Volume 305, number 2, 129–132 FEBS 11217 June 1992

Characterization of carbohydrates in the α_2-macroglobulin receptor

Poul H. Jensen[a], Jørgen Gliemann[a] and Torben Ørntoft[b]

[a]*Institute of Medical Biochemistry, University of Aarhus, and* [b]*Department of Clinical Chemistry, University Hospital of Aarhus, DK-8000 Aarhus C, Denmark*

Received 8 May 1992

Carbohydrates were characterized in the human placental α_2-macroglobulin receptor and its associated protein. Carbohydrates, largely N-linked, contributed to about 18% of the size of the receptor α-chain and to about 25% of the β-chain. The 40 kDa receptor-associated protein also contained carbohydrate. The α- and β-chains contained a wide variety of carbohydrates as judged by binding of lectins. Monosaccharide-competing inhibition of α_2M-methylamine binding by WGA suggested a functional significance of sugars in binding of ligands to the α-chain.

α_2-Macroglobulin receptor; Low density lipoprotein receptor-related protein; Glycosylation; Lectin

1. INTRODUCTION

The α_2-macroglobulin receptor (α_2MR) [1–3], identical with the low density lipoprotein receptor-related protein (LRP) [4,5], is a glycoprotein with an approximately 500 kDa ligand binding α-chain and a 85 kDa membrane spanning β-chain [4–7]. This multifunctional receptor binds α_2M-proteinase complexes and α_2M-methylamine [7], and β-migrating very low density lipoprotein activated by enrichment with apolipoprotein E [8]. Moreover, it binds a 40 kDa [1,5,7] or 39 kDa [3,4,8] α_2MR-associated protein (α_2MRAP) copurifying with the receptor when using α_2M-methylamine affinity chromatography [1,3]. α_2MRAP modulates binding of α_2M-proteinase complexes [7] as well as lipoproteins [8].

α_2MR is synthesized as single chain precursor containing high mannose N-linked carbohydrates sensitive to Endo-β-*N*-acetylglucosaminidase H (Endo-H). In cultured cells, cleavage to the two chain form occurs at about the same time that the carbohydrates are converted to the endo H-resistant form [6]. The mature α_2MR contains terminal sialic acid in both chains [6], and the α-chain contains about 150 glucosamine residues per molecule [9].

The purpose of the present report was to further characterize the carbohydrates of the affinity purified human placental α_2MR preparation and their possible significance for binding of α_2M-proteinase and α_2MRAP.

Abbreviations: α_2M, α_2-macroglobulin; α_2MR, α_2-macroglobulin receptor; α_2MRAP; α_2-macroglobulin receptor-associated protein; LRP, low density lipoprotein receptor-related protein; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; *O*-glycanase, endo-α-*N*-acetylgalactosaminidase, Endo-H, endo-β-*N*-acetylglucosaminidase H; PNGase-F, peptide *N*-glycohydrolase F. Lectins (agglutinins): ACA, amarantus caudatus; PNA, arachis hypogeae; BS-I, bandeiraea simplicifolia I; ConA, canavalia ensiformis; DSA, datura stramonium; ECG, erythrina crista galli; GNA, galanthus nivalis; LCA, lens culinaris; MAA, maackia amurensis; SNA, sambucus nigra; WGA, triticum vulgaris; PWM, phytolacca americana; VVA, vicia villosa.

Correspondence address: P.H. Jensen, Institute of Medical Biochemistry, University of Aarhus, Universitetsparken, DK-8000 Aarhus C, Denmark. Fax: (45) (86) 131160.

2. MATERIALS AND METHODS

2.1. *Reagents*

Lectins – native, biotinylated and horseradish peroxidase conjugated – were from Sigma (USA) [Canavalia ensiformis (ConA), Triticum vulgaris (WGA), Arachis hypogaea (PNA), Lens culinaris (LCA), Erythrina crista galli (ECG), Bandeiraea simplicifolia-I (BS-I)], Boehringer Mannheim (Germany) [Galanthus nivalis (GNA), Sambucus nigra (SNA), Maackia amurensis (MAA)], Pharmavit (Copenhagen, Denmark) [Amarantus caudatus agglutinin (ACA)] and United States Biochemicals (USA) [Vicia villosa (VVA)]. The Glycan detection kit, arthrobacter ureafaciens neuraminidase (EC 3.2.1.18), peptide-*N*-glycosidase F (PNGase-F) (EC 3.2.2.18), endo-α-*N*-acetylgalactosaminidase (O-glycanase) (EC 3.2.1.97) and Endo-β-*N*-acetylglucosaminidase H (Endo-H) (EC 3.2.1.96) were from Boehringer Mannheim.

