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ISOLATION AND CHARACTERIZATION OF A MANNAN-BINDING PROTEIN FROM RABBIT SERUM

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SUMMARY: A binding protein which recognizes mannose and N-acetylglucosamine has been isolated from rabbit serum to apparent homogeneity. The serum binding protein was nearly identical to the mannan-binding protein isolated previously from rabbit liver [Kawasaki, T., Etoh, R. and Yamashina, I. (1978) Biochem. Biophys. Res. Commun. 81, 1018-1024] in respect of immunochemical properties and subunit profiles, but could be differentiated from the liver protein in its larger molecular size and inferior sensitivity to monosaccharides as haptenic inhibitors of the binding to ^{125}I-mannan. A postulation was made that the plasma was, comparable with the liver, a major locus of mannan-binding protein in the rabbit.

Carbohydrate-mediated endocytosis in mammals has been the subject of intensive studies (1). These include those on the galactose specific recognition system in hepatocytes (2 - 5), the mannose/N-acetylglucosamine specific recognition system in sinusoidal cells in the liver and alveolar macrophages (6 - 12) and the mannose-6-phosphate recognition system in fibroblasts (13 - 17). In our previous studies, mannan-binding protein, which is a binding protein specific for mannose and N-acetylglucosamine, was isolated and characterized from rabbit and rat livers (18, 19). During the course of these studies, we noticed dual functions of antiserum induced against the binding protein. Thus, preincubation of the binding protein with a small amount of antiserum caused a marked reduction of the binding activity, whereas on increasing the amount of antiserum, the binding activity recovered progressively to the control value and eventually reached an augmented value. The reason for this was readily seen on detecting a remarkable mannan binding activity in pre-immune serum. The present paper describes the isolation and characterization of a mannan-binding protein from rabbit serum.

MATERIALS AND METHODS

Rabbit serum was purchased from Pel-Freez Biochemical Inc. Saccharomyces cerevisiae yeast mannan was isolated from Baker's yeast obtained from the Oriental Yeast Co., Tokyo, Japan, according to the procedure of Lee and Ballou (20). Mannan was iodinated with Na^{125}I (carrier free, Radiochemical Centre) by a modification of the procedure of Greenwood et al. (21) to specific activities of 1 to 4 μCi per μg. Sepharose 4B and CL-6B were obtained from Pharmacia.

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The binding assay was carried out as described previously (18) except that 900 ng of ^{125}I-mannan was incubated with 0.2 to 1.0 µg purified binding protein or an equivalent amount of crude preparation. Blank values determined by incubation of the ligand without added binding protein were less than 1% of the total radioactivity added and were subtracted from the bound radioactivities. A unit of activity was defined as 1 ng of labeled mannan bound under the standard conditions. Specific activity is expressed as units per µg protein.

The rabbit liver mannan-binding protein was isolated as described previously (18). Antibodies against this binding protein were produced in a goat by multiple muscular and subcutaneous injections of an emulsified mixture of an aqueous solution of the purified binding protein (0.5 mg) and Freund's complete adjuvant (Difco Laboratories Inc.) three times every three weeks. The IgG fraction was recovered from the antiserum by ammonium sulfate precipitation and DEAE-cellulose chromatography as described by Fahey (22).

Preparation of affinity resins of Sepharose 4B-mannan and Sepharose 4B-anti-liver mannan-binding protein IgG were carried out as described previously (18). Mannan (150 mg) or anti-liver mannan-binding protein IgG (500 mg) was coupled to 50 ml aliquots of Sepharose 4B which had been activated with cyanogen bromide (23).

The isolation of a mannan-binding protein from rabbit serum was carried out as follows. Frozen rabbit serum (50 ml) was thawed and diluted with an equal volume of a buffer consisting of 0.02 M imidazole-HCl, pH 7.8, 0.04 M $CaCl_2$ and 2.5 M NaCl. The mixture was applied to a Sepharose 4B-mannan column (15 ml) which had been equilibrated with a loading buffer consisting of 0.01 M imidazole-HCl, pH 7.8, 0.02 M $CaCl_2$ and 1.25 M NaCl. The column was washed with the loading buffer until the absorbance at 280 nm became less than 0.01, and the binding protein was eluted with an eluting buffer consisting of 0.01 M imidazole-HCl, pH 7.8, 1.25 M NaCl and 2 mM EDTA. The eluate was made to 0.02 M $CaCl_2$ and applied to a second smaller affinity column of Sepharose-4B-mannan. This column was washed with the loading buffer and the binding protein was eluted as described above. To the eluate, Triton X-100 was added to make a 0.05% solution and the mixture was dialyzed against a dialyzing buffer consisting of 5 mM imidazole-HCl, pH 7.8, 0.15 M NaCl, 0.25 mM EDTA and 0.05% Triton X-100. The non-diffusable material was applied to a Sepharose 4B-anti-liver binding protein IgG column which had been equilibrated with the dialyzing buffer. After washing the column with the same buffer, the binding protein was eluted with 0.05 M diethylamine, pH 11.8, containing 0.05% Triton X-100. Immediately after collection, the eluate was adjusted to pH 7.8 by the addition of adequate amounts of 2 M imidazole-HCl, pH 7.8. All the procedures described above were carried out at 4°.

