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Subcellular Fractionation and Subcellular Localization of Aminopeptidase N in the Rabbit Enterocytes

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Summary. A fast and easy procedure is proposed for preparing concomitantly from the same sample of intestinal mucosa of A^+ rabbits, four fractions high enriched in the brush-border and basolateral plasma membrane domains, rough endoplasmic reticulum, and smooth endoplasmic reticulum plus Golgi apparatus membranes, respectively. This is the first time the technique of flow fluorometry has been applied to characterize the brush-border and basolateral membrane fractions using polyclonal or monoclonal antibodies against antigens common to or specific for these two plasma membrane domains. This technique definitely proves the presence of aminopeptidase in at least 60% of the basolateral membrane vesicles, where its level is about 4.5% of that in the brush-border membrane vesicles. The endoglycosidase H-sensitive intermediate of glycosylation of aminopeptidase N in the steady state is accumulated in both the rough and smooth endoplasmic reticulum membranes. Although the rough membrane is more extensive it contains only about 40% of this transient form.

Key Words aminopeptidase N · enterocyte · cell polarity · fluorometry · biosynthesis

Introduction

The plasma membrane of enterocytes is composed of at least two regions characterized by their morphological aspect 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 [20]. The basolateral domain is characterized by other specific markers, such as the Na^+,K^+-ATPase [11, 29] or the histocompatibility antigens [14, 21]. How and where these various pools of membrane proteins are sorted out and transported to their final specific location, are key questions in the studies of epithelial cell polarity.

To approach these questions we decided to investigate the biosynthesis and processing of aminopeptidase N, a major brush-border protein [8], during its intracellular transport [2, 7, 9, 26]. As in the case of many glycoproteins [18], only one early intermediate of glycosylation bearing N-linked high mannose oligosaccharides has been detected. This transient form in the steady state represents about 1% of the total cellular aminopeptidase [9]. One aim of the present study was to identify the subcellular compartment in which this transient form accumulates. Microscopy techniques have shown that aminopeptidase is present in the Golgi apparatus where it acquires human blood group A determinants in the trans cisternae and absent from the basolateral membranes [2, 7]. By contrast, subcellular fractionation points to the presence of small amounts of aminopeptidase in this plasma membrane domain [4, 35]. These conflicting results still give rise to much debate. Indeed, relationship may well exist between the presence of a small amount of apical membrane proteins in the basolateral membrane and their sorting and intracellular route [4, 16, 26, 28]. In particular, transit through the basolateral membrane of newly synthesized aminopeptidase could explain its presence, in low amount, in this plasma membrane domain.

To study the intracellular traffic of membrane constituents and, in particular, an eventual passage of brush-border hydrolases through basolateral membrane, no suitable subcellular fractionation procedure was available. The brush-border membrane was easily highly purified because of its particularly high density and the absence of any tendency to aggregate in presence of divalent cations [25, 33]. By contrast, complete separation of basolateral membrane and smooth intracellular membranes, in particular from the Golgi apparatus, was particularly difficult and never achieved by centrifugation through sucrose gradient [1, 35]. Here we used the presence on the basolateral membrane vesicles of human blood group A determinants [2, 7] to separate them from the Golgi and smooth endoplasmic reticulum membranes by specific immunoprecipitation. Simultaneously, the brush-border and the

