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STUDIES ON PHYTOHEMAGGLUTININS

XXII. ISOLATION AND CHARACTERIZATION OF A LYMPHOCYTE RECEPTOR FOR CONCANAVALIN A

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SUMMARY

A receptor glycopeptide for concanavalin A was isolated from calf thymocytes by a method originally devised for the isolation of a lectin receptor from human erythrocytes (Kubánek, J., Entlicher, G. and Kocourek, J. [1973] Biochim, Biophys. Acta 304, 93–102). The method consisted of pronase digestion of the lipid-depleted thymocyte membrane material followed by ethanol fractionation, separation on Sephadex and preparative paper electrophoresis. The isolated glycopeptide contains 10.4 % of neutral sugar. The molar ratios of the sugar components mannose, galactose, glucosamine, glucose, fucose and sialic acid are 3 : 2 : 2 : 1 : 1 : 1. The minimum molecular weight calculated from the sugar composition is about 12 000.

Concanavalin A receptor activity of the glycopeptide was demonstrated in three different ways: (i) Reduction of the ^{125}I-labeled concanavalin A binding to thymocytes. (ii) Prevention of concanavalin A induced agglutination of calf thymocytes. (iii) Inhibition of concanavalin A stimulated DNA synthesis in calf and rabbit thymocytes and rabbit lymph node lymphocytes cultivated in vitro.

The isolated glycopeptide seems to be involved in the interaction of lymphocytes with concanavalin A and the subsequent stimulation.

INTRODUCTION

The appropriate application of lectins can give valuable information about the nature of the carbohydrate substances of the outer membrane surface. Interaction with carbohydrate receptors of the cell membrane is obviously the first step which is necessary for various activities exhibited by lectins in many biological systems. Agglutination of erythrocytes, agglutination of tumor cells and stimulation of the mitosis of lymphocytes are the most important and most widely studied lectin activities [1, 2]. Relatively well characterized lectin receptors or receptor sites were isolated from red blood cells [3–6] and tumor cells [7, 8]. Recently, also lectin receptors of

lymphocytes which could contribute to the understanding of the mechanism of lymphocyte stimulation were isolated. The concanavalin A receptor was isolated from pig lymphocyte plasma membrane by biospecific adsorption on concanavalin A or lentil lectin bound to Sepharose [9, 10]. However, a heterogeneous glycoprotein fraction was obtained the carbohydrate composition of which was not studied in detail.

Our effort was directed towards the isolation and characterization of a carbohydrate receptor site which is directly involved in the interaction with a lectin rather than the receptor as an intact molecule. For this reason extensive proteolytic digestion was employed as the first step of the isolation procedure. A similar approach has been successfully chosen in the isolation of a lectin receptor site from human and rabbit erythrocytes [5, 6].

MATERIALS AND METHODS

Preparation of thymocyte membrane material

Immediately after slaughter the calf thymus was iced, cut into pieces and gently homogenized in phosphate-buffered saline pH 7.3 on an MSE homogenizer at a low speed. The slurry was pressed through a stainless steel mesh. The cell suspension thus obtained was centrifuged 20 min at $85 \times g$. The sediment was washed with cold phosphate-buffered saline and the cell supernatants were centrifuged for 30 min at $1400 \times g$. The cell pellet was lyzed with acidified water (0.4 ml of acetic acid/l). The resulting gelatinous material was separated from the supernatant, homogenized and centrifuged at $250 \times g$ for 20 min. The pellet was discarded and the supernatant was centrifuged for 30 min at $1400 \times g$ and the resulting pellet was washed three times with water and lyophilized. The crude membrane material isolated in this way contained 5.25 % of neutral sugar and was obtained in a yield of 7 g per 1 kg of fresh thymus. The extent of contamination of this material by membranes of intracellular organelles was not studied.

