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(Citation)
The Kobe journal of the medical sciences,24(1):17-30

(Issue Date)
1978-03

(Resource Type)
departmental bulletin paper

(Version)
Version of Record

(URL)
https://hdl.handle.net/20.500.14094/0100488898

Kobe J. Med. Sci. 24, 17 - 30, March 1978

STUDIES ON MANNOSYL GLYCOPROTEINS OF CULTURED FIBROBLASTS*

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INDEXING WORDS

rat fibroblasts; endo-β-N-acetylglucosaminidases; mannosyl glycoproteins

SYNOPSIS

[^{3}H]mannose-labeled glycoproteins were prepared from cultured rat fibroblasts by solubilization with Triton X-100. They were separated into two fractions by affinity column chromatography on con A-Sepharose, namely weakly adsorbed fraction (those eluted by methyl α-mannoside) and strongly adsorbed fraction (those recovered only by sodium dodecyl sulfate). Glycopeptides were prepared from each fraction by pronase digestion and were analyzed by digestion with endo-β-N-acetylglucosaminidases. The result indicated that most of [^{3}H]mannose-labeled glycoproteins were adsorbed to con A-Sepharose, and that separation into the two fractions depended at least partly on the size of oligomannosyl cores, i.e. mannosyl glycoproteins with larger cores were strongly adsorbed to the column and those with smaller cores were weakly adsorbed.

Molecular species of the mannosyl glycoproteins adsorbed to the column of con A-Sepharose were analyzed by SDS disc gel electrophoresis. The electrophoretogram of the both fractions showed

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*This work has been supported by research grants from The Scientific Research Fund (No. 201554) of the Ministry of Education, Science and Culture of Japan.

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Received for publication February 18, 1978

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broad peaks, indicating the complexity of mannosyl glycoproteins. However, the major peak in each fraction was different to each other, i.e. molecular weight about 100,000 for strongly adsorbed fraction and molecular weight about 55,000 for weakly adsorbed fraction.

Then, the iodinated cell surface proteins from rat fibroblasts were separated by con A-Sepharose column and analyzed by disc gel electrophoresis. Molecular species in the strongly adsorbed fraction and weakly adsorbed fraction were again found to be different.

INTRODUCTION

In the previous reports,[7,10,11] we described the general properties of sugar moieties of mannosyl glycoproteins in cultured fibroblasts. The aim of the present report is to characterize the glycoproteins containing mannosyl residues of a rat fibroblast.

MATERIALS AND METHODS

Labeling of 3Y1B Cells.

3Y1B cells, cloned fibroblasts,[6] were kindly supplied by Prof. G. Kimura, Tottori University and were maintained in the culture medium described in the previous paper.[7] The cells (4.5×10^5 cells) were innoculated into a Falcon screw capped plastic flask, (surface area, 75 cm^2). The culture medium 10 ml was exchanged every 2 days. Six days after plating, when the cell density was 4×10^4 cells/cm^2, the culture medium was removed, and the monolayer was washed three times with phosphate buffer saline (PBS). Iodination reaction was performed essentially as described by Pouyssegur et al.[13] Two millilitres of PBS containing 5 mM of glucose and 800 μCi of Na [^{125}I] (carrier free, New England Nuclear) was added into the flask. Then 5 μl of PBS containing lactoperoxidase (40 μg, Behringer Co.), and glucose oxidase (0.2 unit, Warrthington Biochemical Co.) was added to start iodination reaction. After 10 minutes at room temperature, the reaction medium was removed and the monolayer was washed once with PBI (PBS where NaCl was replaced by NaI at the same molality)[13] and twice with PBS containing 2 mM of phenylmethyl-sulfonylfluoride to inhibit proteases action. Thus I obtained radioactive material (2×10^7 cpm) from 8×10^5 cells.

3Y1B cells were also labeled by [^{3}H]mannose according to the procedure described in the previous paper.[7)]

Fractionation by Affinity Column Chromatography on Con A-Sepharose.

