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Biosynthesis and Intracellular Pool of Aminopeptidase N in Rabbit Enterocytes

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Summary. A papain treatment at 15°C and pH 7.3 of a microsomal fraction from rabbit enterocytes quantitatively releases the aminopeptidase N integrated in the plasma membranes without solubilizing the enzyme integrated in the intracellular membranes. Working on A^+ rabbits, characterized by the presence on the brush-border hydrolases of glycans corresponding to the human blood group A-determinant structure, it was possible to separate the intracellular aminopeptidase into two major molecular forms with or without these determinants. The molecular form devoid of human blood group A antigenicity corresponds to the only stable intermediate of glycosylation, bearing N-linked high mannose oligosaccharides. This endoglycosidase H-sensitive form is fully active and represents in the steady state about 1% of the total cellular aminopeptidase. It contains a cytoplasmic sequence of about 3000 daltons that has not yet been detected in the mature form. The A antigenicity is acquired simultaneously with processing of high mannose glycans to complex glycans. Pulse chase labeling of jejunum loops with [^{35}S]-methionine showed that the complete processing of the transient form synthesized during 10 min takes 1 hr. During the last 30 min of processing, all the newly transformed molecules are transported to the plasma membrane.

Key Words intestine · aminopeptidase N · biosynthesis · glycoproteins · A antigenicity

Introduction

Aminopeptidase N comprises 3.5% of the membrane proteins of the brush border of rabbit intestinal absorbing cells. It is a monomeric glycoprotein anchored in the membrane by its hydrophobic N terminal sequence that spans the membrane only once [15]. The hydrophilic domain located on the external surface of the membrane represents almost the entire molecule and bears the active site and the sugars [14]. Sugars account for 17% of the total molecular weight (120,000 daltons) and are thought to be distributed as eight N-linked complex glycans and two O-linked glycans bearing A or H human blood group antigenicity in A^+ or A^- animals, respectively [13, 16, 26].

As early as 1969, Whur et al. [34] had obtained autoradiographic evidence that addition of mannose to glycoproteins occurred in the rough endoplasmic reticulum whereas other sugars, in particular fucose, were incorporated in the Golgi complex of the intestinal absorbing cells [28]. Direct evidence for the presence of aminopeptidase N in the Golgi apparatus has been obtained using immunocytochemical techniques [13]. In A^+ rabbits the human blood group A antigenicity seems to be acquired only in cisternae on the *trans* side of the Golgi stacks [2].

Recently, the biosynthesis of a transient endoglycosidase H sensitive form of the brush-border aminopeptidase N has been demonstrated in pig and rat intestinal enterocytes [1, 9, 10]. Such intermediate was now pointed out in the biosynthesis of a lot of N-glycosidically linked glycoproteins [22]. However, in any case, no information on the biological activity such as the expression of the activity for the enzymes and the intracellular level of this transient form have been obtained.

Here we use the acquisition of A antigenicity as a signal for the completion of glycans which are probably O-linked, to study the different steps of glycosylation preceding this final step. We show that the A antigenicity is acquired simultaneously with the processing of high mannose glycans of the first intermediate of N-glycosylation in complex glycans of the mature aminopeptidase. Then, taking advantage of the absence of A-determinants in the transient form of aminopeptidase N we have completely separated it from the mature form and shown that it was already active and represented 1% of the total cellular enzyme. The kinetics of the transformation and of the transport of the newly synthesized enzyme to the plasma membrane have been studied by pulse-chase labeling, the enzyme located in the intracellular membranes being separated from that integrated in the plasma membrane by a papain treatment of a microsomal fraction. This treatment provided new information on the in-

tegration of the transient form in the intracellular membrane(s).

Materials and Methods

Materials

[^{35}S]-Methionine was from Amersham. Peroxidase-labeled antiguinea pig immunoglobulins were from Cappel laboratories (Cochranville, Pa.). Papain was from Boehringer and endoglycosidase H from Miles.

The procedure of typing rabbits was as previously described [13].

Pulse-chase Labeling

Rabbits (1.2 to 1.7 kg) were fasted overnight and anesthetized intravenously with the analgesic Imalgene 500 (Merieux, France). A jejunum loop about 30 cm long was isolated, and 1 mCi of [^{35}S] methionine, specific radioactivity about 1100 Ci/mmol in 2 ml of 10 mM phosphate buffer, pH 7.4, 0.15 M NaCl (PBS), was injected in the lumen. After 10 min of incorporation, radioactivity was chased with 1 ml of 50 mM methionine in PBS. Rabbits were killed at different times after the chases. The radioactive loop was removed and washed with cold PBS. Microsomal fraction was immediately prepared from the mucosal scraping.

Microsomal Fraction and Papain Treatment

Mucosal scrapings were homogenized in 8 times their weight of a 50 mM Tris-HCl buffer, pH 7.3, containing 0.25 M sucrose, 25 mM KCl and 1 mM phenylmethylsulfonyl-fluoride by 20 then 30 vertical strokes with, respectively, the A and B pestles in a Dounce homogenizer. The resulting homogenate was centrifuged at 750 × g for 10 min then at 9500 × g for 10 min. The microsomal fraction was obtained by centrifugation at 105,000 × g during 30 min of the 9500 × g supernatant. It contains 38% of the brush-border membrane, 20% of the basolateral membrane, 28% of the Golgi membrane and 20% of the endoplasmic reticulum (average of seven assays). These different types of membranes were characterized by their respective enzymatic markers: aminopeptidase [27]; Na^+,K^+-ATPase [7]; galactosyltransferase [5]; arylsulfatase C [11].