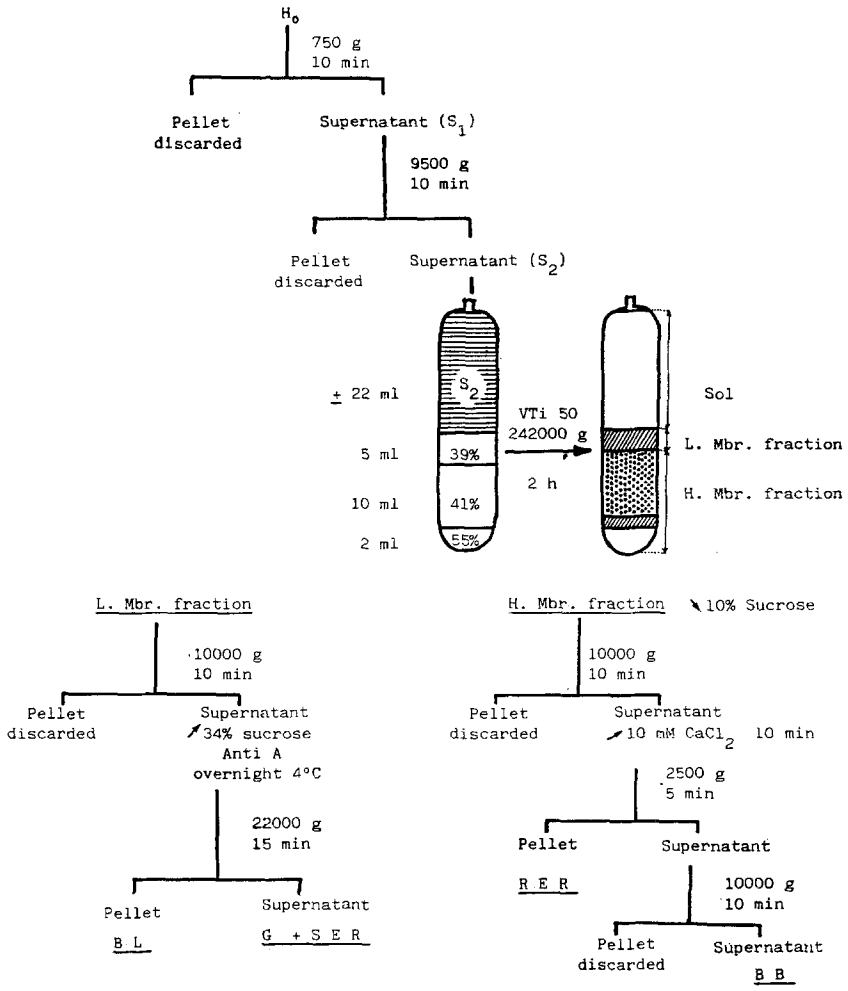

Fig. 1. Schematic representation of the subcellular fractionation of A^+ rabbit enterocytes. Fractions highly enriched in brush-border (BB) and basolateral (BL) plasma membrane domains, rough endoplasmic reticulum (RER) membrane and smooth endoplasmic reticulum (SER) plus Golgi apparatus (G) membranes were purified concomitantly

rough endoplasmic reticulum membranes were purified. For the first time the flow microfluorometry technique was applied to test the homogeneity of these membrane fractions using monoclonal and polyclonal antibodies which are specific for brush-border and basolateral membrane antigens. With this technique, it was possible to definitively conclude that a small amount of aminopeptidase N is present in the basolateral domain.

The transient form of aminopeptidase was found to have accumulated in both the rough and smooth endoplasmic reticulum.

Materials and Methods

Materials

Peroxidase-labeled anti-goat immunoglobulins were from Sigma, peroxidase-labeled anti-guinea pig immunoglobulins, rabbit anti-rat immunoglobulins and anti-mouse immunoglobulins were from Cappel laboratories, trypsin inhibitor from chicken egg white type II-O from Sigma and leupeptin from Bachem. Fluorescein isothiocyanate (FITC) and tetramethyl rhodamine isothiocyanate R isomer (TRITC) were purchased from Sigma.

The rabbit typing procedure was as previously described [7].

Subcellular Fractionation

Mucosal scrapings from A^+ rabbits were homogenized in 8 times their weight of a 20 mM Tris-HCl buffer (pH 7.3), containing 0.25 M sucrose, 10 mM KCl, 1 mM $MgCl_2$, 1 mM phenylmethylsulfonylfluoride, 0.1 mg/ml of trypsin inhibitor and 2 μg/ml of leupeptine by 20 then 30 vertical strokes with the A and B pestles, respectively, in a Dounce homogenizer. The resulting homogenate was then centrifuged as described in Fig. 1.

Aminopeptidase N [27], Na^+,K^+-ATPase [4], galactosyl transferase [3] and arylsulfatase C [5] were used as marker enzymes for brush-border (BB), basolateral (BL), Golgi apparatus (G) and endoplasmic reticulum (ER) membranes, respectively. During subcellular fractionation of pig intestinal mucosa Ahnen et al. [1] observed that the microsomal arylsulfatase C distribution was identical to that of the cytochrome *c* reductase, a more classical marker of ER [25]. The enzyme activities were determined without modification as described elsewhere [3–5, 27]. In

the case of aminopeptidase, Na^+,K^+-ATPase and arylsulfatase, enzyme units were defined as the amount of enzyme inducing the hydrolysis at 37°C of 1 nmol of substrate per min. In the case of galactosyltransferase, unit was defined as the amount of enzyme transferring at 37°C, 1 nmol of galactose on ovomucoid per hour.

Sucrose step gradient centrifugation was performed in a Beckman centrifuge, model L8-70, equipped with a vertical rotor V Ti 50. All sucrose solutions contained 20 mM Tris-HCl buffer (pH 7.3), 10 mM KCl and 1 mM $MgCl_2$. As shown in Fig. 1, 22 ml of S_2 were loaded on the following step gradient setup in each tube (the first figure indicates the volume and the second in parentheses, the sucrose concentration, Wt/Wt: 2 ml (55%), 10 ml (41%), 5 ml (39%). Centrifugation for 2 hr at 50,000 rpm gave the maximal concentration of the Na^+,K^+-ATPase and aminopeptidase in the light and heavy membrane fractions (L. Mbr and H. Mbr), respectively. After removal of the soluble fraction, the 10/39 interface light membrane fraction and the top of heavy membranes were collected from the top using a Buchler Auto densiflow. Each 1-ml fraction was assayed for sucrose concentration and those having sucrose concentrations lower than 38% were pooled. Only fractions of sucrose concentration equal to at least 39% were pooled with the heavy membrane fraction.

The heavy membrane fraction was diluted four times with a 1-mM $MgCl_2$ 10-mM KCl solution to lower the sucrose concentration to 10% and that of Tris to 5 mM and centrifuged 10 min at 10,000 × *g* before adding Ca^{2+} for RER membrane precipitation as described by Schmitz et al. [33]. The pellet was washed once with 20 mM Tris-HCl buffer (pH 7.3) containing 1 mM $MgCl_2$, 10 mM KCl, 10 mM $CaCl_2$.

The most successful immunoprecipitation of the basolateral membrane from the low density membrane fraction was obtained by adding per 100 units of Na^+,K^+-ATPase, 3.4 mg of Ig from rabbit immunsera against human blood group A erythrocytes (Anti A). After an overnight incubation the immunoprecipitate was obtained by centrifugation for 15 min at 22,000 × *g* using a Beckman 60 Ti rotor. It was then washed with 20 mM Tris-HCl buffer (pH 7.3) containing $MgCl_2$ 1 mM, KCl 10 mM and NaCl 0.14 mM and spun down by centrifugation for 5 min at 2,000 × *g* in a Sorvall SS 34 rotor.