The cells labeled with either [^{125}I] or [^{3}H]mannose were solubilized by 3 ml of 1% Triton X-100 in 0.01 M Tris-HCl buffer, pH 7.5, containing 0.1 M NaCl, 0.02% bovine serum albumin and 2 mM of phenylmethyl-sulfonylfluoride. This material was centrifuged at 3000 x *g* for 30 minutes and the supernatant was pooled as [^{125}I]-labeled cell surface material and as [^{3}H]mannose-labeled cell material. These radioactive material obtained from about 8 x 10^{5} cells were applied to a column of concanavalin A-Sepharose (Seikagaku Kogyo Co., 0.5 x 5 cm) equilibrated with 1% Triton X-100 in 0.01 M Tris-HCl buffer, pH 7.5, containing 0.1 M NaCl and 0.02% bovine serum albumin. The column was washed with 10 ml of the above medium (Wash fraction), and then eluted with 10 ml of the above medium containing 0.1 M methyl α-mannoside (Met α-Man fraction). One millilitre fraction was collected. Then the con A-Sepharose was extracted with 3 ml of 1% sodium dodecyl sulfate at 100° for 2 minutes (SDS fraction). Both the "Wash" fraction and "Met α-Man" fraction were concentrated to 3 ml by dialysis against polyethylen glycol 200,000.

The amount of the acid insoluble material was determined as follows. The radioactive material was precipitated by adding 0.75 ml of ice cold 50% trichloroacetic acid. The precipitate collected by centrifugation at 500 x *g* for 5 minutes was washed by 3 ml of 10% trichloroacetic acid, and then by 5 ml of acetone. The washed precipitate was dissolved by 1% sodium dodecyl sulfate in 0.5 ml of 0.05 M Tris-HCl buffer, pH 6.8.

Disc Gel Electrophoresis.

SDS disc gel electrophoresis was performed as described by Maizel,[9)] using 10% and 7.5% polyacrylamide gel. Thus aliquats (40 to 100 μl containing 1 x 10^{5} cpm for [^{125}I]-labeled material and 5 x 10^{4} cpm for [^{3}H]mannose-labeled material) of each fraction separated by con A-Sepharose were dissolved in 1% sodium dodecyl sulfate and were dissociated to subunits by heating at 100° for 2 minutes in the sample buffer, 0.05 M Tris-HCl buffer, pH 6.8, containing 1% sodium dodecyl sulfate and 0.1% β-mercaptoethanol. The protein markers and their assigned molecular weight were as follows: ovalbumin (46,000), gammaglobulin (heavy chain, 51,000,

light chain, 23,500), phosphorylase-b (92,000), a gift from Dr. S. Matsumura and Prof. K. Nishizuka, Kobe University, and myosin (220,000), a gift from Dr. M. Kasai, Osaka University.

Analysis of Mannose-Labeled Glycopeptides by Digestion with Endo-β-N-Acetylglucosaminidases.

Mannose-labeled glycopeptides were digested by endo-β-N-acetylglucosaminidase D[8] and H[15] and the products were analyzed by paper chromatography as described previously.[7] Radioactive material eluted in the region of $(Man)_9GlcNAc$ - $(Man)_7GlcNAc$ was assigned to be from large oligomannosyl cores. Material in the region of $(Man)_6GlcNAc$ - $(Man)_5GlcNAc$ was assigned to be from medium oligomannosyl cores, and those in the region of $(Man)_3GlcNAc$ were assigned to be from a small oligomannosyl core. Mannosyl cores classified to the three groups, large, medium and small, did not depend on any structural characteristics.

Other Materials and Methods.

Descending paper chromatography was carried out on Whatman No. 1 paper in ethylacetate/pyridine/H_2O (12 : 5 : 4). Standard oligosaccharides, $(Man)_6GlcNAc$, $(Man)_5GlcNAc$ and $(Man)_3GlcNAc$, were prepared as described previously.[7]

RESULTS

Preparation of [^{3}H]mannose-Labeled Glycopeptides.

