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INTERACTION OF CONCANAVALIN A WITH RABBIT THYMOCYTE PLASMA MEMBRANES

DISTINCTION BETWEEN LOW AFFINITY ASSOCIATION AND POSITIVELY COOPERATIVE BINDING MEDIATED BY A SPECIFIC GLYCOPROTEIN

R. SCHMIDT-ULLRICH*, D. F. H. WALLACH and J. HENDRICKS

Tufts-New England Medical Center, Radiobiology Division, 171 Harrison Avenue, Boston, Mass. 02111 (U.S.A.)

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SUMMARY

1. We have analyzed the interaction of the mitogenic lectin, concanavalin A, with purified plasma membranes isolated from rabbit thymocytes.

2. Scatchard analyses show that in native membranes binding is positively cooperative at low concanavalin A concentrations and non-interacting at high lectin levels.

3. In contrast, membranes treated with 0.0064 M glutaraldehyde exhibit diphasic Scatchard plots, indicating the presence of high- and low-affinity binding sites. The high-affinity zone corresponds to the region of positive cooperativity in native membranes.

4. The number of high-affinity binding sites per cell-equivalent corresponds approximately to the number of glycoprotein (mol. wt. 55 000) molecules ($1 \cdot 10^6$/cell), but account for $< 25\,\%$ of the total lectin binding.

5. Treatment of membranes with 0.0064 M glutaraldehyde selectively cross-links the glycoprotein (mol. wt. 55000) and its multimers, correlating directly with the modifications of concanavalin A-binding.

6. We conclude that high-affinity binding of concanavalin A to thymocyte membranes is a cooperative process mediated by the glycoprotein (mol. wt. 55 000). We further conclude that the bulk of concanavalin A binding is through low-affinity associations, not involving specific membrane macromolecules.

INTRODUCTION

The interaction of the jack bean lectin with cell surfaces is of great interest for at least two reasons. The protein induces blastogenic transformation of thymocytes [1], and it often differentially agglutinates normal and neoplastic cells [2, 3].

Because the biological effects of concanavalin A can be blocked or reversed by

* To whom correspondence should be sent.

certain "hapten-sugars", e.g. α-methyl-D-manno- or glucopyranosides [4], it is assumed that the action of concanavalin A derives from the binding of the lectin to specific membrane receptors. However, we know little detail about the properties of concanavalin A receptors.

We initiated our attack on this problem by following the protein metabolism in thymocyte plasma membranes after blastogenic stimulation with concanavalin A [5]. We found that mitogenic doses of concanavalin A markedly enhanced the turnover of a glycoprotein (mol. wt. 55000) and induced its secretion into the culture medium. We subsequently purified this protein from Triton X-100 extracts of thymocyte microsomal membranes by affinity chromatography on immobilized concanavalin A and demonstrated that it could exist in mono- and dimeric forms [6].

We now show that the glycoprotein (mol. wt. 55000) accounts for the specific, positively cooperative, high-affinity binding of concanavalin A by thymocyte plasma membranes. We also show that more than 75 % of the concanavalin A bound to thymocytes at saturation is associated through a low affinity process not involving specific membrane receptor molecules.

MATERIALS AND METHODS

Reagents. All chemicals used were of highest purity grade available. Concanavalin A (3 × recrystallized) was purchased from Miles Laboratories (Elkhart, Ind.). Lactoperoxidase Boehringer (Mannheim, G.F.R.) in 3.2 M $(NH_4)_2\ SO_4$ was freed of $(NH_4)_2\ SO_4$ by dialysis against the buffer used for labelling. Carrier-free Na ^{125}I (17 Ci/mg), in 0.1 M NaOH and [*Me*-^{3}H] thymidine in H_2O (specific activity 1.0 mCi/0.12 mg) were obtained from New England Nuclear, (Cambridge, Mass.). Concanavalin A-Sepharose and Dextran-150 were purchased from Pharmacia (Sweden), as was Ficoll, which was extensively dialyzed against water and lyophilized before use. Leuko-Pak nylon wool was obtained from Fenwal Laboratories (Morton Grove, Ill.), Hypaque (sodium diatrizoate, 50 %) from Winthrop (New York, N.Y.), minimum essential medium containing 0.025 M HEPES from Gibco (Grand Island, New York), 4-(hydroxymethyl)-1-piperazinylethane-2-sulfate (HEPES), α-methyl-D-glucopyranoside, Coomassie blue and Triton X-100 from Sigma (St. Louis, Mo.), acrylamide, *N,N'*-methylenebisacrylamide, *N,N'*-diallytartardiamide, *N,N,N',N'*,-tetramethylethylenediamine (TEMED) and ammonium persulfate from Bio Rad Laboratories (Richmond, Calif.). Millipore filters (type GS; pore size 0.22 μm) were purchased from Millipore Corp. (Bedford, Mass.), and Diaflo PM10 ultrafilters from Amicon Corp., (Lexington, Mass.). ^{125}I activity was counted in a Packard Autogamma Spectrometer.

