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Investigation of asialoglycoprotein receptor glycosylation by lectin affinity methods

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The asialoglycoprotein receptor belongs to the family of calcium-dependent (C-type) animal lectins. The purified receptor is a glycoprotein in which 10 % of the dry weight consists of sialic acid, galactose, *N*-acetylglucosamine and mannose. The carbohydrate content of the asialoglycoprotein receptor was investigated by lectin affinity methods. The usefulness of plant lectin affinity methods in the characterization of the saccharide content of the asialoglycoprotein receptor, as an animal lectin, is demonstrated. RCA I, ConA, PHA, SNA I and WGA showed greater affinity toward the asialoglycoprotein receptor, while PSL, AAA and PNA showed negligible interactions with the asialoglycoprotein receptor. The obtained results correlated well with the carbohydrate content of the asialoglycoprotein receptor as determined by chemical methods.

Keywords: asialoglycoprotein receptor (ASGP-R), glycosylation, lectins, lectin affinity methods

INTRODUCTION

The asialoglycoprotein receptor (ASGP-R), originally discovered during studies on the metabolism of serum asialylated glycoproteins, is one of the most extensively investigated vertebrate membrane lectins.[1] Structurally it belongs to the calcium-dependent animal lectin family (C-type lectins) and to the type II subrgoup of endocytic receptors.[2]

The purified rabbit liver receptor is a water-soluble glycoprotein containing about 10 % carbohydrate by weight which consists of sialic acid, galactose, *N*-acetylglucosamine and mannose.[3] Pronase digestion of the intact protein yields two variant glycopeptides, distributed on two protein subunits with molecular masses of 40 and 48 kDa. The purified receptor exhibitis a high degree of aggregation in aqueous solution, forming a 250 kDa oligomer, with a subunit ratio of 2:1.[4] The relative abundance of these two glycopeptides has been estimated to be 14 and 2 mol per mol of intact protein, respectively. The larger component, glycopeptide I, comprises sialic acid, galactose,

mannose, glucosamine and aspartic acid in a molar ratio of 3:3:2:5:1. The smaller, glycopeptide II, contains only two sugars, mannose and glucosamine, together with aspartic acid in the mole ratio 8:2:1.[5]

ASGP-R specifically recognizes and binds galactose and *N*-acetylgalactosamine-terminated oligosaccharides.[6] The liver receptor mediates endocytosis and degradation of several plasma proteins. It has been proposed that the normal pathway of serum glycoprotein turnover might be mediated by ASGP-R, through the action of endogenous neuraminidase.[7]

The present investigation was undertaken in an attempt to characterize the carbohydrate chains of isolated ASGP-R by lectin affinity methods, solid-phase lectin assay, lectin affinity chromatography and lectin blot. The results presented here show that it is possible to characterize the carbohydrate moiety of ASGP-R using lectin affinity methods to complement the data provided by classical chemical analysis.

EXPERIMENTAL

Materials

$Na^{125}I$ was purhased from the Isotope-Institute of Isotopes Corp. (Budapest, Hungary). The sugars D-galactose, D-mannose, D-lactose, and grade V bovine serum albumin were products of ICN Biochemicals, Costa Mesa, while horseradish peroxidase type VI, Concanavalin A-agarose, WGA-agarose and Ricinus lectin were products of ICN Biochemicals, Cleveland, Ohio. Sigma Chemicals, St. Louis was the source of *N*-acetyl-D-glucosamine. Sepharose 4B was purchased from Pharmacia, Uppsala, Sweden. Polystyrene removawell microtiter plates (Immulon 4) were from Dynatech. All other chemicals were of p. a. grade.