[^{3}H]mannose-labeled 3Y1B cells were solubilized in 1% Triton X-100. The solubilized radioactive material was separated into three fractions, "Wash", "Met α-Man" and "SDS" by affinity column chromatography on con A-Sepharose as shown on Fig. 1. "Wash" fraction was the material passed through the column, "Met α-Man" fraction was the material which adsorbed to the column and eluted by 0.1 M methyl α-mannoside. "SDS" fraction was the material which was adsorbed strongly, but extracted by 1% sodium dodecyl sulfate at 100° for 2 minutes.

About 41% of radioactivity from the solubilized total radioactive material was recovered in the latter two fractions. Then the three fractions, "Wash", "Met α-Man" and "SDS" were digested with pronase, heated at 100° for 5 minutes to inactivate the pronase and lyophylized to dryness. Each lyophylized fraction was dissolved in water (0.3 ml), separated from Triton X-100 by paper chromatography in a Solvent described in MATERIALS AND METHODS for

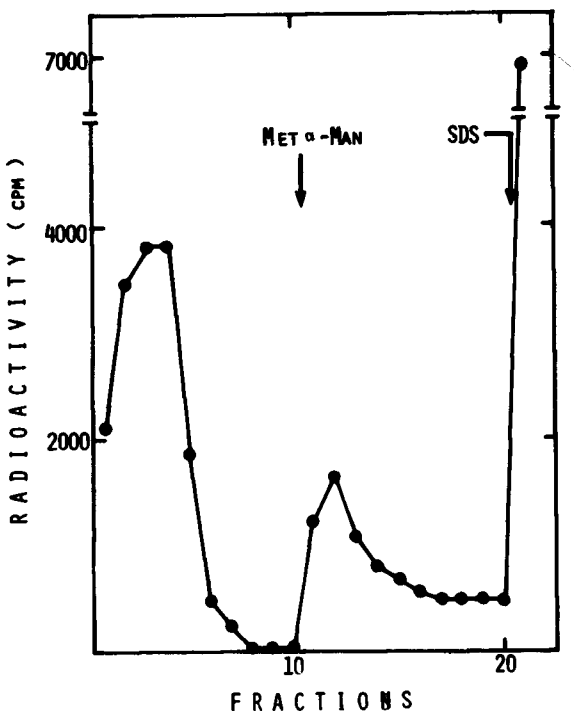

Fig. 1 Fractionation of [^{3}H]mannose-labeled glycoproteins from 3Y1B cells by affinity column chromatography on con A-Sepharose. [^{3}H]mannose-labeled 3Y1B cells (2.6×10^7 cells labeled with 200 μCi of [^{3}H]mannose) were solubilized by 1% Triton X-100, and the supernatant was fractionated by affinity column chromatography on con A-Sepharose described in MATERIALS AND METHODS.

15 hours. Then they were purified by Sephadex G-50 column chromatography, as shown in Fig. 2. No distinct peak was detected in the "Wash" fraction, while typical glycopeptides material was observed in "Met α-Man" and "SDS" fractions. The result indicates that most of mannosyl glycoproteins from the cells are adsorbed to the column of con A-Sepharose.

