Isolation of a Concanavalin A Receptor from Mouse L Cells†

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ABSTRACT: A cell surface glycoprotein receptor for concanavalin A (Con A) has been isolated from mouse L cells. The isolation procedure involved dissolving whole L cells in 0.3 M lithium diiodosalicylate and extracting with aqueous phenol. The Con A receptor, which was found in the aqueous phase of this extract, was further purified by affinity chromatography on a column of Con A-Sepharose; the receptor was adsorbed to Con A-Sepharose and eluted with 0.1 M methyl α-D-glucopyranoside or with 0.1 M methyl α-D-mannopyranoside, but not with other monosaccharides. The cell surface location of the Con A receptor purified in this way was confirmed by showing that it can be isolated from purified L cell plasma membranes and by demonstrating that it can be labeled from the exterior surface of intact L cells by the nonpenetrating galactose oxidase-KB^3H_4 system. Biochemical studies of the Con A receptor have shown that it migrates on sodium dodecyl sulfate-polyacrylamide gels as a single component having an apparent molecular weight of approximately 100,000. Its N-terminal amino acid is valine and it has carbohydrate attached at several (at least five) different sites along the polypeptide chain.

It has been known for many years that the outer surface of mammalian cells is coated with a "fuzzy," carbohydrate-containing layer or glycocalyx which is composed mostly of glycoprotein. Although no biological function for this layer has yet been firmly established, circumstantial evidence has implicated the surface glycoproteins in many different cellular functions including cell–cell recognition processes (Kemp *et al.*, 1973), the regulation of cell growth (Noonan and Burger, 1970), and maintenance of plasma membrane structure (Bretscher, 1973). In attempting to distinguish among these and other possibilities we have taken the view that it would be of use to isolate individual cell surface glycoproteins in pure form suitable for further biochemical analysis. One could then, for instance, examine them structurally and assay them for enzymatic activity. To this end we have initiated studies designed to identify and isolate the surface glycoproteins of a model cell type, mouse L cells. Here we show how one L cell surface glycoprotein, a cell surface receptor for concanavalin A, can be extracted from whole L cells by the lithium diiodosalicylate method (Marchesi and Andrews, 1971), and purified by affinity chromatography on a column of Con A-Sepharose. Biochemical studies on the Con A [1] receptor isolated in this way were then undertaken.

Materials and Methods

(*a*) *Cell Growth.* Monolayer cultures of mouse L-929 cells were grown in 100-mm Falcon plastic tissue culture plates as described previously (Hunt and Brown, 1974). Cellular proteins and glycoproteins were selectively labeled by including 4 μCi/ml of D-[^{3}H]glucosamine (Amersham, 25 Ci/mmol) and 2 μCi/ml of ^{14}C-labeled amino acid mixture (Calatomic Chlorella hydrolysate, 2 Ci/g) in the growth medium. For some experiments cells were grown in the presence of 1 μ Ci/ml of D-[^{14}C]glucosamine (New England Nuclear, 56 Ci/ml) only. Cells were grown for 48 hr (two cell generations) in the presence of radioactive precursors to a final density of approximately 3×10^6 cells/plate. 98% or greater of the cells were found by Trypan Blue exclusion to be live at this state; they were harvested by scraping the monolayer with a rubber policeman.

(*b*) *Preparation of Con A Receptor.* (i) LIS SOLUBILIZATION AND PHENOL EXTRACTION. Preparation of the Con A receptor was carried out beginning with between 3×10^7 and 6×10^7 L cells labeled by growth in the presence of radioactive precursors. These cells were first washed twice in Dulbecco's phosphate-buffered saline (PBS) and then dissolved in 10 ml of 0.3 M lithium diiodosalicylate (Eastman-Kodak) containing 4 mM phenylmethanesulfonyl fluoride (Sigma Chemical Co.), 0.5 mg/ml of DNase I, 0.5 mg/ml of RNase A and 0.05 M Tris-HCl buffer (pH 7.6). This solution was stirred at room temperature for 30 min or until the viscosity decreased, diluted with two volumes (20 ml) of deionized water, and then stirred for a further 15 min at 4°. The cells were completely dissolved at this stage. An equal volume (30 ml) of ice cold 50% (w/v) phenol was then added to the LIS solubilized cells and the mixture was shaken for 15 min at 4°. The resulting emulsion was centrifuged at 4000*g* for 15 min at 4° to separate the aqueous and phenolic phases. The aqueous phase was removed and the phenolic phase was reextracted with 15 ml of water. The two aqueous phases were combined and dialyzed against deionized water for 48 hr. Likewise, the phenolic phase was also dialyzed against deionized water. The total amount of radioactivity present in the dialyzed aqueous and phenolic phases was determined by counting a 1-ml aliquot in 10 ml of a liquid medium containing 1:2 Triton X-100–toluene using a Packard 3320 liquid scintillation counter. When samples contained both ^{3}H and ^{14}C labels the results were corrected for "spillover" of ^{14}C counts in the ^{3}H channel. After dialysis the aqueous phase, which was found to contain the Con A receptor, was concentrated by rotary evaporation.