Preparation of thymocyte glycopeptide

Isolation of the receptor thymocyte glycopeptide was carried out essentially according to the method used originally for the isolation of an erythrocyte glycopeptide [5]. The isolation procedure involved pronase digestion of the lipid-depleted thymocyte membrane material, ethanol fractionation of the digest, separation on Sephadex G-25 column and preparative vertical descending paper electrophoresis. The electrophoresis was repeated once more under the following conditions: the glycopeptide preparation (16 mg) obtained from the first electrophoresis was applied to a washed dry sheet of Whatmann No. 1 paper along a 30 cm origin line. Electrophoresis was carried out in pyridine–acetate buffer, pH 5.6 (4 ml pyridine, 1.2 ml acetic acid, water up to 1 l.) for 2 h at 1500 V using an apparatus designed by Mikes [11]. Zones of separated glycopeptides were cut out according to guide strips, detected with ninhydrin and the substances were eluted with water and lyophilized.

Chemical characterization of the isolated glycopeptide

Carbohydrate analysis. The total amount of neutral sugar was determined

during the fractionation procedure by the phenol–sulfuric acid method of Dubois et al. [12] with mannose as a standard. Identification and determination of all neutral sugars of the glycopeptide was carried out by gas–liquid chromatography. For the hydrolysis of the sample (2 M trifluoroacetic acid, 1 h) and preparation of alditol acetates the method of Albersheim et al. [13] was employed. Alditol acetates were separated at 200 °C, H_2 flow rate was approx. 50 ml/min, on a 3 mm × 3 m column of Chromosorb W containing 0.2 % ethyleneglycol succinate–0.2 % ethyleneglycol adipate–1 % silicone XE 60. Fucose was alternatively determined with unhydrolyzed samples of the glycopeptide by the cysteine–H_2SO_4 method of Dische and Shettles [14]. Sialic acid was determined by the method of Svennerholm [15] also on a sample of the unhydrolyzed glycopeptide. Glucosamine was identified and determined in hydrolyzates of the isolated glycopeptide on an automatic amino acid analyzer. The glycopeptide was hydrolyzed in 4 M HCl (0.5 mg/0.5 ml) for 16 h at 110 °C. Under these conditions the maximum release of glucosamine was observed.

Amino acid analysis. Amino acids were determined on an automatic amino acid analyzer with a sample of the glycopeptide (0.5 mg) hydrolyzed in 0.5 ml of 6 M distilled HCl at 110 °C for 47 h in a sealed tube under N_2.

Tests of glycopeptide receptor activities

Preparation of cell suspensions. Cell suspensions of calf and rabbit thymocytes were prepared by cutting the organs, washed previously with phosphate-buffered saline, into small fragments and pressing them through a stainless steel mesh. Large cell aggregates were removed by a 30 min sedimentation or by a short centrifugation at a very low speed. Cell suspensions were prepared with the use of cold Eagle's medium supplemented with 10 % of the inactivated calf or rabbit serum, respectively, penicillin (100 I.U./ml) and streptomycin (100 μg/ml). For agglutination assays calf thymocyte suspensions were prepared using saline instead of the medium.

Binding study. Concanavalin A (Calbiochem.) was labeled with ^{125}I ($Na^{125}I$) by the chloramine T method modified as described by Allan and Crumpton [16].

A suspension of thymocytes was transferred into Hanks'balanced salt solution without glucose. The cell suspension was incubated with [^{125}I]concanavalin A (spec. act. 0.2 Ci/g) at 20 °C on a reciprocal shaker. The isolated thymocyte glycopeptide or methyl-α-D-mannopyranoside was added to the cell suspension before the concanavalin A addition. During the incubation of the cells with concanavalin A samples were taken and quickly filtered through discs of Whatmann No. 3 paper (24 mm in diameter) and washed twice with 4 ml of ice-cold phosphate-buffered saline. The radioactivity of the dried filter discs was measured on a γ-counter.