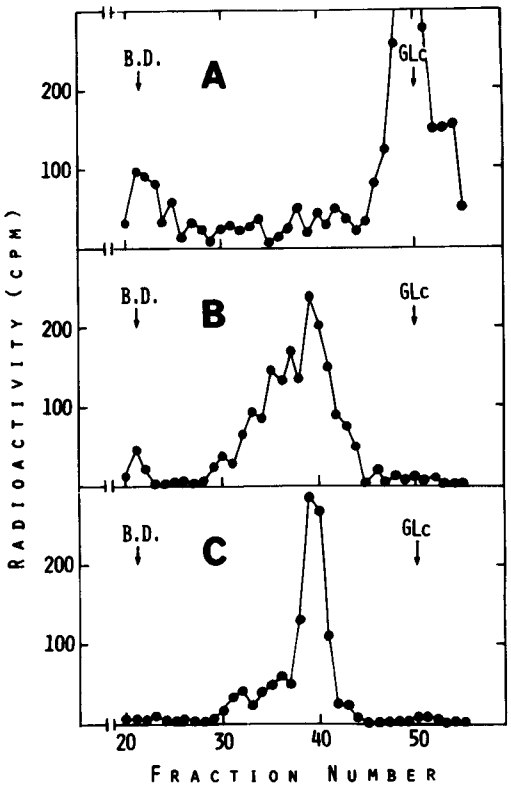

Fig. 2 Sephadex G-50 column chromatography of mannose-labeled glycopeptides from glycoproteins fractionated by affinity column chromatography on con A-Sepharose. The three fractions, "Wash", "Met α-Man" and "SDS" separated by con A-Sepharose were digested with 1 mg of pronase in 0.5 ml of 1 M Tris-HCl buffer, pH 8.4, containing 0.1 M $CaCl_2$ at 37° for 24 hours under toluene layer.[2] One milligram of pronase was added 24 hours' intervals and digestion was continued for 5 days. After the digestion, pronase was inactivated by heating at 100° for 5 minutes. Then, the digests were lyophylized in 3 ml of 0.1 M pyridine-acetate buffer, pH 5.4, and were applied to a column of Sephadex G-50 fine (3 x 90 cm). The column was equilibrated and eluted with the above buffer. Ten millilitres of fraction was collected. Aliquats (0.2 ml) were removed for counting radioactivity. B.D. and Glc represent the elution position of blue dextran and glucose, respectively. A: [^{3}H]mannose-labeled glycopeptides from "Wash" (fraction 1 to 12 in Fig. 1). B: [^{3}H]mannose-labeled glycopeptides from "Met α-Man" (fraction 13 to 23 in Fig. 1). C: [^{3}H]mannose-labeled glycopeptides from "SDS" (fraction 24 to 26 in Fig. 1).

Table 1 Analysis of oligomannosyl cores of glycopeptides by digestion with endo-β-N-acetylglucosaminidases.

Glycopeptides fractions	Percentage of oligomannosyl cores analyzed by endo-β-N-acetylglucosaminidases digestion					
	H*			D*		
	Large cores	Medium cores	Resistant material	Medium cores	The small core	Resistant material
"Met α-Man" fraction**	31.0	28.2	40.8	31.2	21.3	47.5
"SDS" fraction***	43.9	29.8	26.3	22.2	12.5	67.3

*: The glycopeptides were digested with endo-β-N-acetylglucosaminidase D or H, and the products were analyzed as described previously.[3] Results were expressed as percentage radioactivity in respective oligosaccharide region to total radioactivity in the paper chromatogram as described in MATERIALS AND METHODS.

**: [^{3}H]mannose-labeled glycopeptides in fraction 28 to 45 in Fig. 2-B.

***: [^{3}H]mannose-labeled glycopeptides in fraction 28 to 45 in Fig. 2-C.

Comparison of Oligomannosyl Cores between "Met α-Man" and "SDS" Fraction.

In order to know whether carbohydrate structure is different between mannosyl glycoproteins in "Met α-Man" fraction and those in "SDS" fraction, the glycopeptides prepared from each fraction were analyzed by endo-β-N-acetylglucosaminidases digestion. Digestion with endo-β-N-acetylglucosaminidase D was performed in the presence of β-galactosidase, β-N-acetylglucosaminidase and neuraminidase, and the products were analyzed by paper chromatography. Medium mannosyl oligosaccharides $(Man)_{6-5}GlcNAc$, and the small mannosyl oligosaccharide, $(Man)_3GlcNAc$ were released by digestion with the above enzymes as described previously.[7] More $(Man)_3GlcNAc$ was released from glycopeptides of "Met α-Man" fraction than from those of "SDS" fraction (Table 1).