Methods

Preparation of rabbit ASGP-R. ASGP-R was purified from Triton X-100 extracts of acetone liver powders by affinity chromatography using two columns of D-Gal-Sepharose successively, according to a method previously described.[8] The molecular mass and purity of the isolated protein was determined by SDS-PAGE in 12.5 % gels. The gels were stained by Coomassie brilliant blue and calibrated with conventional low molecular mass standards.[9]

Lectin affinity methods. Plant lectins with different carbohydrate specificities were used: concanavalin A (ConA) isolated from *Canavalia ensiformis*,[10] pea lectin (PSL) isolated from *Pisum sativum*,[11] phytohemagglutinin (PHA) isolated from *Phaseolus vulgaris*,[12] peanut lectin (PNA) from *Arachis hypogaea*,[13] wheat germ lectin (WGA) isolated from *Triticum vulgare*,[14] *Sambucus nigra* agglutinin (SNA I)[15] and a lectin from *Artocarpus altilis* (AAA).[16] *Ricinus communi*s agglutin (RCA I) was commercially obtained. All lectins were conjugated in our laboratory with horseradish peroxidase (HRPO).[17]

a) *Solid-phase lectin assay*. This assay was performed in microtiter plates previously coated with 2.5 μg of ASGP-R per well. After incubation of the plates with ASGP-R overnight at 4 ºC and washing with 0.05 % (w/v) Tween 20 in 1 mM Tris-HCl buffer pH 7.0 (loading buffer), nonspecific binding was inhibited by 0.5 % (w/v) bovine serum albumin in the loading buffer. The plates were then incubated for 1 h at room temperature (0.2 ml/well), washed, dried under vacuum and kept in sealed plastic bags with silica until required. In the lectin-binding studies, 0.1 ml lectin-HRPO conjugates were added to each well and the coated plates incubated for 2 h at room temperature in the dark. The wells were washed twice with Tris-Tween buffer. Peroxidase substrate (urea-peroxide) and chromogen (3, 3', 5, 5'-tetramethylbenzidine) were added, the reaction was stopped with 2 M H_2SO_4 and the absorbance of the resulting colour was measured at 450 nm.[18] The specificity of the interac-

tions beteween ASGP-R and the lectins was also determined. Lectin-HRPO conjugates were previously saturated with 0.2 M specific sugar (inhibitor) and then added to the wells containing ASGP-R. The reaction was carried out for 2 h at room temperature in the dark.

b) *Lectin affinity chromatography*. The lectin affinity columns used in this chromatography were: ConA- and WGA-agarose, as well as PSL-, AAA-, PNA- and SNA I-Sepharose 4B (Bed volume = 5 ml). The columns were equilibrated with the following buffers:

Affinity column	Equilibrating buffer	Specific sugars
Con A-agarose	0.1 M acetic buffer pH 6.0 (1 M NaCl, 0.001 M Ca^{2+}, Mg^{2+} and Mn^{2+})	0.2 M Man
WGA-agarose	0.05 M PBS pH 7.4	0.2 M GlcNAc
PSL-Sepharose 4B	1 M NaCl pH 6.5 (0.02 M Ca^{2+}, 0.02 % (w/v) NaN_3)	0.2 M Glc
AAA-Sepharose 4B	0.05 M PBS pH 7.4	0.8 M Gal
PNA-Sepharose 4B	0.05 M PBS pH 7.4	0.2 M Gal
SNA I-Sepharose 4B	0.05 M PBS pH 7.4	0.1 M Lac

The lectins were bound to the column matrices according to Hudson and Hay.[19] Purified ASGP-R was iodinated with 1mCi of $Na^{125}I$, by a modification of the method proposed by Greenwood *et al.*[20] A specific radioactivity of 2.9×10^5 cpm/μg ASGP-R was achieved and the radiolabeled ASGP-R was diluted with 0.05 M PBS buffer (pH 7.4) supplemented with 1 % (w/v) bovine serum albumin, 0.1% (w/v) NaN_3 and 0.5% (w/v) Tween 20 to achieve the concentration of 2.0×10^5 cpm/0.1 ml of buffer. ^{125}I-ASGP-R (0.2 mL) was applied to each affinity column and allowed to stand for 2 h at room temperature. Non-adsorbed ^{125}I-ASGP-R was then washed out with equilibrating buffer. ^{125}I-ASGP-R, specifically bound to the column, was slowly eluted with an appropriate sugar (Flow rate = 0.2 ml/min) and the adsorbed radioactivity was measured on a Micromedic 4/200 γ-counter.[21] Fractions of 2 ml were collected. The columns were further eluted with 0.1 M Gly-HCl (pH 3.0), except for the SNA I-column which was eluted with 0.02 M ethylenediamine. The columns were regenerated with equilibrating buffers, containing 0.1 % NaN_3 (w/v) and kept at 4 ºC until required.