Solubilization and Immunoprecipitation of Aminopeptidase N

Aminopeptidase present in membranes was solubilized by adding Triton X-100 and SDS to final concentrations of 2 and 0.1%, respectively, and incubating for at least 4 hr at 4°C. To improve immunoprecipitation of very low amounts of aminopeptidase in highly diluted solution, immunoglobulins from goat anti-aminopeptidase serum coupled to Ultrogel ACA 22 (3 mg/ml) using the technique described by Ternynck and Avrameas [34] were used. The immunogel was first incubated under vigorous agitation for 1 hr at 4°C in 10 mM phosphate buffer (pH 7.3), 0.15 M NaCl (PBS) containing 10% bovine serum for saturation of unspecific binding sites, then overnight at 4°C with the aminopeptidase solution. The gel was centrifuged for 30 sec in an IEC bench centrifuge operated at full speed (about 600 × *g*), washed and prepared for gel electrophoresis as previously described for direct immunoprecipitates [9].

SDS slab gel electrophoresis, immunoblotting and rocket immunoelectrophoresis techniques have been described elsewhere [9, 13].

Antisera, Immunoglobulins and Monoclonal Antibodies

Rabbit anti-human blood group A (anti-A) antisera and guinea-pig anti-aminopeptidase sera were previously used and their specificity was determined [7, 9].

We also used a goat antiserum raised against pure aminopeptidase and depleted of anti-human blood group antibodies by incubation with mucus as previously described [7]. Immunoglobulins were prepared according to Harboe and Ingild [15]. Their specificity was assayed here by immunofluorescence labeling, immunoblotting, immunoabsorption and rocket immunoelectrophoresis. Rat IgM monoclonal anti-RLA from 8 A 3-1-1 clone is specific for the Rabbit Lymphocyte Antigens of the major histocompatibility complex class I (*manuscript in preparation*). It was purified from culture supernatant by immunoabsorbant chromatography on rabbit anti-rat immunoglobulins coupled to Ultrogel ACA 22 [34]. Murine IgG 1 monoclonal anti-BB Ag from 26 E 122_1 clone is specific for a brush-border antigen (*manuscript in preparation*). It was purified from culture supernatant by immunoadsorbant chromatography on rabbit anti-mouse immunoglobulins coupled to Ultrogel ACA 22 [34].

Conjugation of Antibodies to Fluorescein and Rhodamine

Immunoglobulins from immunsera or monoclonal antibodies were conjugated to FITC or TRITC as described by Le Bouteiller et al. [22, 23]. After extensive dialysis against PBS, the molar fluorochrome/protein (F/P) ratios were: FITC-anti-aminopeptidase: 1.3; TRITC-anti-aminopeptidase: 5; FITC-anti-A: 2; TRITC-anti-A: 1.3; FITC-anti-RLA: 4; TRITC-anti-RLA: 1; FITC-anti-BB Ag: 1; TRITC-anti-BB Ag: 1; FITC-anti-HLA (from B 9-12.1) given by Le Bouteiller: 1.1.

Immunofluorescence of Ultrathin Frozen Sections

Immunofluorescence assays were performed as previously described [7, 13], except that here fluorochrome was directly conjugated to the antibodies raised against the tissue antigens. Tissue sections were therefore incubated with only 100 μl of FITC-anti-aminopeptidase (100 μg/ml) or FITC-anti-human blood group A (30 μg/ml) or FITC-anti-BB Ag (70 μg/ml) or FITC-anti-RLA (50 μg/ml).

Immunofluorescence Staining of Membrane Vesicles

The concentration of each of the conjugated antibodies permitting saturation of surface vesicles was determined by flow fluorometry analysis (*see below*). In the case of anti-aminopeptidase and anti-A, the aggregation of BB vesicles occurred before their surface saturation. This aggregation can be easily detected by light-scatter analysis [12]. The highest concentration of these antibodies which did not induce the BB vesicle aggregation was used. 100 μl of the three membrane fractions studied were incubated in presence of bovine serum albumin 1% for 30 min at 4°C with 40 μg FITC-anti-aminopeptidase or 60 μg of FITC-anti-human blood group A or 14 μg of FITC-anti-BB Ag or 10 μg of

Table 1. Enzyme content of various subcellular fractions from A^+ rabbit intestinal mucosa[a]

	Aminopeptidase		Arylsulfatase C		Gal. Transferase		Na^+,K^+-ATPase	
	Yield	Enrich.	Yield	Enrich.	Yield	Enrich.	Yield	Enrich.
S1	49	0.8	50	0.8	60	1	41	0.7
S2	36	0.8	36	0.8	44	0.9	31	0.6
H. Mbr.	*27*	2.9	*17*	1.8	6.5	0.7	5.9	0.6
L. Mbr.	4.5	0.3	*9.3*	0.6	*17*	1.1	*17*	1.2
BB	*12*	*13*	0		0		0	
RER	0.9	0.5	*7.3*	*4*	2.2	1.2	1.4	0.8
G + SER	0.6[b]	0.5	*5*	*2.5*	*7*	*3.5*	2.7	1.5
BL	0.5	0.50	0.4	0.2	3	1.6	*8.5*	*4.7*[c]

[a] The separation of the fractions is described in Fig. 1. For each enzyme the yield was calculated as the number of units found in the fraction per 100 units in the homogenate. Enrichment was the ratio of specific activities in the fractions to those in the homogenate. The figures are the average of five independent assays.

[b] Only immunoreactive enzyme was taken into consideration since this fraction contains an appreciable amount of an enzyme hydrolyzing alanine-*p* nitroaniline which is different from the aminopeptidase N.