Human α_2M was prepared [10], made receptor-active by incubation with methylamine [1] and iodinated [11]. The human placental α_2MR preparation (containing α_2MRAP) was produced from detergent solubilized third trimester placental membranes by affinity chromatography using Sepharose-immobilized α_2M-methylamine [1]. Two chain α_2MR was prepared from the α_2MR preparation by heparin-Sepharose (Pharmacia, Sweden) chromatography, which removes α_2MRAP [7]. α_2MRAP was purified from Mono-S (Pharmacia) cation exchange chromatography of the α_2MR preparation and iodinated using chloramine-T [7].

2.2. *Glycosidase treatment*

Purified α_2MR preparation (2–20 μg) in 10 μl 1% SDS was heated to 95°C for 3 min, diluted with 90 μl 20 mM NaH_2PO_4, pH 7.2, 10 mM EDTA, 10 mM Na-azide, 0.5% Nonidet P-40, reheated and fi-

nally incubated with glycosidases for 18 h at 37°C using enzyme concentrations of 0.2 U/ml (neuraminidase), 4 U/ml (PNGase-F), 16 mU/ml (Endo-H) and 10 mU/ml (O-glycanase). The incubations were terminated by heating in 2% SDS-Laemmli sample buffer containing 20 mM dithioerythritol [12].

2.3. *Electrophoresis and electroblotting*

Native or glycosidase-treated α_2MR preparation was resolved by 4–16% or 8–16% gradient SDS-PAGE [1] and electroblotted onto polyvinylidene difluoride membranes (Immobilon-P) from Millipore (USA) at 200 mA for 2 h in 25 mM Tris, 192 mM glycine, pH 8.50. The membrane was blocked by a 2 h incubation at room temperature in 140 mM NaCl, 10 mM NaH_2PO_4, 1% Tween-20, pH 7.40. Molecular weight markers (Sigma, USA): 180 kDa, α_2M; 116 kDa, β-galactosidase; 84 kDa, fructose-6-phosphate kinase; 58 kDa, pyruvate kinase; 48.5 kDa, fumarase; 36.5 kDa lactic dehydrogenase and 26.6 kDa, triose-phosphate isomerase. Glycans were detected by an enzyme immunoassay after oxidation of adjacent hydroxyl groups in sugars, followed by covalent binding of spacer-linked digoxigenin according to the manufacturer (Boehringer Mannheim). Immunostaining was performed using a rabbit anti-α_2MR serum (1:1500) that binds to the α_2MR α-chain, β-chain and the α_2MRAP [7], and horseradish peroxidase-conjugated porcine anti-rabbit IgG. Lectin binding to electroblotted α_2MR was performed with approximately 50 μg/ml of the peroxidase-conjugated lectins WGA, PWM, DSA, ConA, LCA, PNA, ACA, ECG, BS-I, VVA or the biotinylated lectins GNA, MAA, SNA, and reacted with horseradish peroxidase-conjugated streptavidin (Amersham, UK).

2.4. *Effect of lectins on binding of ligands to α_2MR*

The buffer contained 140 mM NaCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, pH 7.8. Two chain α_2MR was immobilized on nitrocellulose discs [9] and incubated in 200 μl buffer in the absence or presence of unconjugated lectins at 4°C for 4 h. The discs were washed in 3×1 ml buffer to remove excess lectin, followed by incubation with labeled ligand (10,000 cpm/ml) for 16 h at 4°C in buffer supplemented with 1% bovine serum albumin.

3. RESULTS

Fig. 1, lanes 1 vs. 2 confirm that both chains of α_2MR contain carbohydrates, and show for the first time that α_2MRAP is a glycoprotein. Lanes 3 vs 4 show that terminal sialic acid contributes to about 20 kDa of the α-chain and 5 kDa of the β-chain, whereas neuraminidase had no detectable effect on the mobility of α_2MRAP. Lanes 3 vs. 5 demonstrate that PNGase-F, capable of cleaving high mannose as well as hybrid and complex N-linked glycans, reduced the α-chain by 90 kDa and the β-chain by about 20 kDa. The effect of PNGase-F treatment on the β-chain is further demonstrated in lanes 3a vs. 5a. The fast migrating β-chain band in the non-treated α_2MR preparation (lane 3a), previously noted in [5], might represent a non-glycosylated species since PNGase-F treatment converted all the β-chain immunoreactivity to this band (lane 5a). This treatment also caused a small reduction (about 2.5 kDa) in the apparent size of α_2MRAP (lanes 3a vs. 5a).