Upon digestion with endo-β-N-acetylglucosaminidase H, large mannosyl oligosaccharides, $(Man)_{9-7}GlcNAc$, and medium mannosyl oligosaccharides, $(Man)_{6-5}GlcNAc$, were released.[7] Table 1 shows that large oligosaccharides were released more from "SDS" fraction than from "Met α-Man" fraction.

Thus, this may indicate that con A-Sepharose column chromatography described above could separate glycoproteins, at least partly, according to their size of oligomannosyl cores. Furthermore, it was possible to concentrate partly glycoproteins with large oligomannosyl cores by collecting the "SDS" fraction.

Sodium Dodecyl Sulfate Gel Electrophoresis of [^{3}H]mannose-Labeled Glycoproteins.

Glycoproteins in "Met α-Man" and "SDS" fraction were analyzed by SDS disc gel electrophoresis (Fig. 3). Both fractions contained several heterogeneous glycoproteins, indicating their complexity in the fibroblastic cells. However, the profiles showed that the "Met α-Man" fraction and "SDS" fraction were different. The "Met α-Man" fraction has a major peak near the region where ovalbumin (mw. 46,000) migrated, while "SDS" fraction has a major peak near the region where phosphorylase-b (mw. 92,000) migrated.

Analysis of Mannosyl Glycoproteins from the Surface of 3Y1B Cells.

Then, cell-surface proteins of 3Y1B cells were analyzed by affinity column chromatography on con A-Sepharose. 3Y1B cells were surface iodinated, and [^{125}I]-labeled cell surface proteins were separated by affinity column chromatography on con A-Sepharose,

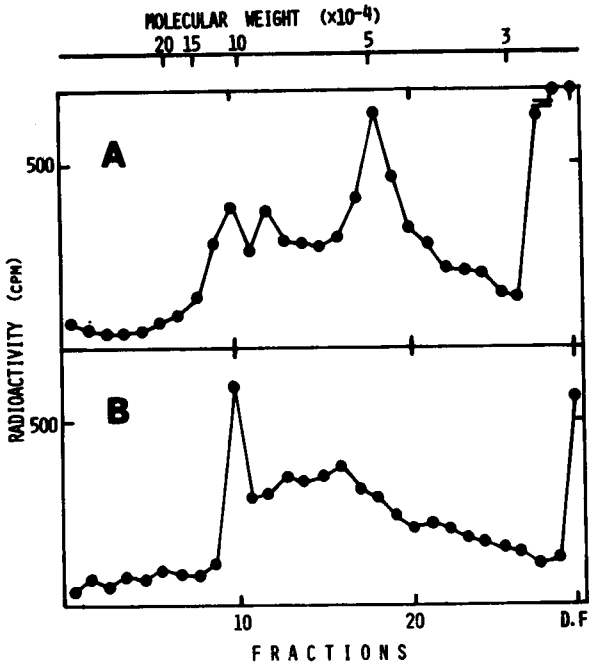

Fig. 3 Sodium dodecyl sulfate slab gel electrophoresis of [^{3}H]mannose-labeled glycoproteins from 3Y1B cells. [^{3}H]mannose-labeled glycoproteins from 3Y1B cells were separated by affinity column chromatography on con A-Sepharose, and analyzed by sodium dodecyl sulfate slab gel electrophoresis. Gel electrophoresis was performed in 7.5% polyacrylamide gel described in MATERIALS AND METHODS. After electrophoresis, gel was cut into 5 mm slices, which were extracted by shaking in 1% sodium dodecyl sulfate for 15 hours and counted for radioactivity. Estimation of molecular weights was performed using molecular weight standards described in MATERIALS AND METHODS. D.F. indicates the peak position of bromphenol blue. A: [^{3}H]mannose-labeled glycoproteins from "Met α-Man" fraction. B: [^{3}H]mannose-labeled glycoproteins from "SDS" fraction.

as shown in Table 2. Although, most of radioactivity (85.4%) was passed through the column (pooled as "Wash" fraction), a part of it (2.6%) was retained and eluted by 0.1 M methyl α-mannoside (pooled as "Met α-Man" fraction) and a part of it (2.2%) was retained and extracted by 1% sodium dodecyl sulfate (collected as "SDS" fraction). However, large amount of radioactivity in "Wash"

Table 2 Recovery of radioactivity after TCA and Acetone precipitation in the fractions separated by con A-Sepharose.