(ii) SEPHADEX G-200 CHROMATOGRAPHY. After concentration the aqueous phase from the LIS–phenol extraction step was dissolved in 7 ml of 0.3% NaCl and 0.05% NaN_3 and applied to a 2.5 × 75 cm column of Sephadex

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[1] Abbreviations used are: PBS, phosphate-buffered saline; Con A, concanavalin A; LIS, lithium diiodosalicylate; α-MG, methyl α D-glucopyranoside.

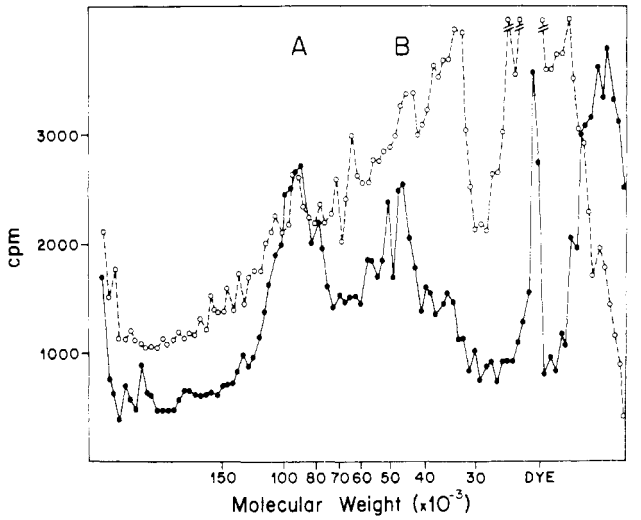

FIGURE 1: Polyacrylamide gel electrophoresis of whole L cells dissolved in 1% sodium dodecyl sulfate and 1% 2-mercaptoethanol. Cells were radioactively labeled by growth in the presence of D-[^{3}H]glucosamine (—) and ^{14}C-amino acids (- - -).

G-200 which had been equilibrated with the same solution. The equilibration solution was also used to elute the column and 5-ml fractions of eluate were collected. Emergence of glycoprotein material from the column was monitored by counting an aliquot of each fraction as described above. Excluded and retarded peaks of the column eluate were pooled separately, dialyzed against deionized water at 4° overnight, and concentrated. Aliquots of the concentrated fractions were used for sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

(iii) CON A SEPHAROSE AFFINITY CHROMATOGRAPHY. The dried excluded fraction from the Sephadex G-200 column, which was called the "crude class A glycoprotein" fraction, was dissolved in 0.05 M Tris-HCl (pH 7.6) containing 10^{-3} M $CaCl_2$, $MgCl_2$, and $MnCl_2$ (CMM buffer), and applied to a 1 × 10 cm column of Con A-Sepharose which had been equilibrated with CMM buffer. The column was then washed with CMM buffer until no further radioactivity emerged from the column; the amount of glycoprotein material which did not adsorb to the column was determined by counting an aliquot of the eluate at this stage. The Con A receptor was then eluted from the column with 50 ml of 0.1 M methyl α-D-glucopyranoside (α-MG) in CMM buffer. The amount of receptor eluted was determined by counting an aliquot of this fraction as described above. Finally, the Con A receptor was freed of α-MG by dialysis against deionized water for 1 week at 4° and lyophilized.

(*c*) *Polyacrylamide Gel Electrophoresis.* Whole cells and fractions obtained during glycoprotein extraction and purification were dissolved in 1% sodium dodecyl sulfate and 1% 2-mercaptoethanol and boiled for 2 min. Polyacrylamide gel electrophoresis of these fractions, slicing the gels, and counting the slices were carried out as previously described (Hunt and Brown, 1974).

The glycopeptides produced by CNBr digestion of the Con A receptor were analyzed by polyacrylamide gel electrophoresis according to the procedure of Swank and Munkres (1971). The dried CNBr digest (prepared as described in the text) was dissolved in 100 μl of 8M urea containing 1% sodium dodecyl sulfate, 1% 2-mercaptoethanol, and 0.01 M Tris–phosphoric acid buffer (pH 6.8). The sample was boiled for 2 min and then subjected to electrophoresis on a 9.5% polyacrylamide gel containing 8M urea, 0.1% sodium dodecyl sulfate, and 0.01 M Tris–phosphoric acid buffer (pH 6.8). The 5 × 140 mm gels were run for 9 hr at 2 mA/gel and then sliced and counted as usual.

(*d*) *Amino acid analyses* were performed beginning with 400–600 μg of purified Con A receptor. This material was dissolved in 0.5 ml of constant boiling 6N HCl and hydrolyzed for 16 hr in a sealed, evacuated tube. Excess HCl was removed by lyophilization and the amino acid composition of the hydrolysate was determined using a Beckman Model 120C automatic amino acid analyzer.

(*e*) *N-Terminal Amino Acid Determination.* The N-terminal amino acid of the Con A receptor was determined beginning with 500 μg of purified receptor. This was dissolved in 100 μl of 0.1 M $NaHCO_3$ and mixed with 100 μl of 1 mg/ml dansyl chloride (Sigma Chemical Co.) in acetone. The resulting solution was incubated for 6 hr at room temperature and dried *in vacuo*. The dried, dansylated protein was then dissolved in 0.5 ml of constant boiling 6N HCl and hydrolyzed for 12 hr at 105° in a sealed, evacuated tube. HCl was then removed by lyophilization and the hydrolysate was dissolved in chloroform. Fluorescent components of the hydrolysate were identified by their chromatographic mobility on silica gel G thin-layer plates developed with three solvent systems, 30:10:1 chloroform–benzyl alcohol–glacial acetic acid (David *et al.*, 1963), 75:20:5 chloroform–methanol–glacial acetic acid (Seiler and Wiechmann, 1964), and 30:50:20:5 chloroform–ethyl acetate–methanol–glacial acetic acid (Seiler and Wiechmann, 1964).