Fig. 1, lanes 6 vs. 7 show that Endo-H, capable of cleaving high mannose N-linked glycans not possessing terminal sialic acid, did cause a reduction in the size of the β-chain. A similar minor effect was seen with the α-chain (not shown). No significant reduction in size of either chain was observed after treatment with *O*-glycanase (data not shown). However, binding of PNA, particularly to the β-chain, was reduced following treatment with neuraminidase plus *O*-glycanase (lane 9) as compared with neuraminidase alone (lane 8). No PNA binding sites were exposed on either of the receptor chains without neuraminidase treatment (not shown). After removal of sialic acid, PNA binds preferentially to the core disaccharide in mucin-type O-linked glycans [13]. Both α- and β-chains may therefore contain small amounts of O-linked carbohydrates.

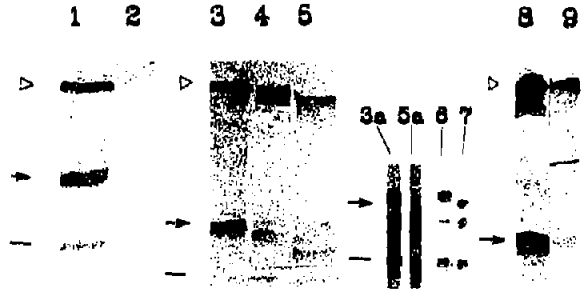

Fig. 1. Carbohydrate analysis of the α_2MR preparation. Native or glycosidase treated α_2MR preparation (about 0.5 μg) was resolved by SDS gradient PAGE under reducing conditions (4–16% polyacrylamide, lanes 1–5, 8, 9; 8–16% polyacrylamide, lanes 3a, 5a, 6, 7), electroblotted and analysed by glycan detection (lane 1), immunodetection using rabbit anti-α_2MR serum (lanes 3–7) and PNA binding (lanes 8 and 9). Lane 2 shows non-glycosylated *E. coli* creatinase (3 μg) as a negative control for glycan detection. Deglycosylation prior to the electrophoresis was performed with neuraminidase (lane 4), PNGase F (lanes 5, 5a) or Endo-H (lane 7) with the non-treated controls in lanes 3, 3a and 6. The binding of peroxidase-conjugated PNA shown in lanes 8 and 9 was performed on electroblotted receptor treated on the Immobilon-P membrane with neuraminidase (lane 8) or neuraminidase plus *O*-glycanase (lane 9). The positions of the α-chain (open triangle), the β-chain (arrowhead) and the α_2MRAP (bar) are indicated.

Table I displays the binding of a series of lectins to the components of the receptor preparation. The β-chain bound all lectins suggesting a broad spectrum of glycans. Both chains bound GNA confirming the presence of high mannose glycans. ACA did not bind to the α-chain suggesting the absence of α-anomeric Galβ(1–3)GalNAc [14]. A further characterization of the carbohydrate structures was attempted by testing a panel of monoclonal antibodies raised against lacto-series carbohydrate structures (Lewis antigens types a, b, x, y) [15]. However, none of the monoclonals bound to the α_2MR α- or β-chain or to α_2MRAP. WGA and ConA bound to α_2MRAP (Table I) confirming its glycoprotein nature.

We next asked the question whether the carbohydrate chains might modulate ligand binding. We were unable to obtain reliable data from experiments designed to remove the carbohydrates since the necessary incubations at 37°C caused marked reductions in ligand binding even in the absence of enzyme (not shown). Fig. 2, upper panel, shows that WGA inhibited binding of α_2M-methylamine to two chain α_2MR by about 30% in

a *N*-acetylglucosamine (and *N*-acetylgalactosamine, not shown) competing way. Glucose, galactose and α-methyl-mannoside did not prevent the inhibitory effect of WGA (not shown). The effect was caused by WGA binding to the receptor since free lectin was removed before the addition of labeled α_2M-methylamine. Fig. 2, lower panel, shows that WGA inhibited the binding of α_2MRAP by about 40% in a monosaccharide competable way. None of the other lectins displayed in Table I showed significant inhibitions as exemplified in Fig. 2 for SNA and LCA, but ConA caused a doubling of α_2M-methylamine binding (not shown).

