and BL fractions from an A^+ rabbit were loaded in 4, 5 and 6, respectively. In this preparation T form represents 60% of AP in (G + SER) fraction and 40% in L Mbr

amount of cellular AP present in the G fraction can be estimated to be 4%.

Flow Kinetics of Aminopeptidase N Through Different Subcellular Fractions

Constant amounts of AP labeled with [^{35}S]-Met for 10 min were immunoprecipitated from microsomal, (G + SER), BL and BB fractions after various times of chase. The T and M forms were separated by SDS polyacrylamide electrophoresis, and their radioactivity was determined after transfer on nitrocellulose as described under Materials and Methods.

The transfer of T form of AP from the endoplasmic reticulum to the Golgi complex has been studied previously (Gorvel et al., 1986). Given that this is the factor limiting the transformation of the T form into the M form, the kinetics of the ER to G transfer are those of this transformation (Gorvel et al., 1986; Hauri et al., 1985). The equation of these kinetics corresponds to that of a first-order reaction until at least 90 min of chase (Gorvel et al., 1986). This result showed that, during this time, newly synthesized M form is stable. Consequently, the specific radioactivity of AP (T form + M form) from the microsomal fraction can be used to calculate the total amount of radioactivity incorporated into the AP by each rabbit. It was checked in some cases that the same specific radioactivity was obtained for AP from the total homogenate.

Considering the labeled enzyme present in the microsomal fraction after various times of chase, the ratio T*/T* + M* gives the proportion of the newly synthesized AP (T form) remaining in the ER (Fig. 2).

Figure 2 (left) gives autoradiograms corresponding to the analysis of labeled AP from (G + SER), BL and BB fractions after different times of chase. It can be seen that the transformation of the T form into the M form in the G fraction began before the end of the 10-min pulse. This agreed with a fast transport of the T form from ER to G, which is the limiting factor in this transformation (Hauri et al., 1985; Gorvel et al., 1986).

After glycan processing, the newly synthesized enzyme rapidly left G, appeared in BL after 15 min of chase, and then reached the BB after 30 min of chase.

The specific radioactivity of AP from these fractions was determined after various times of chase. Then, since in the steady state, BB, BL and G membranes contain 85, 6, and 4%, respectively, of AP present in the homogenate or the microsomal fraction, the total amount of labeled AP present in each fraction after various times of chase was calculated, taking as reference 100 the AP present in the homogenate. At each time, the sum of the radioactivity (Q_t) found in ER (Q_{ER}) and G (Q_G), BL (Q_{BL}) and BB (Q_{BB}) fractions was equal to 100–115%; an average value of 108% was obtained.

To test if our data are compatible with the successive passages of all the newly synthesized AP, through the four fractions considered, we derived the kinetic equations of the system. Our model postulates that AP passes from ER to G with the rate k_1, from the G to the BL with the rate k_2 and from BL to the BB with the rate k_3 and that the three transports are irreversible. Then, the differential equations of the transport system are:

$$dQ_{ER}/dt = -k_1 Q_{ER};\ dQ_G/dt = k_1 Q_{ER} - k_2 Q_G;$$
$$dQ_{BL}/dt = k_2 Q_G - k_3 Q_{BL};\ dQ_{BB}/dt = k_3 Q_{BL}.$$

These equations are readily integrated with the help of the Laplace-Carson transformation (Mouttet et al., 1974). The fact that at time 0 of chase the passage from ER to G has already begun, has been taken into consideration. The phenomenological parameters k_1, k_2 and k_3 may be fitted to the experimental data by the use of Newton-Gauss algorithms (Soulié et al., 1985). It appears in Fig. 2 that the curves simulated with the best set of parameters ($k_1 = 0.024\ \text{min}^{-1}$; $k_2 = 0.046\ \text{min}^{-1}$; $k_3 = 0.049\ \text{min}^{-1}$) are in a reasonably good agreement with the experimental points. It means that these data are consis-