Agglutination assay. 0.1 ml of serially-diluted 1 % solution of the glycopeptide in saline (0.9 % NaCl) and 0.1 ml of the saline solution of concanavalin A (0.3 mg/ml) were mixed. The concentration of the concanavalin A solution was four times higher than the highest dilution at which agglutination was observed macroscopically. After 30 min at laboratory temperature 0.2 ml of about 2 % saline suspension of three times washed thymocytes or rabbit red blood cells was added. Tubes were allowed to stand for 15 min and centrifuged for a short time (100 × *g*, 15 s and 1 min in the case of thymocytes and erythrocytes, respectively). Formation of large macroscopic aggregates which resisted gentle shaking was a criterion of agglutination. The inhibi-

tory activity of the glycopeptide was compared with that of the inhibiting sugar (glucose).

DNA synthesis. Thymocytes were cultivated for 68 h in the medium described above in the presence of both 5 μg/ml of concanavalin A (Pharmacia) and the isolated glycopeptide. 16 h before the termination of the cultivation 0.5 μCi of [*Me*-^{3}H]thymidine (15–20 Ci/mmole) was added. The amount of [^{3}H]thymidine incorporated into the precipitate which was insoluble in trichloroacetic acid was measured by the liquid scintilllation technique [17].

Cell viability tests. The effect of the isolated glycopeptide on cell viability was examined by means of the trypan blue staining and ^{51}Cr release assay [17].

RESULTS

Isolation of the receptor glycopeptide

The crude glycopeptide mixture obtained after pronase digestion of the delipidated thymocyte membrane material and ethanol fractionation was dissolved in 0.1 M acetic acid and fractionated on a Sephadex G-25 column (Fig. 1).

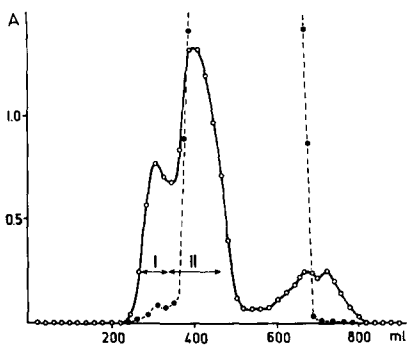


Fig. 1. Gel filtration of the crude glycopeptide mixture on a Sephadex G-25 column. The glycopeptide mixture obtained from 16 g of the membrane material was dissolved in 0.1 M acetic acid and applied to a Sephadex G-25 "fine" column (135 cm × 2.5 cm) equilibrated and eluted with 0.1 M acetic acid at a flow rate of 21 ml/h. ○—○, neutral sugar content ($A_{490\ nm}$); ●—●, content of ninhydrin-positive material ($A_{570\ nm}$). The arrows indicate the pooled fractions (glycopeptide Fractions I and II).

Two glycopeptide fractions (Fractions I and II) were obtained. Fraction II containing apparently lower molecular weight substances was obtained in a much higher yield than Fraction I. Therefore, this fraction was submitted to further fractionation. Components of Fraction II were separated by preparative vertical descending paper electrophoresis on Whatmann No. 3 paper. The electrophoretic pattern is shown in Fig. 2.

To obtain the glycopeptide in an electrophoretically homogeneous form the preparation obtained by electrophoresis on Whatmann No. 3 paper was subjected to an additional electrophoresis on Whatmann No. 1 paper at a higher voltage (1500 V for 2 h). Two glycopeptides were obtained, one of which possessed receptor site activity whereas the other one was practically inactive. The yields and the neutral

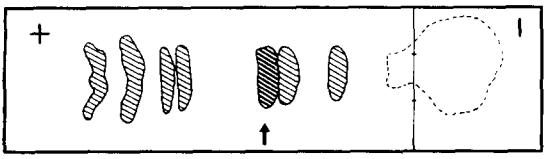


Fig. 2. Vertical descending paper electrophoresis of the glycopeptide Fraction II. About 3 mg of the sample were applied to a Whatmann No. 3 paper. Electrophoresis was carried out in pyridine–acetate buffer (pH 5.6) at 30 mA, 1200 → 800 V for 1.5 h. Ninhydrin was used as a location reagent. The arrow indicates the isolated receptor glycopeptide. The adjacent zone corresponds to the inactive glycopeptide mentioned in Table I.