(*f*) *Other Procedures.* Cells were labeled by the galactose oxidase–KB^3H_4 method as previously described (Hunt and Brown, 1974). Potassium borotritide (6.3 Ci/mmol) used in this procedure was obtained from Amersham. Con A-Sepharose was prepared from Sepharose 4B (Pharmacia) and Con A (Miles Laboratories) by the method of Dufau *et al.* (1972). ATPase activity was measured by the method of Munoz *et al.* (1968); glucose 6-phosphatase was assayed by a similar procedure in which 2 mM glucose 6-phosphate was substituted for ATP. 5′-Nucleotidase was assayed by the method of Bosmann and Pike (1971).

Results

(*a*) *Extraction of Class A Glycoproteins.* The glycoprotein species of mouse L cells can be separated into two molecular weight classes, class A and class B, by electrophoresis on sodium dodecyl sulfate–polyacrylamide gels. For example, Figure 1 shows the results obtained when 3 × 10^6 L cells grown in the presence of D-[^{3}H]glucosamine and ^{14}C-labeled amino acids were dissolved in 1% sodium dodecyl sulfate and 1% 2-mercaptoethanol and subjected to electrophoresis on a 7% polyacrylamide gel containing 0.1% sodium dodecyl sulfate. The distribution of ^{3}H label (solid line) on the gel indicates the positions of class A (MW approximately 100,000) and class B (MW approximately 50,000) glycoproteins, respectively. Our previous studies of L cells have suggested that both class A and class B glycoproteins are heterogeneous in the sense that they consist of more than one species of glycopolypeptide chain and that most, if not all, class A and class B glycoproteins are exposed on the outer surface of the cell. For example, both classes are labeled when intact cells are reacted with the nonpenetrating galactose oxidase–KB^3H_4 probe. (Hunt and Brown, 1974).

The strategy adopted for isolating the L cell Con A receptor was first to prepare a crude glycoprotein fraction

TABLE I: Summary of the Isolation Procedure for Class A Glycoproteins.

	^{3}H cpm Derived from D-[^{3}H]-Glucosamine	%	^{14}C cpm Derived from ^{14}C-Amino Acids	%
1. LIS-solubilized whole cells	406,500	100	260,700	100
2. Phenol extraction of LIS-solubilized cells				
(a) Phenol phase[a]	233,600	57	198,000	77
(b) Aqueous phase[a]	155,700	38	41,900	16
3. Sephadex G-200 chromatography of aqueous phase				
(a) Excluded fraction[b]	53,500	13	11,000	4
(b) Retarded fraction[b]	102,300	25	29,700	11

[a] These values were determined as described under Materials and Methods. [b] These values were determined after pooling excluded and retarded fractions separately.

from whole L cells using the lithium diiodosalicylate (LIS) method of Marchesi and Andrews (1971). The Con A receptor would then be purified from this crude glycoprotein extract by affinity chromatography on a column of Con A-Sepharose; the receptor should be adsorbed to Con A-Sepharose and eluted with methyl α-D-glucopyranoside (α-MG), one of the specific haptens for Con A.

Experimentally, the LIS extraction step was carried out beginning with 5×10^7 L cells which had been grown for two generations in the presence of D-[^{3}H]glucosamine and ^{14}C-labeled amino acids. Cells were harvested by scraping and dissolved in 10 ml of 0.3 M LIS containing 4 mM phenylmethanesulfonyl fluoride, 0.5 mg/ml of DNase, 0.5 mg/ml of RNase, and 0.05 M Tris-HCl buffer (pH 7.6). The solubilized cells were then extracted with aqueous phenol as described under Materials and Methods. The yield of glycoprotein material obtained by this extraction procedure is shown in Table I. Lines 2a and 2b, for instance, indicate that 38% of the original ^{3}H-labeled material was found in the aqueous phase after LIS–phenol extraction while 57% was found in the phenol phase or at the interface between the two layers. Line 2b also shows that the ratio of ^{3}H cpm/^{14}C cpm in the aqueous phase was increased approximately 2.3-fold over the starting material. This suggests that the aqueous phase was, in fact, enriched in glycoprotein material.

Glycoproteins in the aqueous phase were then further purified by chromatography on a column of Sephadex G-200; high molecular weight glycoproteins were found in the excluded volume of the column eluate while smaller glycoproteins and glycopeptides were found in the retarded fraction. Lines 3a and 3b of Table I show that approximately one-third of the ^{3}H label applied to the column was recovered in the excluded fraction while two-thirds was found in the retarded peak. A further increase in the ^{3}H cpm/^{14}C cpm ratio was obtained at this stage and the overall yield with respect to the starting material was 13%.