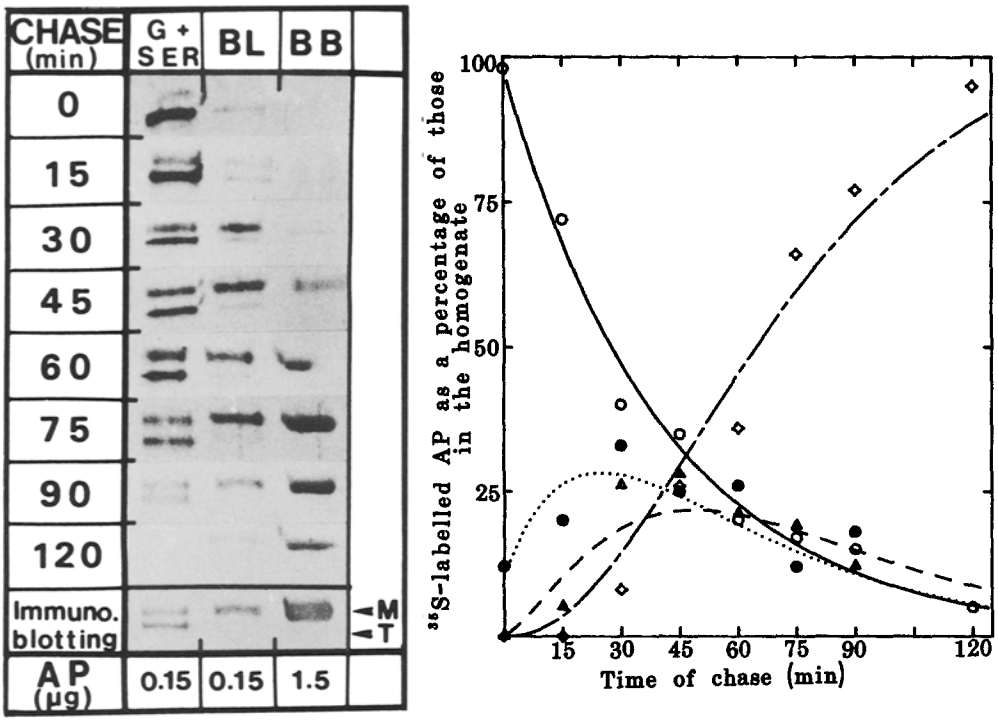

Fig. 2. Kinetics of the flow of newly synthesized aminopeptidase (AP) pulse labeled for 10 min with [^{35}S]Met. After various times of chase a constant amount of AP from detergent extracts of various subcellular fractions was quantitatively immunoprecipitated, subjected to SDS-PAGE and transferred onto nitrocellulose, and the labeled molecules were revealed by autoradiography (6–9 days). Autoradiograms and immunostaining obtained with 0.15 μg of AP from G + SER and BL and 1.5 μg of AP from BB are given on the left. T and M forms of AP present in (G + SER) fraction are located in SER and G membrane, respectively (Gorvel et al., 1986; Moktari et al., 1986). For quantification of radioactivity, twice the amount of material was loaded in larger wells for SDS-PAGE. The bands corresponding to M form were excised and counted (*see* Materials and Methods). The total amount of labeled AP present in G (M form from G + SER fraction) (●····●), BL (▲--▲) and BB (◇---◇) were calculated as described in the text. The newly synthesized AP (T form) remaining in the ER after various time of chase (○—○) is given by the ratio T*/T* + M*, considering the labeled enzyme in the microsomal fraction (Gorvel et al., 1986). Points correspond to experimental data and curves to theoretical equations assuming a successive passage through RE, G, BL and BB (*see* text)

tent with the kinetic model postulating that all the newly synthesized enzyme passing through the G fraction transits through the BL fraction before reaching the BB. The $t_{1/2}$ (0.693/k) of the passages through ER, G and BL fractions are 28, 15 and 14 min, respectively.

Discussion

It has been suggested that in enterocytes, newly synthesized glycoproteins are transported from the Golgi complex to the brush border membrane either directly or after a transient insertion into the basolateral domain (Quaroni et al., 1979*a*,*b*). Both of these pathways may coexist in all epithelial cells.

The direct pathway, which is very similar to that of the polarized exocytosis of secretory proteins (Jamieson & Palade, 1971), seems to be taken by an apical viral protein (hemagglutinin: HA) in Madin-Darby canine kidney (MDCK) cells infected by influenza virus. An imperfect sorting that occurs before HA reaches the cell surface is thought to explain the presence of a small amount of this apical protein in the basolateral domain (Matlin & Simons, 1984; Misek et al., 1984; Rindler et al., 1985).