Brush-border and basolateral membranes were purified as previously described [7]. Papain treatment was performed at 15°C in a 50 mM potassium phosphate buffer, pH 7.3, containing 5 mM cysteine, 0.03 mM dithiothreitol and 0.25 M sucrose. One ml of microsomal fraction containing 8000 units of aminopeptidase N was incubated with 2.5 mg of papain. The hydrolysis was stopped by addition of 20 µl N-ethylmaleimide 0.5 M.

Solubilization and Immunoprecipitation of Aminopeptidase N

Aminopeptidase present in membranes was solubilized by adding Triton X-100 to a final concentration of 2% and incubating overnight at 4°C. After centrifugation at 18,000 × g for 30 min in the angle rotor of a Haereus-Chris centrifuge, aminopeptidase activity is entirely in the supernatant. Generally, 5 µg of enzyme were specifically and quantitatively immunoprecipitated with 15 µl of guinea pig serum containing antibodies raised against pure enzyme. A control experiment involved the same quantity of non-immunized guinea pig serum. After incubation for 2 hr at 37°C and overnight at 4°C, the immunoprecipitates were centrifuged for 3 min in an IEC bench centrifuge operated at full speed (about 600 × g). The precipitates were washed 5 times with 0.5 ml of PBS containing 1% Triton X-100 and 0.5% sodium desoxycholate and twice with 10 mM Tris-HCl, pH 7.4.

Endoglycosidase H Treatment

Samples in 0.2 M citrate buffer, pH 5.5, containing 1 mM phenylmethylsulfonyl fluoride were treated 24 hr at 37°C with 2 additions at 8-hr intervals of 5 mU of endoglycosidase H. Samples were then prepared for gel electrophoresis.

SDS Slab Gel Electrophoresis

Samples were treated as described by Green et al. [17] and immediately applied to a 7.5 to 15% linear polyacrylamide gel in 0.1% SDS with a 5% stacking gel as described by Blobel and Dobberstein [4]. Gels of 1-mm thick and 15-cm long or 0.75-mm thick and 9-cm long were respectively used for subsequent fluorography or immunoblotting.

Fluorography

After staining proteins with Coomassie Blue [13] the SDS-PAGE was prepared for fluorography by incubating in ENHANCE (New England Nuclear) for 1 hr at room temperature. After washing several times in water, the gel was successively incubated for a 15-min period at room temperature in 35% (vol/vol) methanol and in a 2% glycerol, 10% methanol and 2% acetic acid solution. After drying, the gel was exposed to Kodak XO Mat AR film at −80°C.

Immunoblotting

Electrotransfer of proteins from the SDS-PAGE to nitrocellulose was performed as described by Burnette [6]. After transfer, immunoprinting was performed according to Coudrier et al. [8]. All operations were performed at room temperature. The sheet was incubated 30 min in PBS containing 10% bovine serum (buffer I) then 1 hr with anti-aminopeptidase guinea pig serum in a 1/200 dilution in PBS containing 10% bovine serum and 0.2% Triton X-100 (buffer II). After four washings for 10 min in buffer II the nitrocellulose was immersed 1 hr in rabbit antiguinea pig Ig G conjugated to horseradish peroxidase at 5 µg ml^{-1} in buffer II, then washed in buffer II as before. Peroxydase was revealed by 5-min incubation in 3,3′-diaminobenzidinetetrahydrochloride reagent prepared just before use. 50 mg of reagent was dissolved in 100 ml of 50 mM Tris-HCl buffer, pH 7.5, and H_2O_2 added to final concentration 0.025%. Autoradiographies were performed at −80°C on Fuji RX film.

Rocket Immunoelectrophoresis

This quantitative immunological technique [3, 33] and specific staining of aminopeptidase N activity were performed as previously described [14].

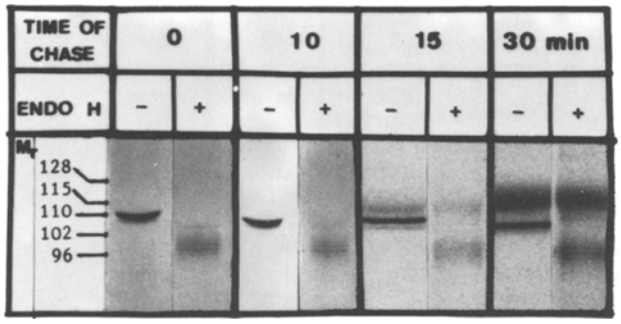

Fig. 1. Endoglycosidase H sensitivity of newly synthesized [^{35}S]-methionine labeled aminopeptidase after different times of chase. 15 μg of aminopeptidase immunoprecipitated from 3% Triton X-100 extract of microsomal fraction were incubated 24 hr at 37°C in citrate buffer pH 5.5 in absence (−) or presence (+) of 10 mU of endoglycosidase H then subjected to SDS-PAGE and fluorography

