The carbohydrate moiety of aminopeptidase N of rabbit intestinal brush-border membrane

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Endoglycosidase F was used to eliminate the *N*-linked complex glycans from intestinal aminopeptidase N. The glycans which were probably *O*-linked remaining after the endoglycosidase F treatment exhibited the human blood group A and H determinants expressed in enzymes from A^+ or A^- rabbits, respectively. The molecular mass estimation of the two types of glycans by SDS-polyacrylamide gel electrophoresis and the sugar composition of aminopeptidase from A^+ and A^- rabbits strongly suggested the presence of eight *N*-linked complex glycans and two *O*-linked oligosaccharides bearing the human group antigenicity.

Aminopeptidase N *A antigenicity* *H antigenicity* *Membrane glycoprotein* *Brush border* *Endoglycosidase F*

1. INTRODUCTION

Intestinal aminopeptidase N is a highly glycosylated membrane glycoprotein exhibiting human blood group A antigenicity in A^+ animals [1,2].

Biosynthesis of an endoglycosidase-H sensitive intermediate of glycosylation that is characteristic of N-glycosylated glycoproteins [3] has been recently reported [4]. In A^+ rabbits, this transient form (T form) is devoid of human blood group A determinants. These determinants are primarily acquired in the trans cisternae of the Golgi apparatus at the same time as the processing of endoglycosidase H-sensitive high mannose glycans into complex glycans [4,5].

Most of the work concerning the structure of human blood group determinants in glycoproteins has been carried out on soluble secreted glycoprotein in particular mucins [6–8]. The A and H determinants have been found to have the following respective structures: α-GalNAc-[α-Fuc(1⟶2)]-βGal and αFuc(1⟶2)-βGal-βGlcNAc, terminal trisaccharides of oligosaccharides with different lengths, *O*-glycosycally conjugated to protein through the linkage α-GalNAc-(1⟶3)Ser or Thr. ABH human blood group determinants have also been shown to exist in glycoproteins of human erythrocytes [9–11]. These were thought to be the terminal trisaccharides of heterogeneous high M_r (3000–11000) glycans with the repeating structure βGal-βGlcNAc. The type of linkage of these glycans to the proteins has not been clearly established but could be of the *N*-glycosylic type.

The aim of this investigation was to establish which type of glycan bears the human blood group in the aminopeptidase N.

2. EXPERIMENTAL

Lectin 1 from *Ulex europeous* and fucosyl-peroxidase were from IBF (Villeneuve-la-Garenne) and endoglycosidase H from Miles.

The *Flavobacterium meningosepticum* strain was a gift from Dr Rosenweig and Dr Jamieson (Yale University, Connecticut). Endoglycosidase F was prepared as described by Elder and Alexander [12] from supernatant of 4 l of stationary phase *F. meningosepticum* culture. Absorbance of the fraction used here was 0.65 at 280 nm.

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Before endoglycosidase F treatment, the various forms of aminopeptidase (0.15 mg per ml of 10 mM phosphate buffer, pH 7.2, containing 0.15 M NaCl and 50 mM EDTA) were treated for 3 min at 100°C. Then, samples containing 1 μg of enzymes were treated for 6 h at 37°C with 3.5 μl endoglycosidase F in a final volume of 20 μl of 10 mM phosphate buffer (pH 7.2) containing 0.15 M NaCl and 50 mM EDTA.

Endoglycosidase H treatment, antisera production and immunoblotting technique with anti-aminopeptidase and anti-human blood group A antisera have been described in [4]. Human blood group H determinants were revealed by *U. europeous* lectin [13] by performing successive incubations of blots on nitrocellulose with lectin at a concentration of 20 μg per ml and then with fucosyl-peroxidase at a concentration of 0.1 mg per ml. Bovine sera albumin was taken here instead of the total bovine serum as used in the incubation medium.

The aminopeptidase N (M form) from A^+ and A^- type rabbits [1] was prepared as in [14]. The endoglycosidase H-sensitive T form of aminopeptidase was extracted from a Golgi and endoplasmic reticulum enriched fraction of intestinal mucosa of A^+ rabbit (in preparation). Fifteen mg membrane proteins were incubated for 2 h at 4°C in 1 ml of 10 mM phosphate buffer (pH 7.2) containing 0.15 M NaCl, 5 mM cysteine, 0.03 mM dithiothreitol, 2% Triton X-114 and 1.5 mg papain. The papain hydrolysis was stopped by adding 20 μl of 0.5 M *N*-ethylmaleimide. The unsolubilized material was eliminated by centrifugation at 15000 $\times$ *g* for 30 min in an angle rotor head at 4°C in a refrigerated Sigma 2 MK centrifuge. The supernatant was brought up to 37°C and the detergent and aqueous phases were separated as described by Bordier [15]. The solubilized aminopeptidase was found in the aqueous phase. The T and M forms were then separated in an anti-human blood group A-immunoabsorbent column as in [4].