The AP present in the basolateral domain of enterocytes (Moktari et al., 1986) is shown in the present study to possibly transit through this membrane before reaching the brush border membrane.

The flow kinetics of the newly synthesized AP through ER, G, BL and BB fractions (Fig. 2) clearly show that on leaving the G fraction, AP did not reach BB directly but passed through membrane vesicles concentrated in the BL fraction. The question then arises as to whether or not the membrane in question is the basolateral domain of the plasma membrane.

From the difference between G and BL kinet-

ics, it can be said that it is not the same Golgi membrane that concentrates in these two fractions.

In view of its post-Golgi position on the intracellular route of newly synthesized AP, the membrane of post-Golgi vacuoles involved in the transport of membrane glycoproteins from the *trans* cisternae to the plasma membrane (Saraste & Kuismanen, 1984; Griffiths et al., 1985) might be a good candidate. However, these post-Golgi vacuoles have been observed only in cultured cells infected by virus after the transport of virus glycoproteins from the Golgi to the plasma membrane was blocked. Under physiological conditions, probably because their turnover is very fast, they seem to be rare and difficult to observe. In enterocytes, no immunogold labeling of this structure was observed with an antisucrase-isomaltase monoclonal antibody capable of labeling the Golgi complex (Fransen et al., 1985). The same antibody also failed to label the basolateral domain of plasma membrane and endoplasmic reticulum, and the authors tentatively concluded that "this finding supports the view that newly synthesized sucrase-isomaltase is transferred directly from the Golgi complex to the microvillus membrane." However, absence of immunolabeling is not sufficient to conclude that an antigen is absent from a membrane since several other anti-sucrase-isomaltase monoclonal antibodies did not react with the Golgi membrane (Fransen et al., 1985).

Our anti-aminopeptidase did not label basolateral membrane of enterocytes on ultrathin frozen sections of rabbit jejunum (Feracci et al., 1982). However, the presence of AP integrated into the basolateral membrane vesicles concentrated in the BL fraction has been definitely proved by double immunolabeling with anti-aminopeptidase and class I anti-histocompatibility antigen, a specific marker of this domain (Moktari et al., 1986).

The arrival of appreciable amounts of labeled AP in the BL fraction after 30 min of chase corresponds to the appearance of labeled AP accessible to papain in the microsomal fraction previously reported (Feracci et al., 1985) and confirmed here on the L Mbr and BL fractions (*results not shown*). Papain accessibility is incompatible with integration into right-side-out intracellular membrane vesicles, in which the glycosylated ectodomain of AP, recognized by the anti-AP antibodies and liberated by papain from membrane vesicles, is located on the intravesicular side (Feracci et al., 1985). Only a very improbable complete transformation of post-Golgi vacuoles or other transporting vesicles into open sheets or inside-out vesicles, during homogenization could explain their concentration in the BL fraction and the accessibility of the AP they contain to papain.

The simplest interpretation of our results is that on leaving G the newly synthesized AP is transiently integrated into the basolateral domain of the plasma membrane before reaching the brush border membrane.

The possibility that brush border aminopeptidase might transit through the basolateral membrane suggests that the sorting of molecules specifically localized in the two plasma membrane domains of enterocytes must occur after their integration into the basolateral membrane. In Madin-Darby canine kidney (MDCK) cells, it has been demonstrated that the apical and basolateral domains of the plasma membrane are connected by a transcellular transport involving endosomes. This pathway effected the redistribution of a basolateral membrane protein which was artificially implanted in the apical domain (Matlin et al., 1983; Pesonen, Ansorge & Simons, 1984). In enterocytes, segregation of aminopeptidase in the upper part of the lateral membrane has been suggested by histochemical studies (Feracci et al., 1982). Intracellular vesicles related to the plasma membrane by the presence on their inner face of human blood group A determinants were found to be mostly localized in the terminal web (Bernadac et al., 1984). These vesicles might be formed of the upper part of lateral membranes where aminopeptidase might be concentrated after its insertion and could be involved in the transport from basolateral to brush border membrane.

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