[c] The specific activity of the BL fraction was underestimated because it was considerably contaminated by A-immunoglobulins inducing the precipitation.

FITC-anti-RLA or 10 μg of FITC-anti-HLA. The volume was brought up to 1 ml with PBS containing BSA 1%. After filtration of vesicles through nylon gauze, the vesicles were injected in the flow system for analysis.

To determine the presence of aminopeptidase N in BL membrane, vesicles were double-stained with a mixed solution of FITC-anti-aminopeptidase (20 μg) and TRITC-anti-RLA (20 μg) for 30 min at 4°C. Vesicles were analyzed by flow fluorometry using the two-color detection system [22]. The number of positive vesicles was determined from the one-dimensional projection histograms on the fluorescein and rhodamine fluorescent intensity axis, respectively, after comparing these with the histograms of the negative control sample stained with FITC-anti-HLA [22].

Flow Flurometry Analysis

The EPICS V Cell sorter (Coulter Electronics, Hialeah, Fla.) was used with the Spectrophysics model 171 argon laser in the single-color analysis and quantitative fluorescent measurements. Each sample was submitted to 10 analyses. For each assay, 10,000 vesicles were analyzed for fluorescence intensity after elimination of disrupted particles by light-scatter gating [12]. During fluorescence measurements, absence of aggregation was simultaneously controlled by light-scatter analysis [11]. For quantitative immunofluorescence measurements, the cell sorter was calibrated with a view to establishing the relation between the number of fluorochromes and the fluorescence emission as described in detail by Le Bouteiller et al. [23]. The density of fluorochromes per surface unit (μm^2) was calculated taking an average diameter of 0.1 and 0.2 μm determined by Gorvel et al. [12] for the brush-border and basolateral vesicles, respectively. In two-color fluorescence analysis we used the spectrophysics model 171 and 164 argon lasers. The dual parameter analysis was performed as described by Le Bouteiller et al. [22]. In these experiments, for each sample, 100,000 vesicles were analyzed.

Results

Subcellular Fractionation

The aim of the procedure shown in Fig. 1 was to separate simultaneously the two plasma membrane domains and the major intracellular membranes known to play a role in the biosynthesis and processing of membrane proteins. As previously described [25], nuclei, mitochondria, nonvesiculized membranes and aggregates were first eliminated by differential centrifugations. Table 1 shows that 60 to 70% of each marker was lost during these preliminary steps probably due to inefficient cell rupture. No homogenization procedure tested permitted to obtain a better yield at this step. Then, the light membranes (L. Mbr.) ranging in density from 1.06 to 1.16 were separated from the heavy membranes (H. Mbr.) ranging in density from 1.17 to 1.26 by centrifugation through a sucrose step gradient. Table 1 shows that the aminopeptidase, marker of the brush-border (BB) membrane, was almost exclusively found in the heavy fraction as expected, whereas the galactosyltransferase and the Na^+,K^+-ATPase, markers of Golgi (G) and basolateral (BL) membranes, respectively, were essentially concentrated in the light fraction. The presence of these two markers in relatively high amounts in the heavy fraction was probably due to an aggregation of BL and G membranes since they were partly eliminated from the pellet resulting from centrifugation at 10,000 $\times$ *g* for 10 min after the sucrose concentra-