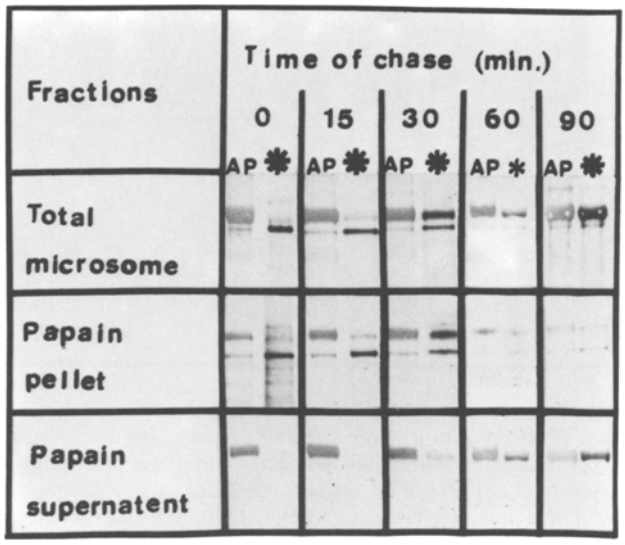

Fig. 2. Papain solubilization from a microsomal fraction of [^{35}S]-methionine-labeled aminopeptidase after different times of chase. After papain treatment (*see* Materials and Methods) the solubilized enzyme was separated from that nonsolubilized by centrifugation. 1 μg of aminopeptidase immunoprecipitated from 3% Triton X-100 extract of total microsomes or from the papain supernatant and 0.4 μg from the 3% Triton X-100 extract of the papain pellet were subjected to SDS-PAGE, transferred to nitrocellulose and immunostained with anti aminopeptidase N (AP column) before autoradiography (* column). The films were exposed for 3 weeks in all cases

Antisera, Immunoglobulins and Immunoabsorbants

Antihuman blood group A antiserum was raised in A$^-$ rabbits by two weekly intravenous injections of 2 ml of a 25% suspension of group A human red blood cells in 0.15 M NaCl [21]. Anti-aminopeptidase sera were obtained as described by Feracci and Maroux [14]. Their specificity has been previously determined [13]. Immunoglobulins and immunoabsorbants were prepared according to Harboe and Ingild [19] and Ternynck and Avrameas [32].

Results

Molecular Forms of Newly Synthesized Aminopeptidase N

Aminopeptidase labeled with [^{35}S]-methionine for 10 min was immunoprecipitated from the microsomal fraction after varying chase times and examined by SDS-polyacrylamide gel electrophoresis (Figs. 1 and 2). The molecular form of the newly synthesized enzyme was shown to change as a function of time.

Until 15 min after chase, the labeled enzyme appeared exclusively as a very thin band with an apparent molecular weight of 110,000. From 15 to 60 min this band disappeared and was replaced by a diffuse band corresponding to an apparent molecular weight of 115,000 to 128,000 which first became visible 15 min after the chase, and then increased in intensity with time. Figure 2 allowed the identification of the lower molecular weight form with a minor band which was revealed by immunoblotting but not by Coomassie Blue staining of polyacrylamide gels, and the diffuse band with the major band of the mature brush-border aminopeptidase. The microheterogeneity of molecular weight observed for this band was assumed to be due to a variability of the sugar moiety also responsible for the microheterogeneity of the isoelectric point [14].

Endoglycosidase H sensitivity of the different forms of [^{35}S]-methionine-labeled aminopeptidase was tested (Fig. 1). The lower molecular weight form was always sensitive to this enzyme whereas the mature form was not. The products of the lower molecular weight form comprised several bands with molecular weights between 96,000 and 102,000. This was probably due to the incomplete cleavage of some glycans in molecules that could still be bound to antibodies. Indeed, when the digestion was carried out on free molecules, as shown in Fig. 5, a homogeneous product was obtained.

Flow Kinetics of Newly Synthesized Aminopeptidase from the Intracellular Pool to the Plasma Membrane

During homogenization the plasma membrane forms closed right-side-out vesicles from which the surface-located hydrophilic head of aminopeptidase bearing the active site and sugars can be released by papain treatment [7, 24]. Membranes of intracellu-

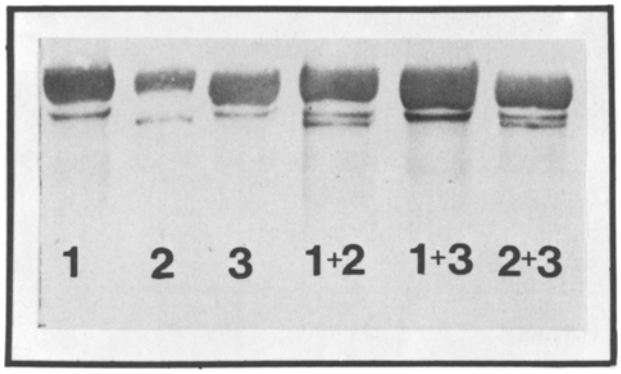

Fig. 3. Electrophoretic migration of the transient endoglycosidase H-sensitive form before and after papain treatment. In (1), 1 μg of aminopeptidase immunoprecipitated from the 3% Triton X-100 extract of the total microsomes. In (2) 0.4 μg of aminopeptidase immunoprecipitated from the 3% Triton X-100 extract of the pellet resulting from papain treatment of microsomes. In (3), 1 μg of aminopeptidase immunoprecipitated from the 3% Triton X-100 extract of total microsomes treated by papain. A better estimation of the differences observed were obtained by mixing the samples. After transfer to nitrocellulose, the aminopeptidase was revealed by immunostaining