Antiserum against rabbit serum mannan-binding protein was produced in guinea pigs as described above, using the binding protein recovered from the eluate of the second smaller Sepharose 4B-mannan column as an antigen.

Protein was determined by the micromethod of Lowry *et al.* (24).

RESULTS AND DISCUSSION

Taking advantage of the dependence on the presence of calcium of the binding, more than 1,000 fold purification of the binding protein was achieved by two consecutive chromatographic steps on Sepharose 4B-mannan columns. The isolated material, however, gave rise to three, one major and two minor, precipitin lines with the antibodies raised against itself upon an Ouchterlony double immunodiffusion test. The final step of the purification, immunoaffinity chromatography, was successfully developed after realization that antibodies against the rabbit liver mannan-binding protein reacted with crude serum binding protein

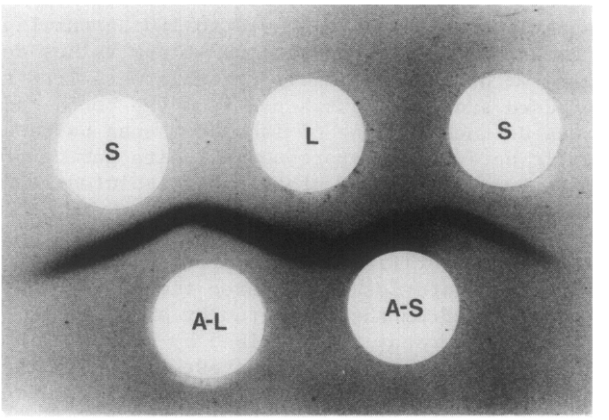

Fig. 1. Double immunodiffusion of binding proteins isolated from serum and liver. Immunodiffusion was carried out in 1% agarose containing phosphate-buffered saline, pH 7.2, 1 mM EDTA and 0.1% Triton X-100 at 37° for 3 h. Subsequently, the plate was washed with phosphate-buffered saline and stained with Amido black 10B. To each well was added the following: S, 1.0 µg of serum mannan-binding protein: L, 1.0 µg of liver mannan-binding protein; A-S, 25 µg of anti-serum mannan-binding protein IgG; A-L, 60 µg of anti-liver mannan-binding protein IgG.

to give a single immunoprecipitin line. By these procedures, 2 to 3 mg of the purified binding protein, with a specific activity of 100 to 130 units per µg protein, was recovered from 100 ml of rabbit serum with a recovery of about 50 per cent, with about 2,000 fold purification.

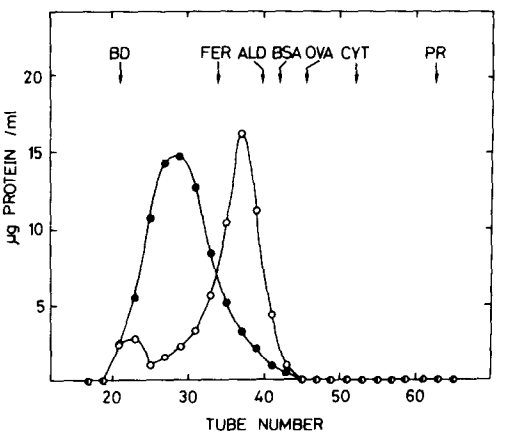

Fig. 2. Gel filtration of binding proteins isolated from serum and liver on Sepharose CL-6B in the presence of Triton X-100. Each sample of serum protein (180 µg) and liver protein (200 µg), which had been dialyzed against a buffer consisting of 20 mM imidazole-HCl, pH 7.8, 0.5 M NaCl, 0.1 % Triton X-100 and 0.5 mM EDTA, was applied to a column of Sepharose CL-6B (0.9 x 100 cm) and eluted with the same buffer at a flow rate of 4 ml/h. Fractions of 1.3 ml were collected. The symbols are: ● , serum mannan-binding protein; o , Liver mannan-binding protein; BD, blue dextran 2000 (void volume); FER, ferritin; ALD, aldolase; BSA, bovine serum albumin; OVA, ovalbumin; CYT, cytochrome C; PR, phenol red.