c) *Lectin blot.* The ASGP-R samples separated by SDS-PAGE were transfered onto nitrocellulose membranes (NC) by semi-dry electrotransfer as previously described.[22] The NC membranes, SDS-acrylamide gels and filter papers were previously equilibrated in blotting buffer- 0.025 mM Tris-HCl pH 8.3 (0.192 M glycine, 20 % (v/v) methanol). After electrotransfer, NC membrane was incubated with 3 % (w/v) bovine serum albumin in 0.05 M Tris-HCl buffer (pH 7.6) containing 0.15 M NaCl (TBS) overnight at 4 ºC. The NC was incubated with lectin-HRPO conjugates for 2 h at room temperature.[23] After washing with TBS-Tween, diaminobenzidine and urea-peroxide were added to develop the colour reaction.

RESULTS AND DISCUSSION

ASGP-R was isolated from rabbit liver according to Hudgin *et al.*[8] and analyzed by SDS-PAGE. The obtained electrophoresogram showed that the ASGP-R consisted of two major polypeptide subunits with molecular masses of 40 and 48 kDa (Fig. 1). In addition, a protein band of 80 kDa molecular mass was visible (possibly a dimer of the 40 kDa subunit). SDS-PAGE also showed the presence of a weak protein band of approximately 60 kDa, which was not of ASGP-R origin. Protein bands smaller than 40 kD were not present.

In an attempt to examine the carbohydrate composition of the purified ASGP-R, lectin affinity methods were developed. Firstly, a solid-phase lectin assay was used to estimate the extent of ASGP-R binding to some plant lectins. The lectin assay used in this work was a modification of the method proposed by Van der Schaal.[24] Briefly, microtiter plates were coated with 2.5 μg of ASGP-R per well overnight at 4 ºC and then incubated with 2 μg lectin-HRPO conjugates. Plant lectins and ASGP-R, as an animal lectin, are glycoproteins. ASGP-R requires calcium ions for its activity. The reaction mixture was free of calcium ions and of ASGP-R lectin activity. Specific interactions between ASGP-R and lectin-HRPO conjugates were determined in the presence of specific sugars. The results obtained by measuring A_{450} are shown in Table I.

TABLE I. Reactivity of plant lectins (labeled with HRPO) with ASGP-R, adsorbed on the solid-phase (A_{450}).*

Lectin	PNA	AAA	PSL	WGA	SNA I	PHA	ConA	RCA I
A_{450}	0.020	0.039	0.052	0.152	0.213	0.243	0.313	0.394

*Microtiter plates were coated with 2.5 μg of ASGP-R per well overnight at 4 ºC, in loading buffer (1 mM Tris-HCl pH 7.0). Nonspecific binding was inhibited by incubation of every well with 0.2 ml 0.5 % bovine serum albumin in the loading buffer, for 1 h at room temperature. Lectin-HRPO conjugates (0.1 ml) were added to each well and allowed to stand for 2 h at room temperature. Peroxidase substrate and chromogen were added and the resulting absorbance was measured at 450 nm. Specific interactions between ASGP-R and plant lectins were determined in the presence of lectin-HRPO conjugates previously saturated with 0.2 M specific sugar.