TABLE I

RESULTS OF THE ISOLATION PROCEDURE

Isolation step	Yield (g)	Neutral sugar content (%)
Crude membrane material	16	5.25
Gel filtration: Fraction I	0.56	50.0
Fraction II	2.48	27.0
Electrophoresis: glycopeptide preparation (1200 → 800 V)	0.045	14.5
Electrophoresis: active receptor glycopeptide (1500 V)	0.012	10.4
inactive glycopeptide	0.008	6.6

sugar content of the preparations obtained at significant stages of the isolation procedure are summarized in Table I.

Characterization of the receptor glycopeptide

The isolated active receptor glycopeptide represented 0.15 % of neutral sugar of the crude thymocyte membrane material. The yield of the glycopeptide was 0.08 % of the weight of the original membrane material. The neutral sugar content of the isolated thymocyte glycopeptide was 10.4 % and the minimum molecular weight calculated from the sugar composition was about 12 000. The composition of the isolated receptor glycopeptide is given in Table II.

Biological activities of the receptor glycopeptide

Due to difficulties in the investigation of the direct interaction of the receptor glycopeptide with concanavalin A, the interaction was examined indirectly. Inhibition of concanavalin A binding to thymocytes, inhibition of agglutination of thymocytes with concanavalin A, and inhibition of concanavalin A stimulated DNA synthesis in thymocytes or lymphocytes was investigated. The effect of the isolated glycopeptide on concanavalin A binding to calf thymocytes is shown in Fig. 3. Besides the glycopeptide the effect of the highly active haptenic monosaccharide inhibitor (methyl-α-D-mannopyranoside) was examined.

As shown in Fig. 3 the steady state of concanavalin A binding was reached after 60 min of incubation. During this period no agglutination was observed macro-

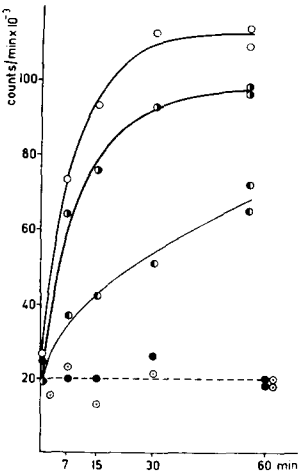
TABLE II

COMPOSITION OF THE THYMOCYTE GLYCOPEPTIDE WITH RECEPTOR ACTIVITY FOR CONCANAVALIN A

	%	Molar ratio to mannose	
		Actual	Nearest integer
Mannose	4.80	3.00	3
Galactose	3.39	2.12	2
Glucose	1.59	0.99	1
Fucose	2.25	1.41	1
Sialic acid	2.90	1.12	1
Glucosamine	3.91	1.64	2
Lysine	5.71	4.46	4
Arginine	3.15	2.21	2
Aspartic acid	6.45	5.56	6
Threonine	7.87	7.96	8
Serine	8.10	9.24	9
Glutamic acid	4.45	3.75	4
Proline	4.36	4.52	5
Glycine	10.75	17.14	17
Alanine	10.72	14.43	14
Valine	3.84	3.41	3
Isoleucine	1.94	2.20	2
Leucine	2.76	2.94	3

scopically. Under the described experimental conditions the first signs of agglutination were revealed after 90 min. The concanavalin A concentration (0.5 μg/ml) and cell density were chosen so that most of the concanavalin A was bound to the cells, i.e. the concanavalin A binding sites were more or less saturated with cell receptors. Preliminary experiments showed that a further increase of cell density (from $1 \cdot 10^8$ cells/ml) did not lead to a significant increase of bound concanavalin A. At the same time the concanavalin A concentration used was found to be optimal for the stimulation of DNA synthesis in mouse [18] and rabbit (Haškovec, C., unpublished) lymphocytes cultivated in a serum-free medium. Methyl-α-D-mannopyranoside in a concentration of 1 mg/ml and 10 mg/ml suppressed the concanavalin A binding (Fig. 3). The radioactivity found in the samples in the presence of the glycoside did not change during the whole incubation period (15 s–60 min) and was independent of the cell amount. This radioactivity level apparently corresponded to a nonspecific adsorption of concanavalin A to the filter discs. Additional experiments showed that the concentration of the receptor glycopeptide necessary for a 50 % inhibition of concanavalin A binding was about three to four times (weight to weight basis) or about 200 times (molar basis) lower than that of methyl-α-D-mannopyranoside.