The glycoprotein species extracted in this way were then analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Aliquots of the excluded and of the retarded fractions from the Sephadex G-200 column eluate were dialyzed against deionized water and taken to dryness by lyophilization. They were then dissolved in 1% sodium dodecyl sulfate and 1% 2-mercaptoethanol and subjected to electrophoresis on polyacrylamide gels containing sodium dodecyl sulfate as described above. The results of this experiment, as shown in Figure 2, indicate that the excluded fraction from the Sephadex G-200 column contained mostly class A glycoproteins (Figure 2a). There was very little contamination with class B glycoproteins (which were extracted into the phenol phase after the LIS–phenol step) or with other cellular components. The excluded fraction will, therefore, be referred to as "crude class A glycoprotein." As shown in Figure 2a the crude class A glycoprotein fraction was found to contain both ^{3}H label derived from D-[^{3}H]glucosamine and ^{14}C label derived from ^{14}C-labeled amino acids.

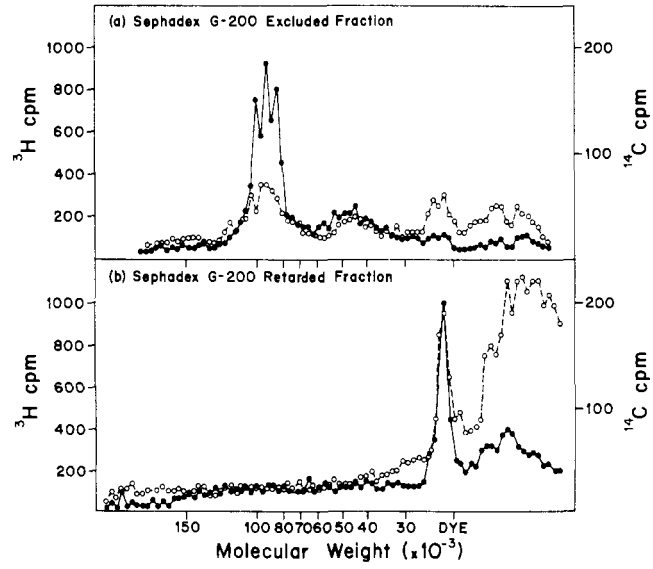

FIGURE 2: Polyacrylamide gel electrophoresis of the excluded (a) and retarded (b) fractions of the Sephadex G-200 column eluate. L cells grown in the presence of D-[^{3}H]glucosamine (—) and ^{14}C-amino acids (- - -) were extracted by the LIS–phenol procedure as described in Materials and Methods. The aqueous phase of this extract was then subjected to chromatography on a column of Sephadex G-200; excluded and retarded fractions of the column eluate were pooled and analyzed by gel electrophoresis.

In contrast to the excluded fraction, the retarded fraction from the Sephadex G-200 column (Figure 2b) was found to consist only of low molecular weight glycopeptides which migrate at the Bromophenol Blue dye region of the gel and glycolipids which migrate just ahead of the dye. No class A or class B glycoproteins were found in this fraction.

(*b*) *Purification of the Con A Receptor.* The Con A re-

TABLE II: Fractionation of Class A Glycoproteins by Affinity Chromatography on a Column of Con A-Sepharose.

	^{3}H cpm Derived from D-[^{3}H]-Glucosamine	%	^{14}C cpm Derived from ^{14}C-Amino Acids	%
1. Total cpm applied to column	197,600	100	51,500	100
2. cpm bound to Con A-Sepharose and				
(a) Eluted with 0.1 M α-MG	62,000	31	10,900	21
(b) Not eluted from column	103,000	52	24,400	47
3. cpm not bound to Con A-Sepharose	32,500	16	13,500	26

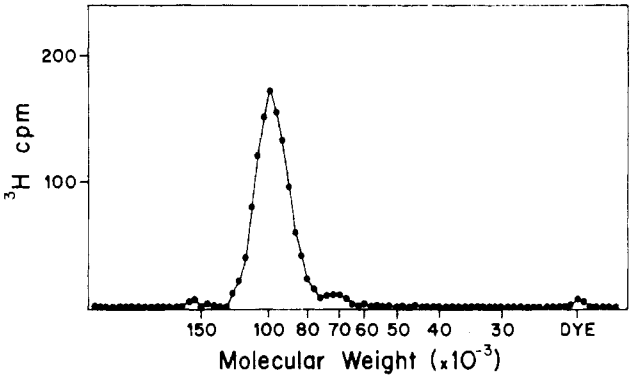

FIGURE 3: Polyacrylamide gel electrophoresis of the purified Con A receptor. The receptor was prepared as described under Materials and Methods from cells which had been grown in the presence of D-[^{3}H]glucosamine.