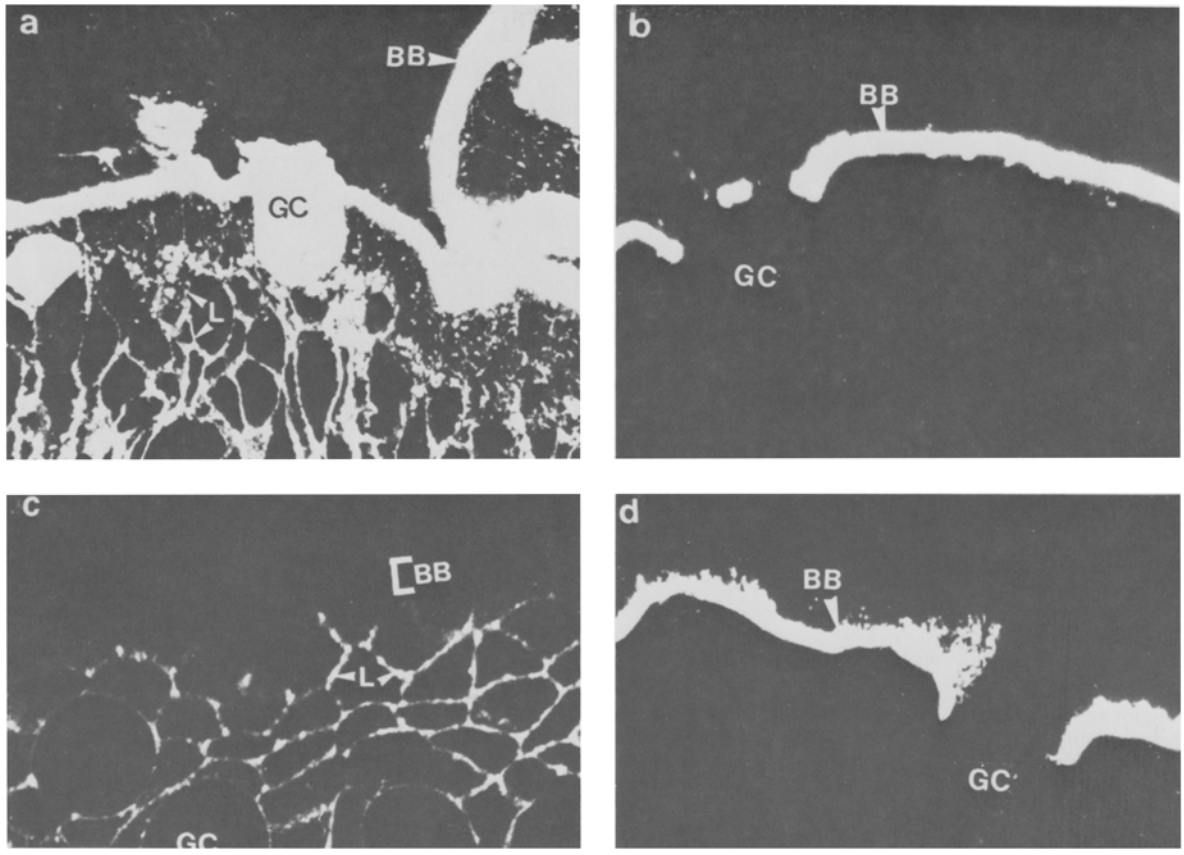

Fig. 2. Immunofluorescence labeling of ultrathin frozen sections of jejunum from A^+ rabbit with FITC-rabbit anti-human blood group A antibodies in *a*; FITC-goat anti-aminopeptidase in *b*; FITC-rat anti-RLA in *c*; FITC-mouse anti-BB Ag in *d*. Identical results were obtained when TRITC conjugates were used. Magnification 750×

tion was brought down to 10%. Arylsulfatase C [5], used here instead of NADPH cytochrome *c* reductase [25] as endoplasmic reticulum (ER) marker, was divided among the light and heavy fractions as expected for smooth and rough endoplasmic reticulum membranes (SER and RER).

By precipitating the RER membrane by Ca^{2+} [19, 33], it was possible to separate it completely from the BB membrane. During this precipitation, the low amounts of BL and G membranes remaining in the H. Mbr. fraction behaved like the RER membrane and consequently constituted the major contamination of the RER fraction. The enrichment factor of aminopeptidase in the present BB membrane fraction was identical to that of the best preparations already obtained [25, 33].

All attempts to separate the three types of light membranes by centrifugation through continuous sucrose gradient were unsuccessful. All three presented the same density heterogeneity [4, 10, 35] and three mixed fractions with different densities were obtained. In the experiments reported below, this fractionation was not performed. Since only BL membranes bear human blood group A determinants on their external surface [2, 7, 13], anti-human blood group A antibodies were used to separate them from the G and SER membranes. To avoid aggregation and sedimentation of these membranes during centrifugation, sucrose concentration was brought up to 34% before adding antibodies. It was not possible to precipitate any more than 75% of the Na^+,K^+-ATPase, which suggests that 25% of the BL membrane vesicles could be inside out or correspond to a domain devoid of A determinants. A very small amount (7%) of SER membrane was carried along with the immunoprecipitated membranes, whereas 30% of the galactosyltransferase was found in this fraction. So, rather than being due to an unspecific carrying, this could be caused either by the presence of inside-out vesicles from trans Golgi cisternae that bear human blood group A determinants on their luminal face [2] and contain the galactosyltransferase [6], or to the presence of galactosyltransferase in the BL membranes [32, 35].

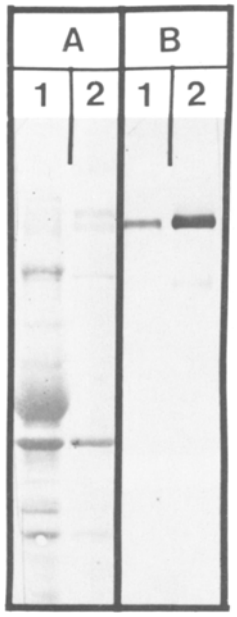

Fig. 3. Specificity of goat-aminopeptidase assayed by immunoblotting technique. Proteins of BL and BB membrane fractions were fractionated by SDS-PAGE and transferred to a nitrocellulose sheet for staining by Ponceau S in *A* and immunostaining with goat anti-aminopeptidase in *B*. BL (115 μg of proteins) and BB (4 μg of proteins) containing 0.3 μg of aminopeptidase were treated in 50 μl as previously described [13] and loaded in (1) and (2), respectively. A nonquantitative extraction of aminopeptidase during treatment of the large amount of BL could be responsible for the weaker specific reaction in *B*. (1). In the BB a small amount of a very often observed degradation product of aminopeptidase is also revealed

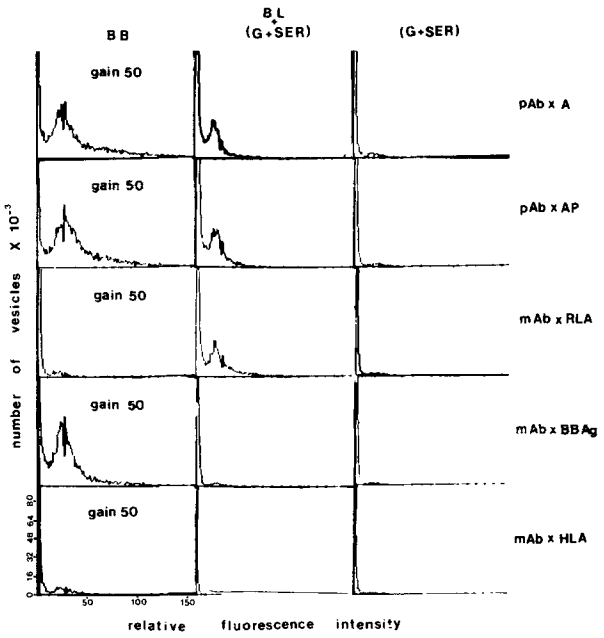

Fig. 4. Surface labeling of various membrane fractions by several antibodies assayed by flow microfluorometry. Vesicles were stained by direct immunofluorescence with FITC conjugated with: rabbit polyclonal anti-human blood group A determinants (pAb × A); goat polyclonal anti-aminopeptidase (pAb × AP); rat monoclonal anti-rabbit lymphocyte antigen of the major histocompatibility complex class I (mAb × RLA); murine monoclonal anti-brush-border antigen (mAb × BBAg). Murine monoclonal anti-human lymphocyte antigen (mAb × HLA) was used as negative control. The cell sorter was set at a gain of 50. Vesicle number was plotted against fluorescence intensity (channel number 0 to 256)