4. DISCUSSION

The present results show that a wide variety of glycans contribute to about 25% of the size of the β-chain. The contribution of about 18% in the α-chain is in agreement with previous estimates based on the content of glucosamine [9]. We show for the first time that α_2MRAP is *N*-glycosylated, probably at Asn^{234} which is the only potential *N*-glycosylation site [16].

The potentiating effect of ConA on α_2M-methylamine binding is most likely related to its tetravalency and agglutinating properties since ConA binds both to α_2MR and to α_2M (unpublished observation) and since LCA with similar specificities [17] had no effect. WGA, here reported to inhibit α_2M-methylamine binding, has previously been observed to inhibit binding of platelet derived growth factor [18], insulin [19] and basic fibroblast growth factor [20] to their receptors by 50–70%. N-Linked sugars are thought to be functionally important for the basic fibroblast growth factor receptor since removal decreases binding dramatically and since WGA inhibits both binding and biological activity [20]. The inhibitory effect of WGA on the binding of both α_2M-methylamine and α_2MRAP to α_2MR is in agreement with the reported partial cross-inhibition by the two ligands in radioligand binding studies [7] suggesting that they bind to overlapping or neighbouring sites.

Table I

Lectin binding to α2MR α-chain, β-chain and α2MRAP

	Carbohydrate specificity*	α-chain	α-chain	α_2MRAP
WGA	GlcNAcβ(1–4)$_n$	+	+	+
PWM	(GlcNAcβ(1–4)GlcNAc)$_n$	+	+	−
DSA	(GlcNAcβ(1–4)GlcNAc)$_2$	+	+	−
ConA	αMan > αGlc	+	+	+
LCA	αMan > αGlc > αGlcNAc	+	+	−
GNA	Manα (1–3 > 6 > 2)Man	+	+	−
PNA	Galβ(1–3)GalNAc	+	+	−
ACA	Galβ(1–3)GalNAc	−	+	−
ECG	Galβ(1–4)GlcNAc	+	+	−
BS-I	αGal, αGalNAc	+	+	−
VVA	GalNAc	+	+	−
MAA	NeuNAcα(2–3)Gal	+	+	−
SNA	NeuNAcα(2–6)Gal	+	+	−

The α_2MR preparation (approximately 0.5 μg), resolved by SDS-PAGE and electroblotted onto Immobilon-P membranes, was incubated with 50 μg/ml each of the peroxidase-labeled lectins; WGA, PWM, DSA, ConA, LCA, PNA, ACA, ECG, BS-I, VVA and the biotinylated lectins GNA, MAA and SNA. Both native and neuraminidase-treated receptor preparations were tested. Each membrane was probed by rabbit anti-α_2MR serum to localize the α-chain, the β-chain and the α_2MRAP. The data are combined from 2 or more experiments with each lectin.

*The lectins might bind to other carbohydrate structures or monosaccharides than those listed.