The data (Table I) indicate that ASGP-R was bound to the applied lectins in the following order of increasing affinity: PNA<AAA<PSL<WGA<SNA I<PHA<ConA <RCA I. Lectin activities of PNA and AAA with ASGP-R were approximately the same, which explains their very similar carbohydrate specificities. Peanut lectin (PNA) binds to the terminal galactose (especially to Galβ1→3GlcNAc and less to Galβ1→4Glc structures) in O-type oligosaccharide chains (25). This O-type glycosylation is absent in ASGP-R which explains their very low reactivity. AAA binds to the terminal galactose in O-linked oligosaccharide chains.[26] The very low reactivity of AAA towards ASGP-R provides additional evidence that O-type oligosaccharides were not attached to the examined ASGP-R. The weak binding may originate from the low affinity of AAA for the terminal galactose in *N*-type glycans. Lectin PSL possesses strong binding affinity for Fucα1→6GlcNAc in *N*-type glycans.[27] Its binding to ASGP-R is relatively weak, due to the absence of fucose in ASGP-R molecules. Lectin WGA has strong affinity for N- and O-type oligosaccharide chains, but also for GlcNAcβ1→4GlcNAcβ1→4GlcNAc and Manβ1→4GlcNAcβ1→4GlcNAc,[28] so the binding of WGA to ASGP-R indicates the presence of one or more of these structures. WGA could also recognize terminal sialic acid in molecules of ASGP-R.

ConA, PHA, RCA I and SNA I showed greater reactivity with ASGP-R, although there were some differences in their binding affinity. ConA and PHA are lectins specific for N-type oligosaccharide chains. As mentioned previously, in both glycopeptides of ASGP-R, the sugars are bound to Asn (N-type).[5] Concanavalin A confirmed the presence of biantennary complex oligosaccharide chains with a trimannosyl core[29] in glycopeptide

I. ConA could also recognize the octamer of mannose in glycopeptide II with a higher affinity. PHA probably binds to the terminal galactose in glycopeptide I, which is a part of the sequence Galβ1→4GlcNAcβ1→2Man found in desialylated ASGP-R. The applied PHA was a mixture of two isolectins known as PHA-E and PHA-L. A bisecting GlcNAc unit linked by a β1→4 bond to the central core mannose residue of biantennary glycoproteins is important for PHA-E binding.[30] This structure is also present in glycopeptide I and explains the strong affinity of PHA towards ASGP-R. RCA I is very useful for the fractionation of *N*-acetyllactosaminic type glycopeptides or oligosaccharides. RCA I recognizes and binds to galactose (with a preference for terminal sugars) connected to the rest of molecule by a β1→4 bond (Galβ1→4GlcNAcβ1→R).[31] RCA I reacts more strongly with the branched oligosaccharides present in glycopeptide I of ASGP-R. SNA I which is specific for NeuAcα2→6Gal[32] (present in glycopeptide I), showed almost the same affinity for ASGP-R.

Lectin affinity chromatography was applied as a more sensitive lectin method to confirm and quantify the results obtained by lectin assay. Lectins with known carbohydrate specificities were bound to the affinity matrices, while ASGP-R was labeled with $Na^{125}I$. All columns were prepared in our laboratory. ASGP-R (4×10^5 cpm) was applied to each affinity column and, after 2 h incubation, it was eluted with the appropriate specific sugar.

TABLE II. Lectin affinity chromatography of ASGP-R*

Lectin bound to the matrix	Bound ASGP-R released by elution with specific sugar	
	cpm	% of loaded
PNA	16640	5.0
AAA	37939	8.8
PSL	40272	9.2
WGA	89318	20.1
ConA	181243	41.9
SNA I	213389	49.9

*Purified ASGP-R was iodinated with 1 mCi of $Na^{125}I$ (specific radioactivity 2.9×10^5 cpm/μg ASGP-R) and diluted with 0.05 M PBS buffer (pH 7.4) containing 1 % w/v bovine serum albumin, 0.1% w/v NaN_3, 0.5% v/v Tween 20, to achieve 2.0×10^5 cpm/0.1 ml. ^{125}I-ASGP-R was applied to each affinity column and allowed to bind for 2 h at room temperature. ^{125}I-ASGP-R, specifically bound to the columns was slowly eluted with appropriate sugars and the adsorbed radioactivity was measured on a γ-counter.