In an alternative method to the receptor activity assay the inhibition of thymocyte agglutination was studied. In our assay system the receptor glycopeptide inhibited the concanavalin A induced thymocyte agglutination at a minimal concentration of 80 μg/ml. Compared on a weight to weight basis and on a molar basis it showed about an 8 and 500 times higher inhibitory activity than D-glucose, respectively.



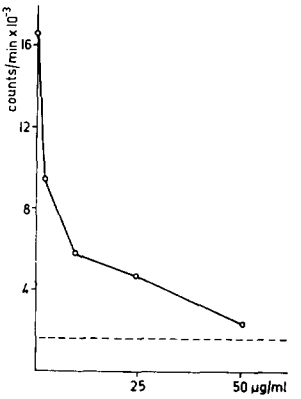


Fig. 3. The effect of the isolated thymocyte glycopeptide and methyl-α-D-mannopyranoside on [^{125}I]concanavalin A binding to calf thymocytes. Calf thymocytes ($1.1 \cdot 10^8$ cells/ml in Hanks' balanced salt solution were incubated with 0.5 µg/ml of [^{128}I]concanavalin A at 20 °C for 60 min. Samples per 0.5 ml. ○—○, control; ◑—◑, 5 µg/ml thymocyte glycopeptide; ◐—◐, 50 µg/ml thymocyte glycopeptide; ●—●, 1 mg/ml methyl-α-D-mannopyranoside; ⊙—⊙, 10 mg/ml methyl-α-D-mannopyranoside.

Fig. 4. The effect of the thymocyte glycopeptide on concanavalin A stimulated DNA synthesis in rabbit thymocytes. $4 \cdot 10^6$ rabbit thymocytes in 2 ml of Eagle's medium was cultivated for 68 h. [*Me*-^{3}H]thymidine incorporation was determined after an overnight pulse. The isolated thymocyte glycopeptide was added shortly before concanavalin A. - - -, level of [^{3}H]thymidine incorporation in nonstimulated cells.

The interaction of the isolated glycopeptide with concanavalin A was also assayed as the effect of this substance on the concanavalin A stimulated DNA synthesis in thymocytes or lymph node lymphocytes (Fig. 4). A considerable inhibitory effect of the isolated glycopeptide on the DNA synthesis in rabbit thymocytes was already observed at a very low concentration (0.5 µg/ml) in some experiments. At the concentration of 50 µg/ml the concanavalin A induction of DNA synthesis was almost completely blocked (Fig. 4). The same sensitivity of the concanavalin A induced DNA synthesis to the glycopeptide was also found in calf thymocytes and a somewhat lower sensitivity in rabbit lymph node lymphocytes.

In order to exclude the possibility of the toxic effects of the glycopeptide itself or trace amounts of contaminating substances on the cells, the effect of the glycopeptide on DNA synthesis in nonstimulated cells and cell survival were investigated. A certain inhibitory effect of the glycopeptide on DNA synthesis in nonstimulated cells was found only in some experiments, particularly in those with calf thymocytes. Nevertheless, this effect was far less pronounced than in cells stimulated with concanavalin A. No significant effect of the glycopeptide on thymocyte survival was observed as measured by the trypan blue staining or ^{51}Cr release method.

DISCUSSION

Intact cells or isolated cell membranes are used as starting material for the

isolation of carbohydrate components of the cell surface. As shown by Jett and Jamieson [19] the material isolated from living (intact) cells and usually considered to originate exclusively from the outer surface of cell membrane can be extensively contaminated by substances excreted by cells. Thus the main reason for the use of intact cells becomes unfounded. On the other hand the use of isolated membrane material is very convenient for large scale work. Therefore, this approach was chosen in our study.