ceptor was purified from the crude class A glycoprotein fraction by affinity chromatography on a column of Con A-Sepharose. Crude class A glycoproteins were prepared as described above from 6×10^7 L cells labeled by growth in the presence of D-[^{3}H]glucosamine and ^{14}C-labeled amino acids. The crude class A glycoprotein fraction which was found to contain 197,600 ^{3}H cpm and 51,500 ^{14}C cpm in a total volume of 40 ml was then dialyzed overnight against 0.05 M Tris-HCl buffer (pH 7.6) containing 1 mM $CaCl_2$, $MgCl_2$, and $MnCl_2$ (CMM buffer) and applied directly to a 1×10 cm column of Con A-Sepharose. After the sample was applied the column was washed thoroughly with CMM buffer. The Con A receptor was then eluted with 50 ml of 0.1 M α-MG in CMM buffer. Table II shows the yield of glycoprotein obtained by this procedure. Lines 2 and 3 indicate that of the ^{3}H cpm applied to the column 83% was adsorbed to Con A-Sepharose while 16% passed through the column. Line 2a shows that a fraction containing 31% of the ^{3}H label and 21% of the ^{14}C was bound to the column and eluted with 0.1 M α-MG. This is the Con A receptor preparation whose properties we have examined. After dialysis to remove α-MG, this preparation was analyzed by electrophoresis on a sodium dodecyl sulfate-polyacrylamide gel. The result shown in Figure 3 indicates that the receptor migrated as a single component on the gel and that it had an apparent molecular weight of approximately 100,000. A similar gel stained by the periodic acid-Schiff method (Zacharius *et al.*, 1969) led to the same conclusion. These results are consistent with the Con A receptor's being a class A glycoprotein species.

The specificity of interaction between Con A receptor and Con A-Sepharose was verified by testing whether monosaccharide groups other than α-MG would elute the receptor from Con A-Sepharose. These tests were performed

TABLE III: Specificity of Interaction between Con A Receptor and Con A-Sepharose.[a]

Wash Solution	% ^{3}H cpm Eluted
0.1 M D-fucose	<1
0.1 M *N*-acetyl-D-glucosamine	<1
0.1 M D-galactose	<1
0.1 M α-methyl D-glucopyranoside	92
0.1 M α-methyl D-mannopyranoside	98

[a] At least 4000 cpm of ^{3}H-labeled Con A receptor was adsorbed to 1×10 cm columns of Con A-Sepharose. Columns were first washed with five column volumes of CMM buffer and then with two column volumes of each of the monosaccharide solutions listed above in CMM buffer. The amount of Con A receptor eluted from the columns was determined by counting an aliquot of the column effluent in a liquid medium as described in Materials and Methods.

by preparing ^{3}H-labeled Con A receptor as described above from cells which had been grown in the presence of D-[^{3}H]glucosamine. The receptor was then adsorbed to a column of Con A-Sepharose and the column was washed with 0.1 M solutions of various monosaccharides in CMM buffer. Elution of the Con A receptor from the column was monitored by counting fractions of the column effluent. The results of these experiments, as shown in Table III, indicate that of the sugars tested only methyl α-D-mannopyranoside and methyl α-D-glucopyranoside were able to elute the receptor from Con A-Sepharose. Since Con A binds specifically to α-mannosyl and α-glucosyl groups in glycoproteins (Poretz and Goldstein, 1970) this suggests that Con A interacts with the Con A receptor through its specific saccharide binding site.

(*c*) *Cell Surface Location of the Con A Receptor.* Two types of experiments were carried out to verify that the Con A receptor is actually located on the outer cell surface. First, a purified plasma membrane fraction from L cells was examined for the presence of the Con A receptor. The membrane fraction employed in these studies was prepared by the $ZnCl_2$ method of Warren *et al.* (1967). In agreement with these authors, the membrane fraction was found by phase contrast microscopy to consist of sheets of membrane and by enzymatic analysis to be enriched in several enzyme activities characteristic of plasma membranes. For example, the membrane fraction was enriched fivefold over the homogenate in the specific activity (measured as enzyme activity per milligram of protein) of Mg^+-dependent ATPase. Enrichment was eightfold in the case of Na^+-K^+-

TABLE IV: Con A-Sepharose Chromatography of Class A Glycoproteins Isolated from L Cell Plasma Membranes.[a]

	^{3}H cpm Derived from D-[^{3}H]Glucosamine	%
1. Total cpm applied to column	11,600	100
2. cpm bound to Con A-Sepharose and		
(a) Eluted with 0.1 M α-MG	6,200	51
(b) Not eluted from column	<200	
3. cpm not bound to Con A-Sepharose	5,900	49

[a] 11,600 cpm of crude glass A glycoproteins prepared from 49,000 ^{3}H cpm of purified L cell plasma membranes was applied to a 1 × 10 cm column of Con A-Sepharose which had been equilibrated with CMM buffer. The column was washed with two column volumes of CMM buffer and the amount of glycoprotein material not bound to the column was determined by counting an aliquot of the column effluent at this stage. The column was then eluted with two column volumes of 0.1 M α-MG in CMM buffer and the amount of eluted glycoprotein determined by counting.

stimulated, ouabain-inhibited ATPase and threefold in the case of 5′ nucleotidase. Conversely, the membrane fraction was found to be depleted in enzymatic activities characteristic of the smooth endoplasmic reticulum fraction. For example, no glucose 6-phosphatase activity could be assayed in the membrane fraction although the cell homogenate had an easily detectable level.