Labels	Fractions	Radioactivity			
		In each fraction		After precipitation by TCA and Acetone	
		cpm	%[b]	cpm	%[b]
[^{3}H][a]	"Wash"	63,380	(56.9)	16,220	(26.6)
	"Met α-Man"	26,372	(23.7)	24,900	(40.8)
	"SDS"	21,657	(19.4)	19,860	(32.6)
[^{125}I][a]	"Wash"	1,768,020	(85.2)	193,260	(65.5)
	"Met α-Man"	53,206	(2.6)	49,269	(18.7)
	"SDS"	45,255	(2.2)	41,499	(15.7)

a: [^{3}H]mannose-labeled 3Y1B cells (3_5x 10^7 cells) and [^{125}I]-labeled 3Y1B cells (6 x 10^5 cells) were solubilized in 1% Triton X-100. The supernatant was separated to the three fractions, "Wash", "Met α-Man", and "SDS" fraction by affinity column chromatography on con A-Sepharose, as described in MATERIALS AND METHODS.

b: Figures in parenthesis are the percentage of radioactivity in respective fraction to the total radioactivity recovered in all of the three fractions.

fraction was soluble in TCA and acetone, while those in "Met α-Man" and "SDS" fraction were not (Table 2). Thus, the ratio of [^{125}I]-label incorporated into "Met α-Man" fraction per total radioactivity incorporated into cell surface proteins was significantly high (18.7%), (Table 2). Similar value (15.7%) could be calculated for "SDS" fraction. This value indicates that about one third of the cell surface proteins are mannosyl glycoproteins if the surface proteins could be iodinated equally.

[^{125}I]-labeled cell surface proteins in the "Met α-Man" and "SDS" fractions were analyzed by SDS slab gel electrophoresis followed by autoradiography of electrophoretogram (Fig. 4). The elctrophoretogram shows that "Met α-Man" fraction has two definite bands at the position of the molecular weight about 120,000 and 52,000