The use of other antibodies which are more specific for the basolateral membrane than anti-human blood group A antibodies, such at the monoclonal anti-RLA used below for specific labeling, could improve basolateral membrane purification and is under investigation.

Localization of Aminopeptidase N on Basolateral Membrane Vesicles Assayed by Flow Microfluorometry

A very low amount of aminopeptidase was found in all these membrane fractions, particularly in BL membrane (Table 1). To test whether this was due to a low contamination by the BB membrane or to the actual presence of the enzyme in this membrane we used the flow microfluorometry technique [12, 17, 22–24]. This analysis could not be performed on the final BL membrane fraction since it is obtained as an immunoprecipitate whereas flow fluorometry can be applied only to free particle suspension. It was achieved by comparing labeling of the light membrane fraction before and after BL membrane immunoprecipitation. BB membrane fraction was studied as control. In Fig. 2 the four antibodies used to label the membrane fractions were tested on ultrathin frozen sections of jejunum. Specificity of anti-aminopeptidase was also tested by immunoblotting as shown in Fig. 3.

Figure 4 shows diagrams of one flow fluorometry analysis of different membrane fractions labeled with the different antibodies. Ten such analyses were integrated to calculate the values given in Table 2. They show that 30% of vesicles in the light membrane fraction bore human group A determinants and that their immunoprecipitation induced by anti-human group A was particularly efficient. The possibility that this vesicle population might be identical with that of BL membrane was confirmed by the observed labeling with anti-RLA, a specific marker of this plasma membrane domain as shown in Fig. 2 and by the absence of BB membrane vesicle labeling. Contrary to what was suggested by im-

Table 2. Surface labeling of various membrane fractions by several specific antibodies coupled with fluorescein, assayed by flow microfluorometry

Fractions	Percent[a] of vesicles labeled in:			Determinant density by μm^2 of labeled vesicles ($\times 10^{-3}$)		
	B.B.	B.L. + (G. + SER)	(G. + SER)	B.B.	B.L. + (G. + SER)	(G. + SER)
Antibody						
pAb × A	78	27	4.3	2700	155	27
pAb × AP	81	30	3.5	2800	124	30
mAb × RLA	5	38	2.5	257	241	21
mAb × BB Ag	74	3	2	2700	29	16
mAb × HLA	8	6	1.6	120	9	15

[a] Calculated from Fig. 4 as described in Materials and Methods. Similar results were obtained on two independent subcellular fractions. Identical results were obtained using the same antibodies coupled with rhodamine. pAb × A: rabbit polyclonal anti-human blood group A determinants. pAb × AP: goat polyclonal anti-aminopeptidase. mAb × RLA: rat monoclonal anti-rabbit lymphocyte antigen of the major histocompatibility complex class I. mAb × BB Ag: murine monoclonal anti-brush-border antigen. mAb × HLA: murine monoclonal anti-human lymphocyte antigen (control).

munofluorescence on tissue sections where BL membranes were not labeled by anti-aminopeptidase, these antibodies reacted with BL membrane vesicles precipitated by anti-human blood group A. The presence of both aminopeptidase and RLA on the same membrane vesicles were tested more closely by the double-labeling experiment reported in Fig. 5. Quantitative analysis (*see* Materials and Methods) of the diagram in this figure showed that all vesicles labeled by anti-aminopeptidase were also labeled by anti-RLA, whereas 40% of the labeled vesicles bound RLA exclusively.

A monoclonal antibody against a brush-border protein (BB Ag) which did not belong to the hydrolase class and was exclusively located in the brush border of enterocytes and proximal tubule kidney cells was recently obtained (*manuscript in preparation*). In contrast to anti-aminopeptidase, this antibody reacted exclusively with BB membrane vesicles. As shown in Table 2 the very small amount of vesicles labeled with this antibody, present in the BL + (G + SER) fraction was not above the control value and their labeling density was not compatible with that of brush-border vesicles. These results showed that the BL and (G + SER) fractions were not contaminated by a detectable amount of brush-border membrane. It can be noted that the three antibodies able to react with the BB membrane labeled 75 to 80% of the vesicles of this membrane fraction. This was the first fine evaluation of the degree of homogeneity of a membrane preparation to be performed. Among the interesting information provided by flow fluorometry analysis is an estimation of the density of labeling [23]. Here it showed that the amount of aminopeptidase per surface unit of the BL membrane was only 4.5% of that of BB membrane. This value was in agreement with the relative specific activities of aminopeptidase in the two membrane fractions: 15,000 for BB membrane and 60 and BL membrane.