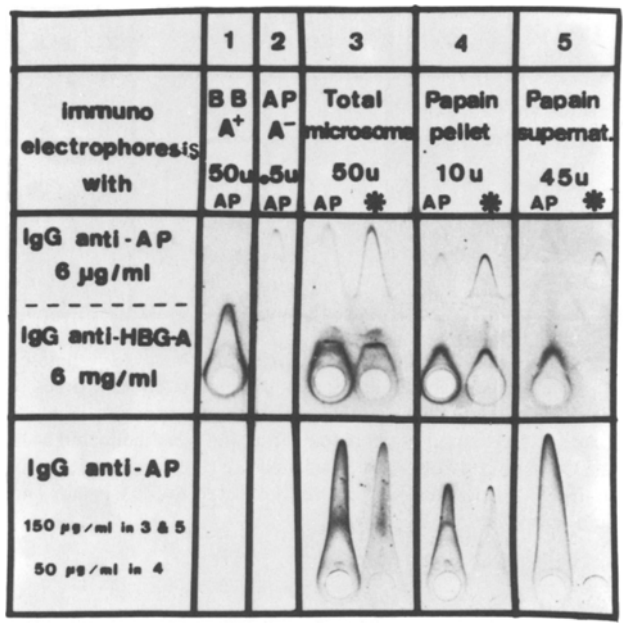

Fig. 4. Separation and specific immunoprecipitation of aminopeptidase devoid of HBG-A determinants by immunoelectrophoresis through agarose gels containing successively anti-HBG-A antibodies and anti-aminopeptidase in concentrations given in the upper part of the figure. Enzyme from the brush-border membrane purified from A^+ rabbit (column 1), from the total microsomes (column 3) and papain pellet (column 4) were solubilized by Triton X-100. AP-A^- (column 2): mature enzyme purified from A^- rabbits. Microsomes were prepared after a pulse-labeling of 10 min. Total aminopeptidase from microsomes and derived fractions after papain treatment were specifically precipitated by anti-aminopeptidase in the lower part of the Figure. Immunoprecipitates were revealed by aminopeptidase activity using chromogenic substrate (AP column). Staining by Coomassie brilliant blue gave the same picture. Labeled enzyme was revealed by autoradiography (* column). The films were exposed for 15 days

lar compartments also form right-side-out vesicles that must contain the newly synthesized aminopeptidase located on the intravesicular side of the membrane and consequently inaccessible to papain. Here, we have used these observations to separate the mature enzyme of the brush border from that still integrated in the intracellular membrane. At 15°C and pH 7.3 where the biomembranes are stable, papain can release 90 to 100% of the aminopeptidase integrated in brush-border and basolateral membrane fractions in 24 hr. Generally, about 10% of the activity remained in the pellet of a papain-treated microsomal fraction. In some cases a second papain treatment of 24 hr was necessary to obtain this value whereas in other rare cases it fell to 3 or 5% after 6 hr. In all cases the transient form of the aminopeptidase was totally found in the papain pellet as shown in Fig. 2. Likewise, Fig. 2 shows that 15 and 30 min after the chase, all the labeled enzyme already processed in N-complex glycan glycoprotein was found in the papain pellet. Its appearance in the supernatant began after 30 min and was almost complete after 60 min.

Papain Proteolysis of the Transient Form

Figure 3 shows that the transient form accumulated in the pellet resulting from the papain treatment of the microsomal fraction migrated faster than before papain proteolysis. The difference observed corresponded to the loss of a fragment of about 3000 daltons. If the papain treatment was performed after solubilization by Triton X-100, this proteolysis did not occur. This suggests that the papain-sensitive peptide bond located at the surface of the membrane vesicles could be masked after solubilization inside the detergent micelles or by conformational change of the molecule in this region.

The A Antigenicity of the Intracellular Pool of Aminopeptidase

The human blood group A (HBG-A) antigenicity is due to the addition of a terminal galactosamine to what are probably O-linked glycan(s) [30]. This addition seems to occur only in the *trans* cisternae of the Golgi stack [2]. So it was interesting to characterize the molecules that had not yet acquired this antigenicity.