Examination of plasma membranes for the presence of Con A receptor was carried out beginning with membranes prepared from 5 × 10^7 cells which had been labeled by growth in the presence of D-[^{3}H]glucosamine. These membrane preparations were dissolved in 0.3 M LIS, extracted with phenol, and subjected to Sephadex G-200 column chromatography as described above. The resulting crude class A glycoprotein fraction was then analyzed for the presence of the Con A receptor by affinity chromatography on a column of Con A-Sepharose. The result of this analysis as shown in Table IV indicates that 51% of the glycoprotein material isolated from plasma membranes was eluted from Con A-Sepharose with 0.1 M α-MG in the Con A receptor fraction. This material was further identified as authentic Con A receptor by its mobility on sodium dodecyl sulfate–polyacrylamide gels; it migrated coincidentally with Con A receptor prepared from whole cells. This result suggests that at least some Con A receptor is associated with the plasma membrane and that it can be isolated from membranes by the same method used to isolate it from whole cells.

The second method employed for showing that the Con A receptor is actually located on the cell surface involved isolating the receptor from cells which had previously been labeled by the galactose oxidase–KB^{3}H$_4$ method of Gahmberg and Hakomori (1973). According to this procedure intact cells are treated with the enzyme galactose oxidase which oxidizes to aldehydes the C-6 hydroxyl functions of exposed galactose and *N*-acetylgalactosamine groups. The aldehydes are then reduced back to hydroxyls with KB^{3}H$_4$. Since the enzyme galactose oxidase does not penetrate into the interior of the cell, only cell surface glycoproteins and glycolipids are labeled by this method. Internal glycoproteins would not be labeled. Thus, the Con A receptor isolated from cells treated in this way ought to be radioactively labeled only if the receptor itself is exposed on the outer cell surface.

This experiment was carried out beginning with 3 × 10^7 L cells which had been grown for two generations in the presence of D-[^{14}C]glucosamine as an internal label for cellular carbohydrate-containing structures. These cells were reacted with the galactose oxidase–KB^{3}H$_4$ system as previously described (Hunt and Brown, 1974). As before, we found that optimal labeling by this method was obtained when cells had been treated briefly with neuraminidase before exposure to galactose oxidase. Crude class A glycoproteins were prepared from the galactose oxidase–KB^{3}H$_4$ labeled cells by LIS–phenol extraction followed by Sephadex G-200 chromatography as described above. This fraction, which was found to contain 21,500 ^{14}C cpm derived from D-[^{14}C]glucosamine and 24,000 ^{3}H cpm derived from KB^{3}H$_4$, was dialyzed against CMM buffer and applied to a column of Con A-Sepharose. The Con A receptor fraction was then eluted from the column with 0.1 M α-MG. The yield of glycoprotein material at this stage of the preparation is presented in Table V. Line 2a shows that 58% of the ^{3}H-labeled glycoprotein applied to the column was found in the Con A receptor fraction. That is, it was bound to Con A-Sepharose and eluted with 0.1 M α-MG; 21% of the ^{14}C label was found in the same fraction. This material was further identified as Con A receptor by the fact that it migrated coincidentally with the Con A receptor on a sodium dodecyl sulfate–polyacrylamide gel. Also, digestion of this material with CNBr followed by electrophoresis of the digest on a sodium dodecyl sulfate–polyacrylamide gel revealed the presence of CNBr glycopeptide subclasses with

TABLE V: Con A-Sepharose Chromatography of Class A Glycoproteins Labeled from the Exterior Cell Surface by the Galactose Oxidase–KB^{3}H$_4$ Method.[a]

	^{14}C cpm Derived from D-[^{14}C]Glucosamine	%	^{3}H cpm Derived from KB^{3}H$_4$	%
1. Total cpm applied to column	21,500	100	24,000	100
2. cpm bound to Con A-Sepharose and				
(a) Eluted with 0.1 M α-MG	4,400	21	14,000	58
(b) Not eluted from column	6,600	31	500	2
3. cpm not bound to Con A-Sepharose	10,300	48	9,400	39

[a] This experiment was carried out as described in Table IV.

TABLE VI: Amino Acid Composition of the Con A Receptor.

Amino Acid	Moles of Amino Acid/Mole of Methionine	Amino Acid	Moles of Amino Acid/Mole of Methionine
Lysine	7.20	Glycine	12.29
Histidine	2.36	Alanine	9.09
Arginine	1.82	Valine	4.49
Aspartic Acid	8.19	Methionine	1.00
Threonine	6.30	Isoleucine	3.43
Serine	12.56	Leucine	5.49
Glutamic Acid	11.07	Tyrosine	2.12
Proline	4.29	Phenylalanine	2.14

electrophoretic mobilities similar to those of authentic Con A receptor as described below. These results suggest that the Con A receptor itself is labeled by the galactose oxidase-KB^3H_4 method and that at least some Con A receptor is ordinarily exposed on the outer cell surface.

(*d*) *Properties of the Con A Receptor.* After establishing a reproducible procedure for purifying the Con A receptor, we began to examine its biochemical properties. Studies were carried out to determine the amino acid composition of the Con A receptor, to identify its N-terminal amino acid, and to decide whether carbohydrate moieties are attached at one or at more than one site along the polypeptide chain. The amino acid composition of the Con A receptor was determined beginning with receptor prepared from 5×10^8 L cells. The isolation procedure for this preparation was the same as described above except that the volumes of solutions at the LIS solubilization and phenol extraction stages were increased threefold. After purification by Con A-Sepharose affinity chromatography, the Con A receptor was desalted by dialysis against deionized water and lyophilized; 500 μg of lyophilized receptor was then dissolved in 0.5 ml of constant boiling 6 N HCl, hydrolyzed, and analyzed for its content of individual amino acids as described in Materials and Methods. The results, expressed as moles of amino acid per mole of methionine, are given in Table VI. They show that the Con A receptor contains significant amounts of two amino acids, serine and threonine, which may contain carbohydrate side chains in the intact molecule. The hydrolysate was also found to contain a significant amount of aspartic acid a portion of which may have arisen as the HCl hydrolysis product of asparagine to which carbohydrate may also be attached. No cysteic acid, the HCl hydrolysis product of cysteine, was found in the hydrolysate.