Isolation of concanavalin A receptor protein: White New Zealand rabbits (female, 2 months old) were employed. Thymocytes, microsomal membranes and plasma membranes were prepared as described in [7, 8]. Extraction of membrane preparations with Triton X-100 was done as in ref. 6. The pelleted membrane material was suspended in 1 % Triton X-100, 0.0038 M Tris/0.01 M glycine, pH 8.7, to a final concentration of 2.5 mg protein/ml. Non-solubilized material was then pelleted at $1.2 \cdot 10^7 \cdot g \cdot$ min. The sediment was reextracted in identical fashion, the supernatant fluids combined, concentrated to a level of about 2 mg protein/ml, by ultrafiltration (PM10) and dialyzed against the buffer used for affinity chromatog-

raphy for 16 h at 6 °C as described in ref. 6. Concanavalin A-Sepharose was washed as described in ref. 6 and packed into a column (10 × 150 mm) and washed with 50 ml chromatography buffer, namely: 0.075 M Tris · HCl/0.0002 M $CaCl_2$/0.0002 M $MnCl_2$, pH 7.5/0.2 % Triton X-100 (v/v). The final fractions eluted during this stage contained no protein. The membrane protein (7–10 mg/4 ml) was then pumped onto the column and, after 90 min equilibration at 6 °C, unabsorbed protein was eluted with 50 ml chromatography buffer. The column was then eluted with chromatography buffer, 0.0016 M in α-methyl-D-glucopyranoside, to elute the monomeric glycoprotein 5.1 [6]. The eluate was concentrated about 10-fold by ultrafiltration using a Diaflo PM10 membrane and dialyzed against 1000 ml 0.02 M Tris, pH 7.5. Since Triton X-100 does not pass readily through PM10 membranes and dialysis tubing, we removed excess detergent by molecular sieving on columns of Sephadex G-25 (10 × 300 mm) preequilibrated with 0.020 M Tris, pH 7.5. The protein-containing fractions [9] were pooled and concentrated by ultrafiltration to 1 mg protein/ml. The concentrated protein was finally dialyzed against phosphate buffered saline for experiments with intact thymocytes or against 0.001 M HEPES, pH 7.5, for studies with isolated membranes.

^{125}I-labelling of glycoprotein (mol. wt. 55 000). The concentrated, dialyzed 55 000 dalton glycoprotein eluted from the concanavalin A-Sepharose column was labelled with ^{125}I by lactoperoxidase-catalyzed iodination at 25 °C in 1.0 ml 0.02 M Tris · HCl, pH 7.5, 10^{-6} M KI, containing 1.0 mg protein, 20 μg lactoperoxidase, 10 μl butylated hydroxytoluene (100 mg/100 ml H_2O), 20 μl Na ^{125}I (0.02 mCi). Iodination was initiated by adding 20 μl of 0.003 % H_2O_2 in 0.02 M Tris · HCl, pH 7.5, and was maintained by three further additions of 10 μl H_2O_2 at 1-min intervals. The ^{125}I was removed by dialysis of the reaction mixture for 36 h (total against) 3 × 1000 ml 0.02 M Tris · HCl, pH 7.5, containing 0.005 M KI and 1000 ml 0.02 M Tris · HCl, pH 7.5.

^{125}I-labelling of concanavalin A. We used a concanavalin A (5 mg/ml) solution in 0.001 M HEPES, 0.0002 M $CaCl_2$ and 0.0002 M $MnCl_2$, pH 7.5 and 0.1 mg lactoperoxidase per ml otherwise proceeding as already described. After labelling the concanavalin A solution was dialyzed against 3 × 1000 ml 0.001 M HEPES 0.0002 M $CaCl_2$ and 0.0002 M $MnCl_2$, pH 7.5, containing 0.005 M KI, and once against the same buffer without KI.

Glutaraldehyde treatment of microsomal membranes. Washed microsomal membranes were suspended in 0.001 M HEPES, pH 7.5, to a protein concentration of 1 mg/ml. The membranes were treated at glutaraldehyde concentrations from 0.0016–0.0256 M for 30 min at 20 °C. The samples were diluted 20-fold and collected by pelleting at $1.2 \cdot 10^7$ $g \cdot$ min. For studies of concanavalin A binding, the membranes were resuspended in the original volume of 0.001 M HEPES, 0.0002 M $CaCl_2$, 0.0002 M $MnCl_2$, pH 7.5.