We obtained a convenient analytical separation of molecules with or without HBG-A antigenicity by rocket immunoelectrophoresis as described in Fig. 4. The aminopeptidase as well as all other glycoproteins of the samples tested, bearing the HBG-A antigenicity were immunoprecipitated by anti-HBG-A antibodies contained in the lower agarose gel. The aminopeptidase devoid of this antigenicity emerged from this gel and was then specifically immunoprecipitated by anti-aminopeptidase. It was clear that in spite of its large microheterogeneity all the aminopeptidase integrated in the brush-border membrane bore A antigenicity. By contrast a small amount of active aminopeptidase devoid of the HBG-A antigenicity could be detected in the microsomal fraction. It was estimated at 1% of the total aminopeptidase content of this fraction since the peak obtained from 50 units was comparable to that given by 0.5 unit of pure enzyme. This form of the enzyme remained essentially in the pellet after papain treatment. However, a very diffuse peak can sometimes be observed in the papain supernatant. When aminopeptidase was labeled during a 10-min pulse with [^{35}S]-methionine, autoradiography shown in Fig. 4 strongly suggested that all the radioactive transient form of the aminopeptidase was contained in the peak of aminopeptidase devoid of HBG-A antigenicity. Indeed, the radioactivity contained in this peak appeared equivalent to that obtained when the total aminopeptidase was specifically immunoprecipitated by antiaminopeptidase antibodies. Counting of the corresponding peaks confirmed this observation.

The molecular form(s) devoid of HBG-A antigenicity have been definitively characterized by their complete separation from that bearing HBG-A determinants by chromatography through an anti-HBG-A immunoabsorbant column. As shown in Fig. 5*A*, the very low amount of unretarded material can be detected by rocket immunoelectrophoresis through successive anti-HBG-A and anti-aminopeptidase gel that also allowed us to control the efficiency of the column to bind molecules bearing A antigenicity. After concentration, this unretarded material was analyzed on polyacrylamide gel electrophoresis and immunoblotting. Figure 5*B* showed that it contained exclusively the transient endoglycosidase H-sensitive form of aminopeptidase. The ratio of the unretarded to the bound aminopeptidase activity obtained from different pellets of papain-treated microsomes depended on the remaining activity in these pellets but the quantity of the unretarded transient form always correspond to about 1% of the starting microsomal activity. This value agreed very well with that estimated by rocket immunoelectrophoresis in Fig. 4.

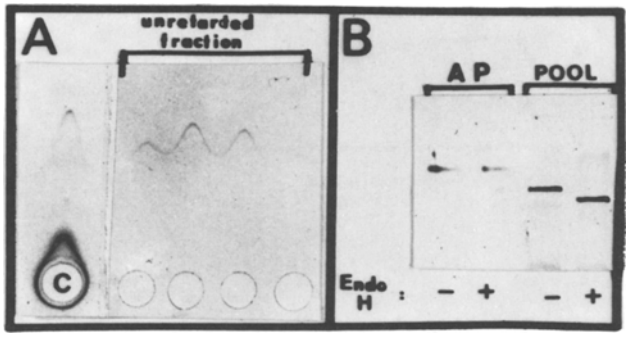

Fig. 5. Characterization of aminopeptidase unretarded on anti-HBG-A immunoabsorbant column. The column (15 ml) was loaded with 0.3 ml of 3% Triton X-100 extract of the pellet resulting from papain treatment of microsomes, containing 270 units of aminopeptidase. Unbound material was eluted during washing of the column with phosphate saline buffer. *A*. Detection of aminopeptidase and control of absence of enzyme-bearing HBG-A determinants in unretarded fractions by rocket immunoelectrophoresis of 20 μl of each fraction in gel containing successively anti HBG-A and anti-aminopeptidase as in the upper part of Fig. 4. Well *c*: 11 μl of the material loaded on the column. The immunoprecipitates were revealed by specific chromogenic substrate. *B*. Analysis by immunoblotting of 0.2 μg of aminopeptidase contained in the pooled unretarded material (pool). Its endoglycosidase H-sensitivity was tested by incubation during 24 hr at 37°C in citrate buffer, pH 5.5, in absence (−) or presence (+) of 10 mU of the glycosidase. As reference 0.1 μg of purified mature aminopeptidase from A$^-$ rabbit (AP)

After correction for the cross activity on the leucine *p*-nitroanilide of the aminopeptidase A [12] contained in the unretarded fraction from the immunoabsorbant column, the same ratio existed between the enzymatic activities and the area of the aminopeptidase peaks obtained by rocket immunoelectrophoresis of the transient and the purified mature forms. This indicated that these two forms had the same specific activity.

Discussion

A pulse-chase labeling study has allowed us to show that the N-glycosylation of the rabbit intestinal aminopeptidase N follows the now classical pathway of synthesis and processing of the asparagine-linked oligosaccharides [22], and allowed us to draw up the kinetic of these events. The cotranslational glycosylation [10] of aminopeptidase synthesized for 10 min, generated a homogeneous transient molecular form with N-linked endoglycosidase H-sensitive glycans assumed to be the high mannose chain, common precursor of all N-linked glycans. This molecular form did not undergo any apparent modifications for at least 10 min (Fig. 1). Its steady-state