The N-terminal amino acid residue of the Con A receptor was identified by the dansyl chloride method of Gray and Hartley (1963); 500 μg of desalted, lyophilized receptor was reacted with dansyl chloride and hydrolyzed as described under Materials and Methods. The hydrolysate was then analyzed by thin-layer chromatography on activated silica gel G plates. Spots corresponding to dansylated amino acids were localized by their fluorescence when viewed under ultraviolet light. Chromatography of the hydrolysate in three different solvent systems revealed the presence of only one amino acid dansylated at the α-amino group, dansylvaline. We conclude, therefore, that the Con A receptor has a valine residue at its amino terminus.

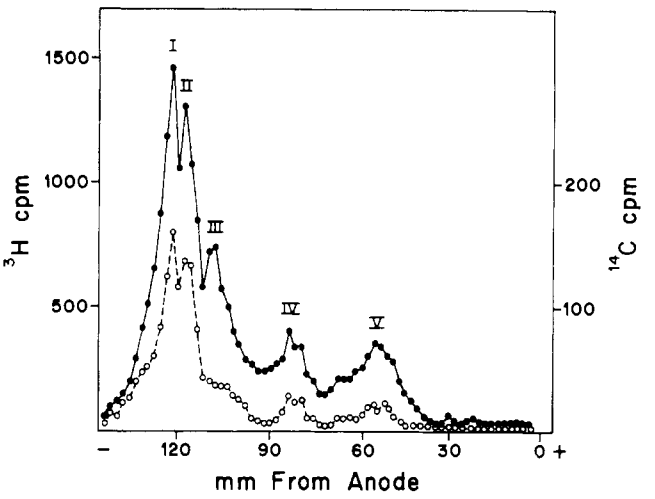

FIGURE 4: Polyacrylamide gel electrophoresis of the Con A receptor digested with CNBr. The Con A receptor was prepared from cells which had been grown in the presence of D-[^{3}H]glucosamine (—) and ^{14}C-amino acids (- - -). The receptor was digested with CNBr as described in the text.

Finally, in order to determine whether there is one or more than one site where carbohydrate is attached to the polypeptide, the Con A receptor was digested with CNBr and then analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Since CNBr cleaves the polypeptide chain specifically at methionine residues, this digest should contain more than one glycopeptide species only if the intact receptor itself has carbohydrate attached at more than one site along the polypeptide chain. This experiment was carried out beginning with approximately 100 μg of Con A receptor prepared as previously described from 5×10^7 L cells which had been grown in the presence of D-[^{3}H]glucosamine and ^{14}C-labeled amino acids. The desalted, lyophilized receptor was dissolved in 0.1 ml of 70% formic acid containing 0.1 mg of CNBr and incubated for 48 hr at room temperature. This solution was then diluted with 0.9 ml of deionized water and dried by lyophilization (Steers *et al.*, 1965). The dried CNBr digest was then analyzed by Sephadex G-200 column chromatography and by electrophoresis on 9.5% polyacrylamide gels according to the procedure of Swank and Munkres (1971). Sephadex G-200 chromatography revealed that all of the glycoprotein material in the digest was found in the retarded volume of the column eluate; none was found in the excluded fraction where the intact Con A receptor is ordinarily eluted. This indicates that all Con A receptor molecules present had been cleaved by the CNBr digestion procedure. Electrophoresis of the CNBr digest on 9.5% polyacrylamide gels led to the separation of five glycopeptide subfractions numbered I to V as shown in Figure 4; all five glycopeptide subfractions were found to contain ^{3}H label derived from D-[^{3}H]glucosamine as well as ^{14}C label derived from ^{14}C-labeled amino acids. No peptides were found to contain ^{14}C label only. These results indicate, first, that there must be at least four methionine residues in the intact Con A receptor molecule and, second, since all five glycopeptide subclasses were found to contain ^{3}H label there must be at least five separate carbohydrate side chains attached at different sites along the polypeptide chain.

Discussion

A very straightforward method has been employed here to isolate a cell surface Con A receptor from mouse L cells.

A crude glycoprotein fraction was first prepared by dissolving whole L cells in 0.3 M LIS and extracting with aqueous phenol; glycoproteins found in the aqueous phase of this extract were then freed of contaminating small molecules by chromatography on a column of Sephadex G-200. The Con A receptor was then purified from this crude glycoprotein preparation by affinity chromatography on a column of Con A-Sepharose. The receptor was adsorbed to Con A-Sepharose and eluted with 0.1 M α-MG, one of the specific haptenic groups for Con A. This procedure is quite a general one that ought to be useful for isolating Con A receptors from other cell types. In fact, quite similar methods have already been employed for isolating Con A receptors from human platelet (Nachman *et al.*, 1973), Ehrlich ascites carcinoma (F. Aull, personal communication), and pig lymphocyte (Allan *et al.*, 1972) cells. This procedure is also general in the sense that it can be easily modified for the isolation of cell surface receptors for other lectin agglutinins. One would simply carry out affinity chromatography on columns, for instance, of wheat germ agglutinin-Sepharose or soybean agglutinin-Sepharose instead of Con A-Sepharose. Studies of this type have already led to the isolation of wheat germ agglutinin receptors from L1210 cells (Jansons and Burger, 1973).