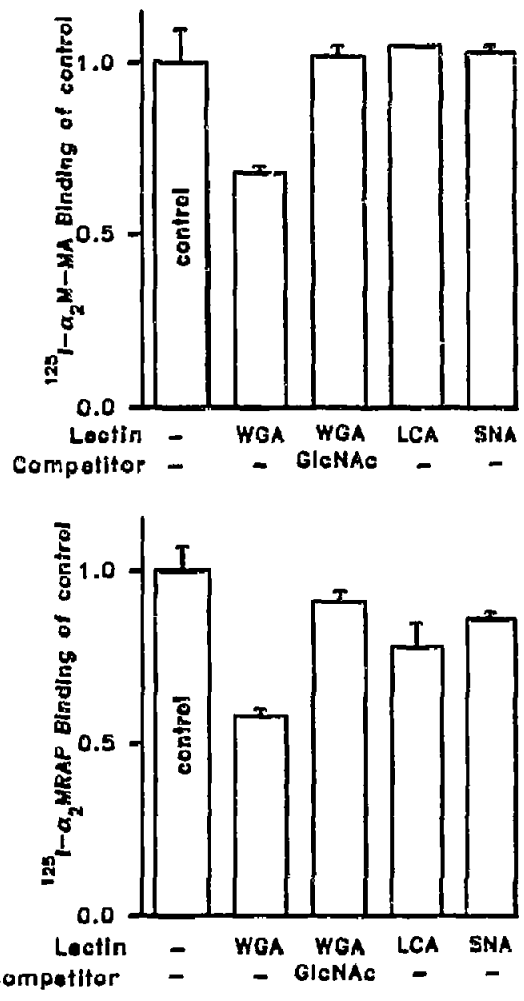

Fig. 2. Effect of lectins on the ligand binding to immobilized two chain α_2MR. The lectins (50 μg/ml) and monosaccharides included in the incubation are indicated below the bars. *Upper panel*: α_2MR immobilized on nitrocellulose discs was preincubated with the lectin for 4 h at 4°C in the absence or presence of 100 mM competing monosaccharide, washed and incubated with 8 pM [^{125}I]α_2M-methylamine at 4°C for 16 h. One of four similar experiments is presented. *Lower panel*: the assay was performed with the lectins present during the incubation with [^{125}I]α_2MRAP (10,000 cpm/ml). One of three similar experiments is presented. Non-saturable binding, measured in the presence of unlabeled α_2M-methylamine (10 μg/ml) or α_2MRAP (5 μg/ml), is subtracted from all values. Data are presented as mean values ± 1 S.D. of triplicate determinations with the control binding in the absence of lectin set at 1.0.

Acknowledgement: This work was supported by grants from the Danish Cancer Society, The Danish Biomembrane Research Centre, Direktør Jakob Madsen og hustru Olga Madsens Fond. Drs. Søren K. Moestrup and Anders Nykjær are thanked for valuable discussions.

REFERENCES

[1] Jensen, P.H., Moestrup, S.K. and Gliemann, J. (1989) FEBS Lett. 255, 275–280.
[2] Moestrup, S.K. and Gliemann, J. (1989) J. Biol. Chem. 264, 15574–15577.
[3] Ashcom, J.D., Tiller, S.E., Dickerson, K., Cravens, J.L., Argraves, W.S. and Strickland, D. (1990) J. Cell. Biol. 110, 1041–1048.
[4] Strickland, D.K., Ashcom, J.D., Williams, S., Burgess, W.H., Migliorini, M. and Argraves, W.S. (1990) J. Biol. Chem. 265, 17401–17404.
[5] Kristensen, T., Moestrup, S.K., Gliemann, J., Bendtsen, L., Sand, O. and Sottrup-Jensen, L. (1990) FEBS Lett. 276, 151–155.
[6] Herz, J., Kowal, R.C., Goldstein, J.L. and Brown, M.S. (1990) EMBO J. 9, 1769–1776.
[7] Moestrup, S.K. and Gliemann, J. (1991) J. Biol Chem. 266, 14011–14017.
[8] Herz, J., Goldstein, J.L., Strickland, D.K., Ho, Y.K. and Brown, M.S. (1991) J. Biol. Chem. 266, 21232–21238.
[9] Moestrup, S.K., Kaltoft, K., Sottrup-Jensen, L. and Gliemann, J. (1990) J. Biol. Chem. 265, 12623–12628.
[10] Sottrup-Jensen, L., Petersen, T.E. and Magnusson, S. (1980) FEBS Lett. 121, 275–279.
[11] Gliemann, J. and Davidsen, O. (1986) Biochim. Biophys. Acta 885, 49–57.
[12] Laemmli, U.K. (1970) Nature 227, 680–685.
[13] Lotan, R., Skutelsky, E., Danon, D. and Sharon, N. (1975) J. Biol. Chem. 250, 8518–8523.
[14] Rinderle, S.J., Goldstein, I.J., Matta, K.L. and Ratcliffe, R.M. (1989) J. Biol. Chem. 264, 16123–16131.
[15] Clausen, H. and Hakamori, S. (1989) Vox Sang. 56, 1–20.
[16] Strickland, D.K., Ashcom, J.D., Williams, S., Battey, F., Behre, E., McTigue, K., Battey, J.F. and Argraves, W.S. (1991) J. Biol. Chem. 266, 13364–13369.
[17] Goldstein, I.J. and Hayes, C.E. (1978) in: The Lectins: Carbohydrate-Binding Proteins of Plants and Animals (Tipson, R.S. and Horton, D. eds) Advances in Carbohydrate Chemistry and Biochemistry, vol. 35, pp. 127–340, Academic Press, New York.
[18] Heldin, C.-H., Ek, B. and Rönnstrand, L. (1983) J. Biol. Chem. 258, 10054–10061.
[19] Rouiller, D.G., Sharon, N., McElduff, A., Podskalny, J.M. and Gorden, P. (1986) Endocrinology 118, 1159–1165.
[20] Feige, J.-J. and Baird, A. (1988) J. Biol. Chem. 263, 14023–14029.