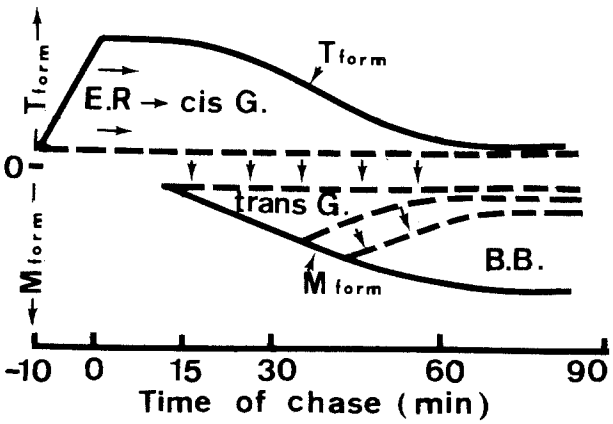

Fig. 6. Kinetic of transformation (full lines) of the transient endoglycosidase H-sensitive form (*T* form) into the mature form (*M* form). Dashed lines delimit areas proportional to the amount of newly synthesized enzyme (*T* form in the upper part and *M* form in the lower part) found at different time of chase in the brush-border membrane (*B.B*) and the intracellular compartments. Considering that the absence of HBG-A antigenicity is characteristic of the *T* form and of the endoplasmic reticulum (*RE*) and *cis* cisternae (*Cis G*) whereas its presence is characteristic of the *M* form and of the *trans* cisternae of the Golgi (*trans G*), we tentatively propose that the *T* form and the *M* form were respectively accumulated in these different compartments and that the limiting rate of the transformation would be set by the transfer (arrows) of the *T* form to the *trans* cisternae of the Golgi. Relative values of *M* and *T* forms and the subcellular localization were estimated from Fig. 2

concentration was sufficient to allow its detection by immunoblotting (Fig. 2). The conversion of its high mannose oligosaccharides into glycans of complex type, endoglycosidase H resistant, occurred between 15 and 60 min after the synthesis. This kinetic of transformation agreed very well with that observed for the rat enzyme [1]. It is difficult to compare this with the results obtained by Danielsen [9] on the porcine aminopeptidase because in that work the chase was defective.

The processing of the high mannose oligosaccharide into glycan of complex type involves first its degradation by specific exoglycosidases then addition to the residual core structure $(GlucNAc)_2$ $(Man)_3$ of N-acetylglucosamine, galactose and fucose residues by specific glycosyl-transferases [22]. The aminopeptidase probably contains both O-linked and N-linked glycans [26] and the synthesis of O-linked glycans also occurs by successive additions of monosaccharides [30]. In spite of these multistep processes, we have not detected by SDS-polyacrylamide gel electrophoresis any corresponding intermediate molecular forms: the first transformed endoglycosidase H-resistant molecules that appeared after 15 min of chase, were indistinguishable from the mature form of the brush border. It was endoglycosidase H-resistant and presented the same microheterogeneity (Figs. 1 and 2).

In the case of rat enzyme, after a labeling of 30 min without chase, an intermediate that migrated with the mature enzyme and was always endoglycosidase H-sensitive, has been detected [1]. It was proposed that the difference in size between this intermediate one and that of lower molecular weight could be due to the O-glycosylation. The expression of the HBG-A antigenicity can be considered as a signal corresponding to the completion of the synthesis of probably O-linked oligosaccharides since the corresponding determinant is the trisaccharide α-Gal NAc-[α-Fuc(1 → 2)]-β-Gal in which the terminal GalNAc is essential [30]. We have tried here, to characterize the transient molecular forms still devoid of this antigenicity. Rocket immunoelectrophoresis through agarose gels containing successively anti-HBG-A antibodies and anti-aminopeptidase N (Fig. 4) permitted a quantitative analysis of a very small amount of this type of molecule in presence of a large quantity of mature enzyme. Complete separation of molecules with A antigenicity from those without the A antigenicity was obtained by passage through an anti-HBG-A immunoabsorbant column. The immunoblotting of the unretarded material revealed the high mannose transient form exclusively (Fig. 5). This molecular form was fully active and its steady-state level was estimated as 1% of the total cellular aminopeptidase. The level of other transient intermediates with incomplete O-linked glycans was very much lower and could not be detected by the very sensitive immunoblotting technique. It can be concluded that the termination of the glycans bearing HBG-A antigenicity occurred in the same very short space of time as the transformation of glycans of high mannose type into complex type.

Much less is known about the biosynthesis of O-linked glycans than is known for N-linked oligosaccharides but in both cases each step of their synthesis and processing could reflect their movement from the rough endoplasmic reticulum through the Golgi stacks [17, 23, 28, 34]. The HBG-A antigenicity seems to be primarily acquired in the *trans* cisternae of the Golgi [2] where the transformation of the N glycans also seems to occur [18, 29, 31]. The newly synthesized endoglycosidase H-resistant molecules bearing A antigenicity that appeared 15 min after the chase were probably accumulated in this subcellular compartment for at least 15 min before its exportation to the plasma membrane that began 30 min after the chase (Fig. 2). This accumulation generated the intracellular pool of mature molecules. Its steady-state level cannot be deduced from the quantity of enzyme remaining in the mem-