The cell surface location of the Con A receptor isolated in this way was verified by preparing it from purified L cell plasma membranes and by showing that it can be labeled from the exterior cell surface by the nonpenetrating galactose oxidase-KB^3H_4 system. Together these results suggest that the receptor molecule is associated with the plasma membrane in such a way that at least some of its carbohydrate groups are exposed to the external environment of the cell. It is these exposed carbohydrate groups that are most likely to constitute the cellular binding sites for Con A. A rough estimate of the number of Con A receptor molecules present on the cell surface can be made from the yield of receptor obtained by the procedure described above. We estimate that one can prepare 1 mg of Con A receptor from 6×10^8 cells. This implies that there are 10^7 Con A receptor molecules per cell or enough to cover approximately 5% of the surface of a growing L cell.

Biochemical studies of the Con A receptor have shown that it migrates as a single component during electrophoresis on sodium dodecyl sulfate-polyacrylamide gels, that its N-terminal amino acid residue is valine and that it has carbohydrate groups attached at several (at least five) different sites along the polypeptide chain. Probably the best estimate for the molecular weight of the Con A receptor is the value of 100,000 derived from its electrophoretic mobility on sodium dodecyl sulfate-polyacrylamide gels although this value can only be regarded as approximate. The preponderance of biochemical evidence suggests that the Con A receptor fraction consists of only a single species of glycopeptide chain. For example, it is most likely that the gel electrophoresis, N-terminal amino acid analysis, and CNBr digestion studies would each have given a more complex result if the Con A receptor fraction actually consisted of more than one species of glycoprotein. In addition, attempts were unsuccessful to resolve the receptor into subfractions by chromatography on a Sepharose 4B column (R. Hunt and J. Brown, unpublished observations). It is clear, however, that the Con A receptor we have isolated need not be the only species of Con A receptor present on the L cell surface. For example, there may be other Con A receptors among the class B cell surface glycoproteins which we have not examined here.

Finally, since many mammalian cell types bind Con A on their exterior surfaces, it is of interest to ask how their Con A receptors will compare to the one we have isolated from L cells. One is especially interested in characterizing the Con A receptors of cells for which Con A has special biological effects. For example, Con A is mitogenic for T lymphocytes and it selectively agglutinates oncogenically transformed but not normal cells. Studies on Con A receptors of these cell types represent areas of research where the methods described here ought to be most useful.

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References

Allan, D., Auger, J., and Crumpton, M. (1972), *Nature (London), New Biol. 236,* 23.

Bosmann, H. B., and Pike, G. Z. (1971), *Biochim. Biophys. Acta 227,* 402.

Bretscher, M. S. (1973), *Science 181,* 622.

David, I. B., French, T. C., and Buchanan, J. M. (1963), *J. Biol. Chem. 238,* 2178.

Dufau, M. L., Tsuruhara, T., and Catt, K. J. (1972), *Biochim. Biophys. Acta 278,* 281.

Gahmberg, C. G., and Hakomori, S. (1973), *J. Biol. Chem. 248,* 4311.

Gray, W. R., and Hartley, B. H. (1963), *Biochem. J. 89,* 59.

Hunt, R. C., and Brown, J. C. (1974), *Biochemistry 13,* 22.

Jansons, V. K., and Burger, M. M. (1973), *Biochim. Biophys. Acta 291,* 127.

Kemp, R. B., Lloyd, S. W., and Cook, G. (1973), *Progr. Surface Membrane Sci. 7,* 271.

Marchesi, V. T., and Andrews, E. P. (1971), *Science 174,* 1247.

Munoz, E., Freer, J. H., Ellar, D., and Salton, M. (1968), *Biochim. Biophys. Acta 150,* 531.

Nachman, R. L., Hubbard, A., and Ferris, B. (1973), *J. Biol. Chem. 248,* 2928.

Noonan, K. D., and Burger, M. M. (1970), *Nature (London) 228,* 512.

Poretz, R. D., and Goldstein, I. J. (1970), *Biochemistry 9,* 2890.

Seiler, N., and Wiechmann, J. (1964), *Experientia 20,* 559.

Steers, E., Craven, G. R., Anfinsen, C. B., and Bethune, J. (1965), *J. Biol. Chem. 240,* 2478.

Swank, R. T., and Munkres, K. D. (1971), *Anal. Biochem. 39,* 462.

Warren, L., Glick, M. C., and Nass, M. (1967), *in* The Specificity of Cell Surfaces, Davis, B., and Warren, L., Ed., Englewood Cliffs, N.J., Prentice-Hall, p 109.

Zacharius, R. M., Zell, T. E., Morrison, J. H., and Woodlock, J. J. (1969), *Anal. Biochem. 30,* 148.