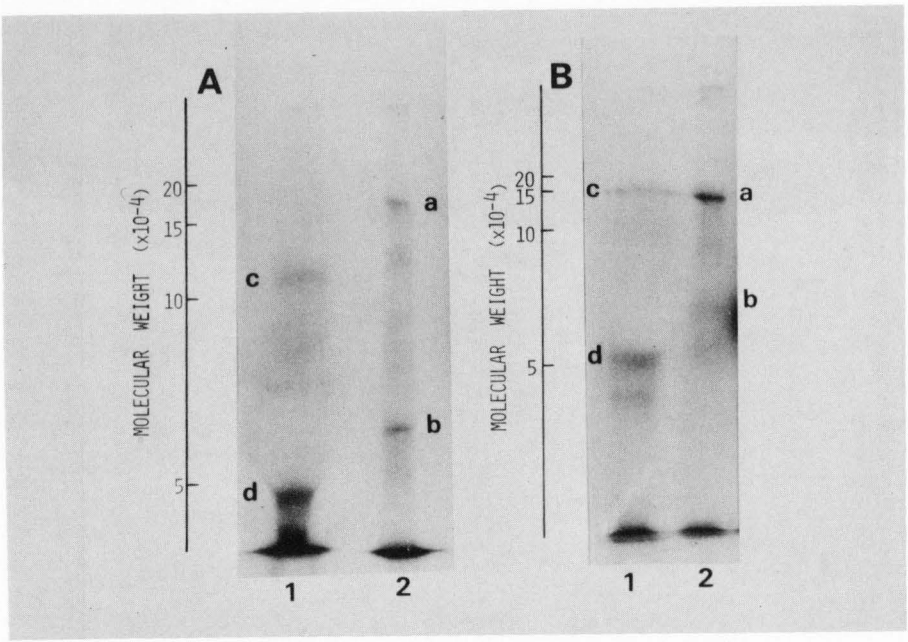

Fig. 4 Sodium dodecyl sulfate polyacrylamide gel electrophoresis of [^{125}I]-labeled cell surface proteins was separated by affinity column chromatography on con A-Sepharose. Both the "Met α-Man" and "SDS" fraction were analyzed by sodium dodecyl sulfate polyacrylamide slab gel electrophoresis, as described in MATERIALS AND METHODS. After electrophoresis, gel was fixed by methanol/acetic acid/H_2O (10 : 7 : 83), dried in vacuum and subjected to autoradiography using FX-Fuji film. Autoradiography was exposed for 24 hours. Estimation of molecular weights was performed using molecular weight standards described in MATERIALS AND METHODS. A: autoradiogram of 7.5% polyacrylamide gel. B: autoradiogram of 10% polyacrylamide gel. 1: "Met α-Man" fraction. 2: "SDS" fraction. a,b,c,d: [^{125}I]-labeled surface glycoproteins.

and "SDS" fraction has two definite bands at the position of molecular weight about 150,000 - 130,000 and 60,000. The bands in "Met α-Man" fraction appear to be different from those in "SDS" fraction, although their molecular weights are rather close. Those glycoproteins on the surface of 3Y1B cells could be called major con A-binding proteins on the surface of these cells.

DISCUSSION

In the present manuscript, I have reported that most of mannosyl glycoproteins from rat fibroblasts were adsorbed to the column of con A-Sepharose and separated into two fractions according to the size of oligomannosyl cores. The concanavalin A binding proteins which could be recovered only by sodium dodecyl sulfate had stronger affinity to concanavalin A than those recovered by methyl α-mannoside and had larger oligomannosyl cores.

Feller et al. have recently reported that concanavalin A receptors on cell surface could be classified into two types, a high affinity group and a low affinity group.[2] Evidence presented in this report agrees with their results. There are also several reports on biochemical analysis of concanavalin A binding sites in cultured fibroblasts. Studies of those from mouse L cells showed that they have an apparent molecular weight of about 100,000 and they are labeled by external cell surface labeling by galactose oxidase-KB[^{3}H]$_4$ system.[3] Burridge has also shown a useful method for detecting concanavalin A receptors.[1] LETS protein (large, external, transformation sensitive protein) is one of the concanavalin A binding glycoproteins of the cell surface.[4] I could not detect any remarkable radioactivities where LETS protein should move in Fig. 3 and Fig. 4. However, several molecular species which interact with concanavalin A were detected in the cell surface proteins of a normal rat fibroblast in Fig. 4.

Concanavalin A has several biological activities, such as blastogenesis for lymphocytes[14] and agglutination of fibroblastic cells, in particular for transformed fibroblastic cells.[5,12] Cell surface concanavalin A receptors responsible for the agglutination induced by concanavalin A have not been fully clarified. Many surface glycoproteins may interact with concanavalin A; however, those having high affinity to concanavalin A should be responsible for cell agglutinability. The iodinated cell surface proteins which were strongly adsorbed to concanavalin A-Sepharose column may be some of the concanavalin A receptors responsible for cell agglutination.

In any event, procedures described in the present report should be valuable in further analysis of mannosyl glycoproteins

and concanavalin A binding sites on the surface of cultured fibroblasts, especially because the glycoproteins with stronger affinity to concanavalin A (those extracted by SDS) are likely to play important roles upon interaction of concanavalin A with fibroblasts.

ACKNOWLEDGEMENTS

The author wishes to express his sincere gratitude to Professor A. Kobata and Associate Prof. T. Muramatsu for their valuable suggestion and generous supports during the course of this work and helpful criticism of the manuscript. He also wishes to thank his colleagues in his laboratory for their encouragement and stimulation.

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