brane fraction of microsomes treated by papain because this fraction contained not only all the intracellular pool but certainly also a residual fraction (less than 10%) of enzyme integrated in the plasma membrane. The kinetic data presented in Fig. 2 and summarized in Fig. 6 suggested that this pool must be less large than that of the transient endoglycosidase H-sensitive form since the transformation of this form was slower than the exportation of the resulting mature form. Previously, using subcellular fractionation, we observed an earlier appearance of the newly synthesized aminopeptidase in the basolateral than in the brush-border membrane fraction, probably owing to contamination of these fractions by intracellular membranes [25]. The kinetic of transport to the plasma membrane we have determined here is in good agreement with that of the insertion in the microvilli of the [^{3}H]-fucose-labeled rat sucrase-isomaltase [20]. In contrast a slower transport of aminopeptidase N to the brush border has been described in pig explants [9] and particularly in the rat [1].

In the endoplasmic reticulum and in cisternae of the Golgi where molecules bearing HBG-A antigenicity have not been detected [2], the presence of high mannose glycans had been strongly suggested by specific labeling with Concanavalin A in different cell types [18, 31]. So it seemed highly probable that the transient form of the aminopeptidase was accumulated in one of these subcellular compartments. A rate-limiting transfer to the *trans* cisternae of the Golgi where the transformation occurred, rather a biochemical limiting step, could be responsible for its accumulation.

During the papain treatment of the microsomes we observed (Fig. 3) that the transient form of the aminopeptidase lost a fragment of about 3000 daltons. In right-side-out intracellular membrane vesicles in which this molecule was integrated, only its cytoplasmic part(s) was accessible. Such a cytoplasmic domain has not been detected in the mature brush-border enzyme. Only a N-terminal tetrapeptide has been characterized as the possible cytoplasmic domain [15]. Contrary to the 3000-daltons cytoplasmic part of the transient form, it was released with the adjacent transmembrane domain by papain proteolysis of the detergent-extracted aminopeptidase [14].

Several hypotheses could explain these observations. The more attractive one would be that the 3000-daltons cytoplasmic domain of the transient form could play the role of a "signal" in its intracellular transport and would be cleaved before or when the enzyme reaches the brush border. However, we cannot rule out the possibility that it was present in the mature enzyme integrated in the brush border but was missed in our previous studies. Further experiments are under investigation to specify its N or C position in the transient form and its presence or absence in the mature molecule.

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References

1. Ahnen, D.J., Mircheff, A.K., Santiago, N.A., Yoshioka, Ch., Gray, G.H. 1983. Intestinal surface aminooligopeptidase. Distinct molecular forms during assembly on intracellular membranes "in vivo." *J. Biol. Chem.* **258:**5960–5966
2. Bernadac, A., Gorvel, J.P., Feracci, H., Maroux, S. 1984. Human blood group A-like determinants as marker of the intracellular pools of glycoproteins in secretory and absorbing cells of A^+ rabbit jejunum. *Biol. Cell.* **50:**31–36
3. Bjerrum, O.J., Lundahl, P. 1973. Detergent-containing gels for immunological studies of solubilized erythrocyte membrane components. *In:* A Manual of Quantitative Immunoelectrophoresis. Methods and Applications. N.H. Axelsen, J. Krøll, and B. Weeke, editors. pp. 139–143. Universitetsforlaget, Oslo
4. Blobel, G., Dobberstein, B. 1975. Transfer of proteins across membranes. II. Reconstitution of functional rough microsomes from heterologous components. *J. Cell Biol.* **67:**852–862
5. Bretz, R., Stäubli, W. 1977. Detergent influence on rat liver galactosyltransferase activities towards different acceptors. *Eur. J. Biochem.* **77:**181–192
6. Burnette, N.N. 1981. "Western blotting," electrophoretic transfer of proteins from sodium dodecyl sulfate-polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. *Anal. Biochem.* **112:**195–203
7. Colas, B., Maroux, S. 1980. Simultaneous isolation of brush border and basolateral membrane from rabbit enterocytes. *Biochim. Biophys. Acta* **600:**406–420
8. Coudrier, E., Reggio, H., Louvard, D. 1983. Characterization of an integral membrane glycoprotein associated with the microfilaments of pig intestinal microvilli. *EMBO J.* **2:**469–475
9. Danielsen, M.E. 1982. Biosynthesis of intestinal microvillar proteins. Pulse-chase labelling studies on aminopeptidase N and sucrase isomaltase. *Biochem. J.* **204:**639–645
10. Danielsen, M.E., Norén, O., Sjöström, H. 1983. Biosynthesis of intestinal microvillar proteins. Processing of aminopeptidase N by microsomal membranes. *Biochem. J.* **212:**161–165
11. Eto, Y.M.D., Wiesmann, U.N., Carson, J.H., Herschkowitz, M.D. 1974. Multiple sulfatase deficiencies in cultured skin fibroblasts. *Arch. Neurol.* **30:**153–156
12. Feracci, H., Benajiba, A., Gorvel, J.P., Doumeng, Ch., Maroux, S. 1981. Enzymatic and immunological properties of the protease form of aminopeptidases N and A from pig and rabbit intestinal brush border. *Biochim. Biophys. Acta* **658:**148–157
13. Feracci, H., Bernadac, A., Gorvel, J.P., Maroux, S. 1982. Localization by immunofluorescence and histochemical la-