Fig. 1 presents the data indicating the immunochemical homogeneity of the purified serum binding protein and also the indistinguishable immunochemical properties of the serum binding protein and the liver mannan-binding protein. Thus, in every one of the four possible combinations of the two binding proteins and the corresponding antisera, a single precipitin line was generated.

Despite their apparent immunochemical identity, the two binding proteins isolated from serum and liver could be differentiated in their molecular size. Upon gel filtration in the presence of 0.1% Triton X-100 and 0.5 mM EDTA (Fig. 2), the serum binding protein eluted as a broad but single peak at a position corresponding to a molecular weight of more than 500,000. On the other hand, the liver mannan-binding protein gave rise to a major peak at a position cor-

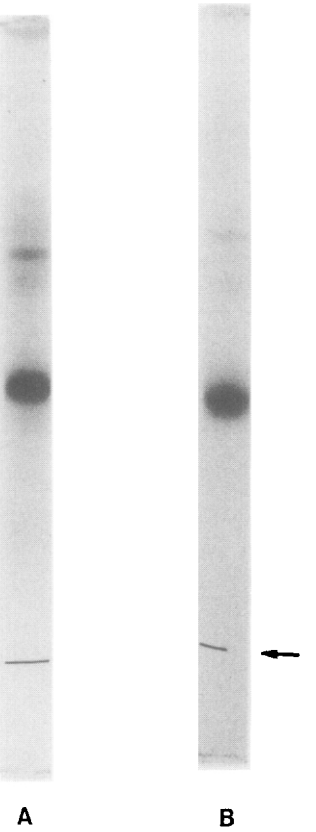

Fig. 3. Polyacrylamide gel electrophoresis of binding proteins isolated from serum and liver in the presence of sodium dodecyl sulfate. Each binding protein (10 μg) freed from detergent by ethanol precipitation was subjected to electrophoresis in 5.3% polyacrylamide gel according to the procedure of Weber and Osborn (29). Protein bands were stained with Coomassie brilliant blue G-250 in 10% trichloroacetic acid. The arrows denote the migration of the marker dye, Pyronine Y. A, serum mannan-binding protein; B, liver mannan-binding protein.

responding to a molecular weight of 250,000 with minor peaks in the higher molecular weight range. In either case, the specific activity was invariable throughout the peaks indicating the binding proteins to be largely free from inactive components.

Upon gel electrophoresis in sodium dodecyl sulfate, the serum binding protein gave rise to a major band, the molecular size of which was estimated to be 29,000 (Fig. 3A) and this value was practically identical to that of the liver mannan-binding protein (Fig. 3B) reported previously as 31,000 daltons (18). A slight difference was detected in the profiles of their minor constituents with slower mobilities between these two proteins, the significance of which is currently unclear. All of these bands were negative on periodic acid-Schiff staining for carbohydrate.

In an attempt to determine the specificity of the binding, a number of sugars were assayed as inhibitors of ^{125}I-mannan binding to the serum binding protein (see Table I). At the sugar concentration of 30 mM, at which the liver binding protein gave a pertinent profile of inhibition, no sugar except N-acetyl mannosamine inhibited the binding significantly. However, at the elevated con-

Table I

Inhibitory activity of various sugars

Compound	% inhibition of binding		
	Liver-BP[a)]	Serum-BP[b)]	
	30 mM	30 mM	100 mM
N-Acetylmannosamine	84	67	98
N-Acetylglucosamine	43	2	88
Mannose	35	0	55
L-Fucose	17	0	21
2-Deoxyglucose	14	0	0
Glucose	13	0	0
Galactose	11	2	10
Mannosamine	5	0	5
N-Acetylgalactosamine	0	0	6
Galactosamine	0	2	0
Glucosamine	0	2	0
D-Fucose	0	4	0
N-Acetylneuraminic acid	0	2	0
Mannose-6-phosphate	0	0	---

a) 1.1 μg of purified liver mannan-binding protein was used.
b) 0.7 μg of purified serum mannan-binding protein was used.

centration of 100 mM, the serum binding protein developed a spectrum of inhibition very similar to that of the liver binding protein, indicating that mannose and N-acetylglucosamine are the common sugars recognized by these binding proteins. Mannose-6-phosphate, which is a postulated recognition component on lysosomal enzymes for pinocytosis in human fibroblasts (25 - 27), was inert under the present conditions with either binding protein.