The carbohydrate composition was determined by Dr Strecker at the Biological Chemistry Laboratory of the University of Lille (France) as in [16]. On the basis of a simultaneous estimation of *N*-acetylglucosamine and amino acids conducted previously with an automatic amino acid analyzer [14], it was possible to calculate the molecular composition.

3. RESULTS AND DISCUSSION

Endoglycosidase F (endo F) cleaves *N*-linked glycans of both the high-mannose and the complex type through hydrolysis of the glycosidic bond of the adjacent *N*-acetylglucosamine residues linking the glycan moiety to the asparagine of the protein backbone [12]. This glycosidic bond is cleaved by endoglycosidase H (endo H) only in the case of high-mannose glycans [17].

The action of these two endoglycosidases on the T and M forms of aminopeptidase was studied. Fig.1 shows that both had the same action on the T form suggesting that *N*-linked glycans of the T form were strictly of the high-mannose type. Although endo H had no effect on the M form [4], its apparent molecular mass changed considerably as a result of endo F treatment.

Much less is known about the biosynthesis of *O*-linked glycans than of the *N*-linked oligosaccharides. It seems likely, however, that only addition of *N*-acetylgalactosamine residues to Ser or Thr residues could precede the conversion of *N*-linked high-mannose type glycan into the complex type [18,19]. So the T form (M_r 106000) can only contain *N*-linked high-mannose glycans and after

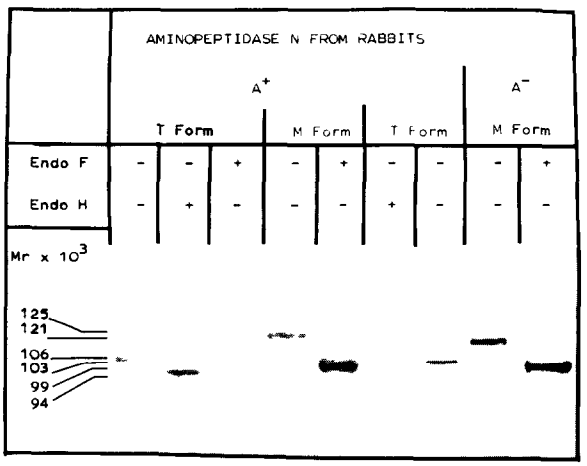

Fig.1. Endoglycosidase H and F sensitivity of the transient form of glycosylation (T form) and mature form (M form) in the intestinal aminopeptidase N from A^+ and A^- rabbits; 0.2 μg of enzymes before (−), and 0.4 μg of enzymes after (+) endoglycosidase treatment were subjected to SDS–PAGE, transferred to nitrocellulose and immunostained with anti-aminopeptidase N.

endoglycosidase treatments the molecular form obtained (M_r 99000) must correspond to the protein backbone with only one *N*-acetylglucosamine on the sites of *N*-glycosylation and at most one *N*-acetylgalactosamine on the sites of *O*-glycosylation. If the M form of aminopeptidase contained only *N*-linked glycans, the molecular form obtained after endo F treatment would be identical to the endoglycosidase treated T form. Fig.1 shows that this was not the case. Even when more drastic conditions were used for endo F treatment, the apparent M_r of the treated M form was always higher than that of the treated T form. This result shows the presence on the M form of glycan(s) that are resistant to the endo F action and hence probably *O*-linked. Fig.2 shows that these glycans exhibit the human blood group A or H determinants in enzymes from A^+ and A^- rabbits, respectively. The thickness of the band corresponding to the endo F treated M form indicates considerable heterogeneity among these glycans. In view of the apparent M_r corresponding to the middle of the band (103000) and that of the protein backbone (99000), the average total M_r of glycans bearing human blood group determinants can be estimated to be 4000. This value is low compared

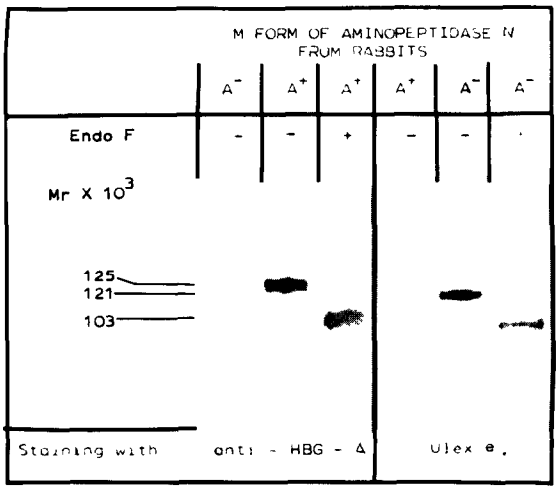

Fig.2. Evidence for the presence of human blood group A and H determinants in endoglycosidase F resistant glycans of aminopeptidase from A^+ and A^- rabbits, respectively. The same blottings as in fig.1 were immunostained with anti-human blood group A (HBG-A) antiserum or *Ulex europeus* lectin as indicated at the bottom of the figure.

to the value of 22000–18000 attributable to the *N*-linked glycans hydrolyzed by endo F. Estimations of the M_r of the glycan moiety based on the electrophoresis on polyacrylamide gel technique require further confirmation [18]. Fig.1 and 2 show that this technique yields different M_r values for enzymes from A^+ and A^- rabbits (125000 and 121000, respectively). This difference cannot be explained by their sugar composition, which is given in table 1, but is probably due to a difference in the structure of their glycans.