beling of aminopeptidase N in relation to its biosynthesis in rabbit and pig enterocytes. *Gastroenterology* **82:**317–324
14. Feracci, H., Maroux, S. 1980. Rabbit intestinal aminopeptidase N. Purification and molecular properties. *Biochim. Biophys. Acta* **599:**448–463
15. Feracci, H., Maroux, S., Bonicel, J., Desnuelle, P. 1982. The amino acid sequence of the hydrophobic anchor of rabbit intestinal brush border aminopeptidase N. *Biochim. Biophys. Acta* **684:**133–136
16. Gorvel, J.P., Wisner-Provost, A., Maroux, S. 1982. Identification of glycoproteins bearing human blood group A determinants in rabbit enterocyte plasma membranes. *FEBS Lett.* **143:**17–20
17. Green, J., Griffiths, G., Louvard, D., Quinn, P., Warren, G. 1981. Passage of viral membrane proteins through the Golgi complex. *J. Mol. Biol.* **152:**663–698
18. Griffiths, G., Brands, R., Burke, B., Louvard, D., Warren, G. 1982. Viral membrane proteins acquire galactose in *trans* Golgi cisternae during intracellular transport. *J. Cell Biol.* **95:**781–792
19. Harboe, N., Ingild, A. 1973. Immunization, isolation of immunoglobulins, estimation of antibody titre. *In:* A Manual of Quantitative Electrophoresis. Methods and Applications. N.H. Axelsen, J. Krøll, and B. Weeks, editors. pp. 161–164. Universtetsforlaget, Oslo
20. Hauri, H.P., Quaroni, A., Isselbacher, K. 1979. Biogenesis of intestinal plasma membrane: Posttranslational route and cleavage of sucrase isomaltase. *Proc. Natl. Acad. Sci. USA* **76:**5183–5186
21. Hinglais, N., Bretton, R., Rouchon, M., Oriol, R., Bariety, J. 1981. Ultrastructural localization of blood group-A antigen in normal human kidneys. *J. Ultrastruct. Res.* **74:**34–45
22. Hubbard, S.C., Ivatt, R.J. 1981. Synthesis and processing of asparagine-linked oligosaccharides. *Annu. Rev. Biochem.* **50:**555–583
23. Johnson, D.C., Spear, P.G. 1983. O-linked oligosaccharides are acquired by Herpes simplex virus glycoproteins in the Golgi apparatus. *Cell* **32:**987–997
24. Louvard, D., Maroux, S., Vannier, Ch., Desnuelle, P. 1975. Topological studies on the hydrolases bound to the intestinal brush border membrane. I. Solubilization by papain and Triton X 100. *Biochim. Biophys. Acta* **375:**236–248
25. Maroux, S., Feracci, H. 1983. Structure and biosynthesis of aminopeptidases. *Methods Enzymol.* **96:**406–423
26. Maroux, S., Feracci, H., Gorvel, J.P., Benajiba, A. 1983. Aminopeptidases and proteolipids of intestinal brush border. *In:* Brush Border Membranes. Ciba Foundation Symposium 95. pp. 34–44. Pitman, London
27. Maroux, S., Louvard, D., Baratti, J. 1973. The aminopeptidase from hog intestinal brush-border. *Biochim. Biophys. Acta* **321:**282–295
28. Michaels, J.E., Leblond, C.P. 1976. Transport of glycoproteins from Golgi apparatus to cell surface by means of "carrier" vesicles, as shown by radioautography of mouse colonic epithelium after injection of ^{3}H-Fucose. *J. Microsc. Biol. Cell.* **25:**243–248
29. Quinn, P., Griffiths, G., Warren, G. 1983. Dissection of the Golgi complex. II. Density separation of specific Golgi functions in virally infected cells treated with monensin. *J. Cell Biol.* **96:**851–856
30. Schachter, H., Roseman, S. 1981. Mammalian glycosyltransferases. *In:* The Biochemistry of Glycoproteins and Proteoglycans. W.J. Lennartz, editor. pp. 85–160. Plenum, New York
31. Tartakoff, A.M., Vassalli, P. 1983. Lectin-binding sites as markers of Golgi subcompartments: Proximal-to-distal maturation of oligosaccharides. *J. Cell Biol.* **97:**1243–1248
32. Ternynck, T., Avrameas, S. 1972. Polyacrylamide-protein immunoabsorbants prepared with glutaraldehyde. *FEBS Lett.* **23:**24–28
33. Weeke, B. 1973. Rocket immunoelectrophoresis *In:* A Manual of Quantitative Electrophoresis. Methods and Applications. N.H. Axelsen, J. Krøll and B. Weeke, editors. pp. 37–56. Universtetsforlaget, Oslo
34. Whur, P., Herscovics, A., Leblond, C.P. 1969. Radioautographic visualization of the incorporation of galactose ^{3}H and mannose ^{3}H by rat thyroids *in vitro* in relation to the stages of thyroglobulin synthesis. *J. Cell Biol.* **43:**289–311

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