From tha data presented here, we conclude that the serum binding protein isolated in this study belongs to the same category of protein as the liver mannan-binding protein, that is, a mammalian lectin which recognizes mannose and N-acetylglucosamine. The binding protein is distinct from the sugar specific immunoglobulins (IgG) described by Sela et al. (28) as shown by the following properties: requirement of calcium for the binding, a single major subunit of 29,000 daltons, the same electrophoretic mobility as α_2- or β_1-globulin, precipitation at between 33 and 50 per cent ammonium sulfate saturation and the incapability of the antiserum against the binding protein to react with IgG.

The yield of mannan-binding protein from serum permits us to postulate that the plasma is, comparable with the liver, a major locus of the mannan-binding protein in rabbit. The relationship of the serum mannan-binding protein to the liver binding protein with regard to physiological functions and metabolism will be a subject of studies in the future.

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REFERENCES

1. Neufeld, E. F., and Ashwell, G. (1980) The Biochemistry of Glycoproteins and Proteoglycans, pp. 241-266, Plenum Publishing Corp., New York.
2. Hudgin, R. L., Pricer, W. E., Jr., Ashwell, G., Stockert, R. J., and Morell, A. G. (1974) J. Biol. Chem. 249, 5536-5543.
3. Kawasaki, T., and Ashwell, G. (1976) J. Biol. Chem. 251, 1296-1302.
4. Tanabe, T., Pricer, W. E., Jr., and Ashwell, G. (1979) J. Biol. Chem. 254, 1038-1043.
5. Doyle, D., Hou, E., and Warren, R. (1979) J. Biol. Chem. 254, 6853-6856.
6. Stockert, R. J., Morell, A. G., and Scheinberg, I. H. (1976) Biochem. Biophys. Res. Commun. 68, 988-993.
7. Stahl, P., Schlesinger, P. H., Rodman, J. S., and Doebber, T. (1976) Nature 264, 86-88.
8. Baynes, J. W., and Wold, F. (1976) J. Biol. Chem. 251, 6016-6024.
9. Achord, D. T., Brot, F. E., and Sly, W. S. (1977) Biochem. Biophys. Res. Commun. 77, 409-415.
10. Stahl, P. D., Rodman, J. S., Miller, M. J., and Schlesinger, P. H. (1978) Proc. Natl. Acad. Sci. 75, 1399-1403.
11. Steer, C. J., and Clarenburg, R. (1979) J. Biol. Chem. 254, 4457-4461.
12. Stahl, P., Schlesinger, P. H., Sigardson, E., Rodman, J. S., and Lee, Y. C. (1980) Cell 19, 207-215.

13. Kaplan, A., Achord, D. T., and Sly, W. S. (1977) Proc. Natl. Acad. Sci. 74, 2026-2030.
14. Neufeld, E. F., Sando, G. N., Garvin, A. J. and Rome, L. H. (1977) J. Supramol. Structure 6, 95-101.
15. Sando, G. N., and Neufeld, E. F. (1977) Cell 12, 619-627.
16. Ullrich, K., Mersmann, G., Weber, E., and von Figura, K. (1978) Biochem. J. 170, 643-650.
17. Rome, L. H., Weissmann, B., and Neufeld, E. F. (1979) Proc. Natl. Acad. Sci. 76, 2331-2334.
18. Kawasaki, T., Etoh, R., and Yamashina, I. (1978) Biochem. Biophys. Res. Commun. 81, 1018-1024.
19. Kawasaki, T., Mizuno, Y., and Yamashina, I. (1979) Fed. Proc. 38, 468.
20. Lee, Y. C., and Ballou, C. E. (1965) Biochemistry 4, 257-264.
21. Greenwood, F. C., Hunter, W. M., and Glover, J. S. (1963) Biochem. J. 89, 114-123.
22. Fahey, J. L. (1967) Methods Immunol. Immunochem. 1, 321-332.
23. Cuatrecasas, P., and Anfinsen, C. B. (1971) Methods Enzymol. 22, 345-378.
24. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) J. Biol. Chem. 193, 265-275.
25. von Figura, K., and Klein, U. (1979) Eur. J. Biochem. 94, 347-354.
26. Distler, J., Hieber, V., Sahagian, G., Schmickel, R., and Jourdian, G. W. (1979) Proc. Natl. Acad. Sci. 76, 4235-4239.
27. Natowicz, M. R., Chi, M. M. Y., Lowry, O. H., and Sly, W. S. (1979) Proc. Natl. Acad. Sci. 76, 4322-4326.
28. Sela, B. A., Wang, J. L., and Edelman, G. M. (1975) Proc. Natl. Acad. Sci. 72, 1127-1131.
29. Weber, K., and Osborn, M. (1969) J. Biol. Chem. 244, 4406-4412.