As was shown in the lectin assay, PNA, AAA and PSL bound to ASGP-R with very low affinity (Table II). This could be related to the fact that PNA and AAA represent lectins specific mostly for O-linked carbohydrate chains. The low reactivity between PSL and ASGP-R suggests the absence of fucose bound by α1→6 links to GlcNAc. The results obtained using this method showed that the lectins WGA, ConA and SNA I bound ASGP-R with stronger affinity. These lectins bind to N-type oligosaccharide chains. Binding of WGA and SNA I can indicate the presence of terminal sialic acid in some ASGP-R molecules, as proposed by Kawasaki and Ashwell.[5] The presence of a GlcNAc polymer connected with β1→4 bonds is responsible for the

binding of WGA to ASGP-R. The binding of ConA showed that the molecule of ASGP-R may contain a trimannosyl core or mannosyl polymer.

Lectin blot analysis with lectin-HRPO conjugates was performed to examine the interactions between plant lectins and protein subunits of ASGP-R or to examine glycosylation of both subunits of ASGP-R (Fig. 1). This method also gave information on whether ASGP-R or maybe only the contaminants of ASGP-R reacted with lectins. These results showed that the lectins PNA, AAA and PSL did not react with ASGP-R which confirmed the results obtained by solid-phase lectin assay. WGA bound to glycoproteins with molecular masses of 25 and 40 kDa. ConA, PHA, RCA I and SNA I recognized protein bands of 40 and 48 kDa, but some of them (ConA) recognized the 25 kDa contaminant. PHA recognized both subunits with the same intensity, while RCA I and SNA I recognized the 40 kDa protein band with greater affinity. The 40 kDa subunit consists of two glycopeptide I units, while the 48 kDa subunit consists of three glycopeptide I units and one glycopeptide II.[5] The differences in the band intensities on the lectin blots could be explained by the different exposure of the relevant carbohydrate moieties in the peptide subunits for reaction with the individual plant lectins. These data supplement the results obtained by other methods.

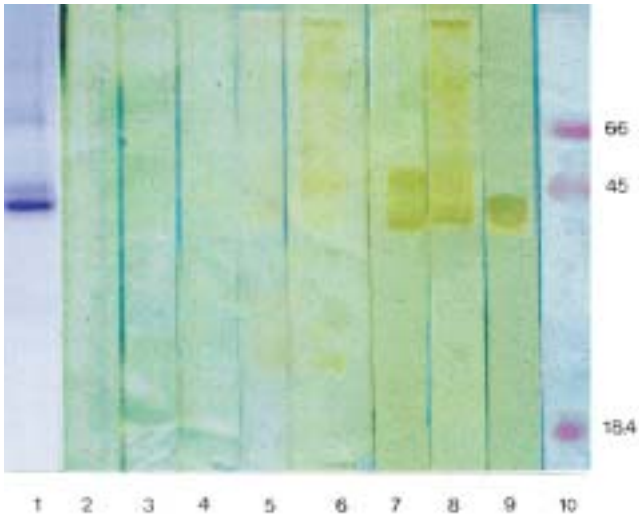

Fig. 1. Lectin blot analysis of ASGP-R (modification of Western blot). 1: Affinity purified ASGP-R (20 μg protein/lane) was separated by SDS-PAGE and stained with CBB-R. 2–9: Purified ASGP-R (20 μg protein/lane; all lines) was separated by SDS-PAGE, electroblotted onto nitrocellulose (NC) and incubated separately with plant lectins in the following order: 2. PNA, 3. AAA, 4. PSL, 5. WGA, 6. ConA, 7. PHA, 8. RCA I, 9. SNA I. 10: Molecular mass standards (BSA-66 kDa, ovalbumin-45 kDa and β-lactoglobulin-18.4 kDa) and their molecular masses are indicated on the right.