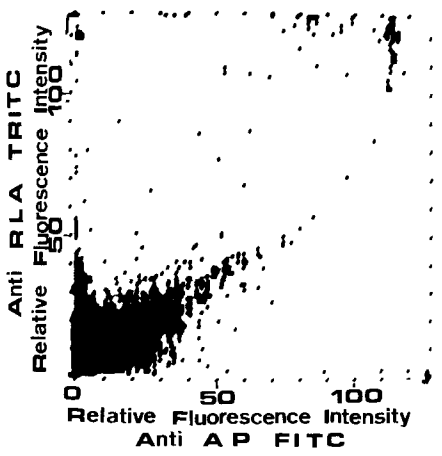

Fig. 5. Double-labeling of basolateral membrane vesicles with fluorescein-anti-aminopeptidase and rhodamine anti-RLA. The relative fluorescence intensity increases linearly to the right (index channel number 0–128 for fluorescein-anti-AP) or to the top (channel number 0–128 for rhodamine-anti-RLA)

Subcellular Localization of the Transient Form of Aminopeptidase

Identification of subcellular compartment(s) where the transient form of aminopeptidase (T form) is accumulated in the steady state was achieved by analyzing the T form content of the various membrane fractions by immunoblotting technique [9]. Since it has been generally accepted that endoglycosidase-

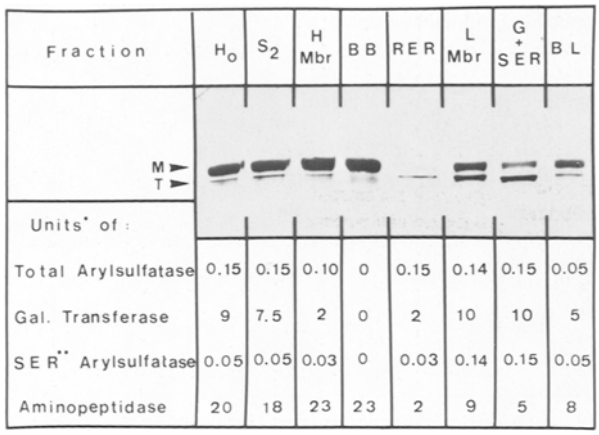

Fig. 6. Molecular form analysis of aminopeptidase along the subcellular fractionation. Aliquots of fractions (*see* Fig. 1) containing, when possible, a constant amount of arylsulfatase (0.15 units) and other markers as indicated in the table at the bottom of the picture, were solubilized and aminopeptidase immunoprecipitated (*see* Materials and Methods). The immunoprecipitates were then subjected to SDS-PAGE, transferred to nitrocellulose and immunostained with guinea-pig anti-aminopeptidase N. M and T indicate the mature and transient forms of the enzyme. In brush-border membrane fraction, the thickness of the faint band observed at the T position suggested it was not T form that always gave a very thin band, but more probably a degradation product of the M form. Absence of T form in this fraction will be confirmed in Fig. 7. * Units as defined in Materials and Methods. ** SER arylsulfatase was calculated from Table 1 that shows that SER Mbr. recovered in the L. Mbr. fraction represented 35% of the total arylsulfatase activity present in the S_2 and consequently in the H_0. Presence of G membrane in H. Mbr. and RER fractions revealed by galactosyltransferase activity, suggested they could also be contaminated by SER membrane. It was assumed that the level of contamination by SER membrane was identical to that by the Golgi membrane and consequently that the ratio of the galactosyltransferase and SER arylsulfatase was the same both in these fractions and in the G + SER fraction

H transient form of glycoproteins are still localized in the endoplasmic reticulum [1, 18], aminopeptidase corresponding to a constant amount of arylsulfatase from each fraction was subjected to analysis. Arylsulfatase is a marker of both the SER and RER membranes. Assuming that the separation of the two types of membranes was almost complete after centrifugation through the sucrose step gradient, the arylsulfatase bound to each type will be approximatively as shown in Fig. 6. This figure strongly suggests that the distribution of the T form of aminopeptidase could be similar to that of the SER membrane. The fraction containing the SER membrane also contains the G membrane. Here this membrane was characterized by the galactosyltransferase activity. This enzyme is a specific marker of only the trans cisternae. However, the result obtained with the present fractionation technique suggests that the probability of the behaviors of membranes from cis and trans cisternae being different seems very slight. It is obvious from Fig. 6 that the T form did not follow the Golgi marker. A more quantitative estimation of the T form contained in each fraction was attempted by means of the quantitative cross immunoelectrophoresis analysis given in Fig. 7 [9]. The results obtained confirm those suggested by immunoblotting but show that RER also contained an appreciable amount of T form. Comparison between T form peaks in the SER and RER membrane fractions indicated that the level of T form in the SER membrane could be about three times that in the RER membrane assuming that the level of arylsulfatase C is equivalent in both types of membrane.

The analysis of molecular forms of aminopeptidase in the various fractions can also be a very useful means of studying the efficiency of the subcellular fractionation. Presence of the mature form of enzyme (M form) in the RER fraction revealed a contamination by BB or G membranes whereas presence of T form in the BL fraction revealed a contamination by SER membrane. Figure 6 showed that these types of cross contamination were very low.

Discussion

In order to study the biogenesis of the various plasma membrane domains of a polarized cell it is important to separate all these membrane regions, as well as the intracellular membranes involved in the biosynthesis of their constituents, from the same starting material. The subcellular fractionation method devised here makes it possible to obtain simultaneously, from a single scraping of A^+ rabbit intestinal mucosa, four membrane fractions highly enriched in brush-border and basolateral plasma membrane domains, rough endoplasmic reticulum, and Golgi plus smooth endoplasmic reticulum membranes of enterocytes.