So far the isolation of lectin receptor sites involved, as the first step, a release of membrane receptors or their large fragments by treatment with specific proteases [3, 4, 20] or membrane solubilizers [7, 9]. Subsequently, digestion with nonspecific proteases, usually pronase, was employed to obtain low molecular weight receptor fragments suitable for carbohydrate analysis. Direct treatment of the membrane material with pronase employed originally for the isolation of erythrocyte receptors [5, 6] considerably simplifies the isolation procedure. The size of the protein moiety of the thymocyte glycopeptide with concanavalin A receptor site activity does not correspond to the expected pronase effect. This may be explained by a rather high contamination of the membranes with DNA which could affect the pronase action.

A prerequisite for the receptor site activity of the isolated thymocyte glycopeptide is its interaction with concanavalin A. This interaction can be inferred from the inhibition of binding of concanavalin A to thymocytes which prevents agglutination or lymphocyte transformation. Significant inhibitory activity of the isolated glycopeptide was found in all of the above mentioned instances even though the amount of the glycopeptide necessary for inhibition varies due to widely different conditions of the assays.

In addition to thymocytes the isolated thymocyte glycopeptide was also found to be active with erythrocytes. Agglutination of rabbit erythrocytes by concanavalin A was inhibited much more (32 times) by the thymocyte glycopeptide than the thymocyte agglutination. The thymocyte glycopeptide even showed a much higher inhibitory activity (16 times) in the agglutination of rabbit erythrocytes than a receptor glycopeptide isolated from these erythrocytes [6]. This would indicate that the thymocyte glycopeptide has a higher affinity to concanavalin A than the erythrocyte glycopeptide.

Although a detailed kinetic study of the binding interaction was not performed we assume that the isolated glycopeptide can be considered a concanavalin A receptor and represents one of those components of the lymphocyte membrane which are responsible for the concanavalin A biological activity in lymphocytes.

REFERENCES

1 Sharon, N. and Lis, H. (1972) Science 177, 949–959
2 Lis, H. and Sharon, N. (1973) Annu. Rev. Biochem. 42, 541–574
3 Kornfeld, R. and Kornfeld, S. (1970) J. Biol. Chem. 245, 2536–2545
4 Akiyama, Y. and Osawa, T. (1972) Hoppe-Seyler's Z. Physiol. Chem. 353, 323–331
5 Kubánek, J., Entlicher, G. and Kocourek, J. (1973) Biochim. Biophys. Acta 304, 93–102
6 Pospišilová, J., Entlicher, G. and Kocourek, J. (1974) Biochim. Biophys. Acta, 362, 593–597
7 Janson, V. K. and Burger, M. M. (1973) Biochim. Biophys. Acta 291, 127–135
8 Smith, D. F., Neri, G. and Walborg, Jr, E. F. (1973) Biochemistry 12, 2111–2118
9 Allan, D., Auger, J. and Crumpton, M. J. (1972) Nat. New Biol. 236, 23–25

10 Hayman, M. J. and Crumpton, M. J. (1972) Biochem. Biophys. Res. Commun. 47, 923–930
11 Mikeš, O. (1957) Collect. Czechoslov. Chem. Commun. 22, 831–850
12 Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A. and Smith, F. (1956) Anal. Chem. 28, 350–356
13 Albersheim, P., Nevins, D. J., English, P. D. and Karr, A. (1967) Carbohydr. Res. 5, 340–345
14 Dische, Z. and Shettles, L. B. (1948) J. Biol. Chem. 175, 595–603
15 Svennerholm, L. (1957) Biochim. Biophys. Acta 24, 604–611
16 Allan, D. and Crumpton, M. J. (1973) Exp. Cell Res. 78, 271–278
17 Haškovec, C. and Hronová, P. (1974) Fol. Biol. in the press
18 Countinho, A. and Möller, G. (1973) Eur. J. Immunol. 3, 531–537
19 Jett, M. and Jamieson, G. A. (1973) Biochem. Biophys. Res. Commun. 55, 1225–1233
20 Winzler, R. J., Harris, E. D., Pekas, D. J., Johnson, C. A. and Weber, P. (1967) Biochemistry 6, 2195–2202