However, these sugar compositions were quite compatible with the proportion of *N*- and *O*-linked glycans given above. Indeed, considering that mannose is exclusively present in the *N*-linked glycans and that in the complex type, only 3 residues remain in the core structure, this composition suggests the presence of eight *N*-linked complex glycans. *N*-Acetylgalactosamine is mainly found in the *O*-linked glycans where it is involved in the linkage with the Ser or Thr residues of the protein [20]. Moreover, it is the terminal additional residue that converts the H antigenicity into A antigenicity [6]. Comparison between the *N*-acetylgalactosamine content of aminopeptidase from A^+ and A^- rabbits strongly suggests that the enzyme contains two *O*-linked glycans containing the A antigenicity in A^+ rabbits.

Human blood group determinants have been shown to exist in several membrane glycoproteins [2,21]. The characterization of glycans bearing these antigenic determinants is important for studies on the sites of protein glycosylation in cells since the expression of these determinants can

Table 1

Sugar composition (residues/mol) of aminopeptidase N from A^+ and A^- rabbits

	A^+ rabbit	A^- rabbit
Galactose	23	22
Glucose	4	4
Mannose	25	25
Fucose	16	21
N-Acetylglucosamine	38	38
N-Acetylgalactosamine	4	2
Amino acids[a]	988	988

[a] From Feracci and Maroux [14]

serve to signal the completion of the synthesis of the bearer glycan.

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REFERENCES

[1] Feracci, H., Bernadac, A., Gorvel, J.-P. and Maroux, S. (1982) Gastroenterology 82, 317–324.
[2] Gorvel, J.-P., Wisner-Provost, A. and Maroux, S. (1982) FEBS Lett. 143, 17–20.
[3] Hubbard, S.C. and Ivatt, R.J. (1981) Annu. Rev. Biochem. 50, 555–583.
[4] Feracci, H., Rigal, A. and Maroux, S. (1985) J. Membr. Biol., in press.
[5] Bernadac, A., Gorvel, J.-P., Feracci, H. and Maroux, S. (1984) Biol. Cell 50, 31–36.
[6] Watkins, W.H. (1972) in: Glycoproteins (Gottschalk, A. ed.) pp.830–891, Biochim. Biophys. Acta Library, vol.5, part B.
[7] Slomiany, B.L., Murty, V.L.N. and Slomiany, A. (1980) J. Biol. Chem. 255, 9719–9723.
[8] Van Halbeck, H., Dorland, L., Haverkamp, J., Veldink, G.A., Vliegenthart, J.F.G., Fournet, B., Ricart, G., Montreuil, J., Gathmann, W.D. and Aminoff, D. (1981) Eur. J. Biochem. 118, 487–495.
[9] Järnefelt, J., Rush, J., Yu-Tech Li and Laine, R.A. (1978) J. Biol. Chem. 253, 8006–8009.
[10] Tsuji, T., Irimura, T. and Osawa, T. (1980) Biochem. J. 187, 677–686.
[11] Viitala, J., Karhi, K.K., Gahmberg, C.G., Finne, J., Järnefelt, J., Myllylä, G. and Krusius, T. (1981) Eur. J. Biochem. 113, 259–265.
[12] Elder, J.H. and Alexander, S. (1982) Proc. Natl. Acad. Sci. USA 79, 4540–4544.
[13] Perieira, M.E.A., Kisailus, E.C., Gruezo, F. and Kabat, E.A. (1978) Arch. Biochem. Biophys. 185, 108–115.
[14] Feracci, H. and Maroux, S. (1980) Biochim. Biophys. Acta 599, 448–463.
[15] Bordier, C. (1981) J. Biol. Chem. 256, 1604–1607.
[16] Fournet, B., Montreuil, J., Strecker, G., Dorland, L., Haverkamp, J., Vliegenthart, J., Binette, J.P. and Schmid, K. (1978) Biochemistry 17, 5206–5214.
[17] Kobata, A. (1979) Anal. Biochem. 100, 1–14.
[18] Cummings, R.D., Kornfeld, S., Schneider, N.J., Hodgod, K.K., Tolleshang, H., Brown, M.S. and Goldstein, J.L. (1983) J. Biol. Chem. 258, 15261–15273.
[19] Roth, J. (1984) J. Cell. Biol. 98, 399–406.
[20] Montreuil, J. (1980) Adv. Carbohydr. Chem. Biochem. 37, 157–223.
[21] Gorvel, J.-P., Rigal, A., Sarles, J. and Maroux, S. (1985) Cell Tissue Res., in press.