During subcellular fractionations, each type of membrane is characterized by specific markers. However, a small amount of a marker considered to be specific for one type of membrane can very often be detected in another after fractionation. It is then difficult to determine whether this reflects a cross-contamination or an actual presence of the same marker on two membrane types but in very different amounts. In the case of enterocytes, the presence of small amounts of brush-border hydrolases in the basolateral domain was still an open question. To settle this problem we applied the flow fluorometry

technique for the first time to membrane vesicles. The results obtained indicate that this method could be as useful and powerful a means of characterizing membrane vesicles as it is for cells [17, 24].

It provided a much more sensitive test of the homogeneity of a membrane preparation than other techniques such as electron microscopy, analysis of protein constituents by polyacrylamide gel electrophoresis, or absence of some subcellular markers. Quantification of the labeling [23] makes it possible to characterize and distinguish two vesicle populations labeled by the same antibodies, such as the vesicles from brush-border and basolateral membrane binding respectively, 2,800 $\times$ 10^3 and 124 $\times$ 10^3 mole of fluorescent anti-aminopeptidase per μm^2. Double-labeling experiments were particularly useful as a means of demonstrating the presence of two antigens on the same membrane domain. Here, we were able to definitely prove the presence of aminopeptidase in the basolateral membranes as we previously reported [4]. Considering that the basolateral fraction was obtained with a yield of 8.5% and contained 0.5% of the starting aminopeptidase (Table 1), the total amount of aminopeptidase in the BL membrane of enterocytes could be estimated to be 6%. However, this estimation must be considered with caution because an important error could be introduced by the value of 0.5%. Why this enzyme cannot be detected by immunofluorecence on ultrathin frozen sections remains an open question. The presence in the basolateral domain of polarized epithelial cells of small amounts of proteins essentially localized in the apical pole, has been described in some other cases, particularly that of cells in virus-infected cultures [28, 30, 31]. It was then found that this did not seem to correspond to the transit through the basolateral domain of the apical proteins as previously suggested for intestinal brush-border hydrolases [4, 16, 26]. An "error" in the sorting and/or in the transport of proteins specific for the two plasma membrane proteins could be responsible for this cross-localization.

Here we used only the analytical possibilities of the flow fluorometry technique, but the sorting of different vesicle populations could be used and will be tested for obtaining small quantities of highly purified membrane fractions. The most limiting factor in the application of this method is that it requires appropriate antibodies. The risk of obtaining from the same membrane two types of closed vesicles, some inside-out and the others right-side-out, should also be kept in mind. Here for example, if basolateral membranes have formed some closed, inside-out vesicles, they escaped immunoprecipitation caused by anti-human blood group A antibodies and labeling by anti-RLA or anti-aminopeptidase,

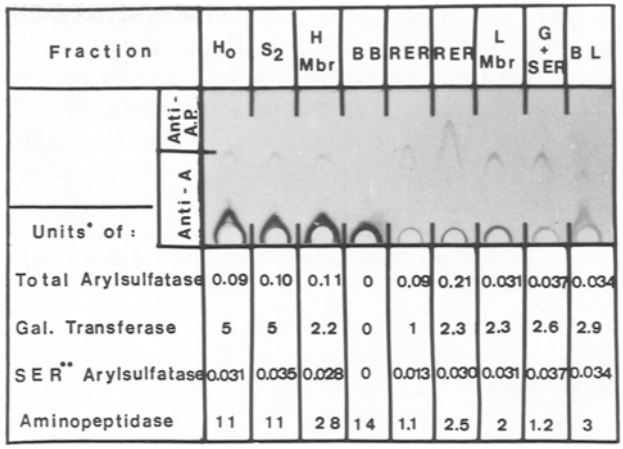

Fig. 7. Quantitative estimation of the transient form of aminopeptidase in various subcellular fractions by rocket immunoelectrophoresis through agarose gels containing successively 6 mg/ml of anti-human blood group A antibodies (anti-A) and 6 μg/ml of goat anti-aminopeptidase (anti-AP). Detergent extracts of different subcellular fractions were analyzed. All molecules bearing human blood group A determinants as the mature aminopeptidase were precipitated in the first gel whereas the transient form devoid of these determinants was specifically precipitated by anti-aminopeptidase of the second gel. Immunoprecipitates were revealed by aminopeptidase activity using chromogenic substrate. The amount of the various markers in each fraction is given. * SER arylsulfatase was estimated as in Fig. 6

all these antibodies being raised against antigens localized on the external side of the membrane. Such vesicles could be responsible for the Na^+,K^+-ATPase activity found in the G + SER fraction.

Glycosylation of glycoproteins is a multistep processing that occurs in various subcellular compartments [6, 18]. However, only an early intermediate of glycosylation can generally be detected in studies of glycoprotein biosynthesis. Recently, we showed that in the case of aminopeptidase this transient form can represent in the steady state at least one percent of the total cellular enzyme [9]. The accumulation of this transient form could be due to an enyzmatic limiting step in the processing or to a limiting transfer rate to the subcellular compartment where it can be transformed into the mature form. This maturation can be followed by the appearance on the molecule of human blood group A determinants and terminated in the trans cisternae of the Golgi apparatus. Here we compare the distribution of the transient form during the subcellular fractionation with that of the specific markers of various intracellular membranes, and we can conclude that it seemed to be essentially localized in both types of endoplasmic reticulum. It seemed to be more highly concentrated in the smooth than in the rough membrane. However, the latter, since it

is more abundant, could contain 30 to 40% of the total amount of the transient form. These results show that the formation of the intracellular pool of the transient form is due to its slow transit through the smooth endoplasmic reticulum where no modification of the intermediate glycans seems to occur.

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