Moreover, the results presented show that, besides conventional chemical methods for the determination of the carbohydrate components of ASGP-R, it is possible to define its saccharide content using lectin affinity methods. Using chemical methods the

exact carbohydrate content of ASGP-R was found to be: 8.9 μmol/100 mg sialic acid, 7.3 μmol/100 mg galactose, 15.3 μmol/100 mg mannose and 16.5 μmol/100 mg glucosamine.[5,8,33] In all the affinity methods, plant lectins were used as the characteristic tool for investigating the carbohydrate content of rabbit ASGP-R, as an animal lectin. According to their reactivity, the applied lectins can be divided into two groups: lectins which show greater affinity towards ASGP-R (RCA I, PHA, ConA, SNA I and WGA), and lectins that show negligible interactions with ASGP-R (PSL, AAA and PNA). A terminal β-D-galactose content in some molecules of ASGP-R was demonstrated by the strong binding of RCA I and PHA. The presence of a mannose octamer and trimannosyl core in ASGP-R enhances the binding of concanavalin A. In addition, strong binding of wheat germ agglutinin (WGA) and *Sambucus nigra* agglutinin (SNA) confirmed the high content of sialic acid reported by others (Kawasaki and Ashwell).[5] WGA recognizes polymers of GlcNAc bound by β1→4 links, which are also present in ASGP-R. According to these results, it can be proposed that the isolate of ASGP-R is a mixture of molecules with terminal sialic acid and others in which the terminal sialic acid had been cleaved during the isolation procedure leaving them with terminal galactose units. Thus, the binding of RCA I and PHA (lectins specific for galactose) was very similar to the SNA binding (lectin specific for sialic acid). The presented results show that these methods could also be useful for the determination of the spatial arrangement of the carbohydrates in ASGP-R.

These findings confirm that the oligosaccharide units of ASGP-R are N-type, containing sialic acid, galactose, *N*-acetylglucosamine and mannose. No direct evidence was found for the presence of O-linked oligosaccharide chains. The proposed carbohydrate structure of ASGP-R obtained after lectin affinity methods is in agreement with the data obtained by Kawasaki and Ashwell.[5] Although the lectin affinity methods have different sensitivities, the results obtained show good correlation with those obtained using chemical methods of analysis and can be used to supplement them.

И З В О Д

ИСПИТИВАЊА ГЛИКОЗИЛОВАЊА АСИЈАЛОГЛИКОПРОТЕИНСКОГ РЕЦЕПТОРА ЛЕКТИНСКИМ АФИНИТЕТНИМ МЕТОДАМА

ИВОНА БАРИЧЕВИЋ, ЉИЉАНА ВИЋОВАЦ, ВЕСНА МАРИНОВИЋ и МАРГИТА ЧУПЕРЛОВИЋ

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Асијалогликопротеински рецептор припада породици калцијум – зависних анималних лектина (лектина типа C). Пречишћени рецептор је гликопротеин са 10 % суве масе сачињене од сијалинске киселине, галактозе, *N*-ацетилглукозамина и манозе. Угљенохидратни садржај асијалогликопротеинског рецептора је испитиван лектинским афинитетним методама. Показана је примењивост афинитетних метода са биљним лектинима у карактерисању угљенохидратног садржаја асијалогликопротеинског рецептора. RCA I, ConA, PHA, SNA I и WGA показују јак афинитет према асијалогликопротеинском рецептору, док PSL, AAA и PNA показују незнатне интеракције са овим

рецептором. Добијени резултати се добро слажу са угљенохидратним садржајем асијалогликопротеинског рецептора који је одређен хемијским методама.

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