Binding of concanavalin A to native and glutaraldehyde-treated microsomal membranes. Millipore filter-membranes were used to collect microsomal membranes, having first demonstrated that these filters retain more than 95 % of ^{125}I-labelled membranes. However, one cannot use more than 20 μg membrane protein per assay, because the filters become obstructed otherwise. Another problem is that concanavalin A binds extremely tightly to the filters in a process that is only 10 % reversible by 0.1 M α-methyl-D-glucoside or 0.1 M α-methyl-D-mannoside. We overcame this

difficulty as follows: the Millipore filters were first treated with a solution containing 2 mg of concanavalin A in 4 ml 0.001 M HEPES, 0.0002 M CaCl, 0.0002 M $MnCl_2$, pH 7.5. Microsomal membranes (maximally 20 μg membrane protein) incubated at a concentration of 0.125 mg/ml with ^{125}I-labelled concanavalin A were then mixed into 5 ml 0.001 M HEPES, 0.0002 M $CaCl_2$ 0.0002 M $MnCl_2$, pH 7.5, containing 0.02 mg concanavalin A/ml and collected by filtration under suction on the Millipore filters. The filters were washed with additional 10 ml of 0.001 M HEPES, 0.0002 M $CaCl_2$, 0.0002 M $MnCl_2$, pH 8.5, containing 0.02 mg concanavalin A/ml. This method allows full recovery of microsomes on the Millipore filters and reduces the amount of non-specifically bound ^{125}I-labelled concanavalin A by 90 to 97 %. To compensate for the remaining, non-specific concanavalin A absorption, binding of labelled lectin to filters was measured under conditions equivalent to those used for membrane binding. Under the most extreme conditions, at 200 μg concanavalin A/ml at a membrane concentration of 0.125 mg/ml 60 % of the radioactivity is retained by the filters. Since without membranes not more than 10 % of the lectin is retained at this high lectin level, the maximal contamination of the retained material by non-specifically bound concanavalin A is approx. 6 %. In the critical range of lectin concentration i.e. below 50 μg lectin/ml the contamination factor is less than 3 %.

In order to determine the amount of concanavalin A binding that is inhibited by the presence of 0.2 M α-methyl-D-glucopyranoside we followed the procedure in ref. 10. The microsomal membranes were incubated in 0.001 M HEPES/0.0002 M $CaCl_2$/0.0002 M $MnCl_2$, pH 7.5, containing 0.2 M α-methyl-D-glucoside present in all buffers used. To construct binding curves, the amount of concanavalin A bound in the presence of 0.2 M "hapten" sugar is then subtracted from the amount bound without "hapten" sugar.

Binding of ^{125}I-labelled glycoprotein (mol. wt. 55 000) to intact thymocytes or microsomal membranes. Thymocytes suspended in HEPES-buffered minimum essential medium containing 1 % fetal calf serum ($5 \cdot 10^7$ cell/ml; > 95 % viability) were equilibrated with purified ^{125}I-labelled glycoprotein (mol. wt. 55 000) at a concentration of 12.5 μg/ml; this corresponds to an approximate 5-fold excess, since $5 \cdot 10^7$ thymocytes contain only approx. 2.8 μg of this protein in their plasma membranes. The amount of protein bound to the cells between 1 and 60 min was measured by taking 1 ml aliquots from the cell suspension, pelleting the cells at 8000 $g \cdot$ min, washing the cells with another 2 ml of medium, precipitating and washing thrice with 10 % trichloroacetic acid and assaying the ^{125}I in the residue. To determine whether the protein incorporated into whole cells localizes in the plasma membrane we isolated these as before [8] and measured the proportion of ^{125}I in the various subcellular fractions.

We have also examined the uptake of glycoprotein (mol. wt. 55 000) by isolated microsomal membranes. For this we used 75 μg glycoprotein (mol. wt. 55 000)/1.0 mg of microsomal membrane protein. The binding kinetics were measured in the same way as described for concanavalin A binding, except that the measurements were done in 0.001 M HEPES, pH 7.5, containing 1 % bovine serum albumin. These conditions avoid non-specific binding of glycoprotein (mol. wt. 55 000) to the filters. To establish that bovine serum albumin does not influence the binding of the 55 000-dalton glycoprotein to microsomal membranes, we performed the binding studies also in 5 % albumin.

To determine whether the glycoprotein (mol. wt. 55 000) is preferentially incorporated into the plasma membrane vesicles in the microsomal fraction, we fractionated microsomal membranes, labelled with ^{125}I-labelled glycoprotein (mol. wt. 55 000), into plasma membrane and endoplasmic reticulum, using dextran gradient ultracentrifugation as before [8].

Dodecyl sulfate polyacrylamide gel electrophoresis. The membrane proteins solubilized in 1 % dodecyl sulfate/40 mM dithiothreitol were separated electrophoretically as in ref. 8 using 7 % acrylamide, cross-linked with 3.75 % *N,N'*-diallyltartardiamide containing 1 % sodium dodecyl sulfate. The electrophoresis buffer was Tris/acetate/EDTA (0.04 M/0.02 M/0.002 M), pH 7.4. Coomassie brilliant blue staining for proteins and reaction with the periodate Schiff reagent were as in ref. 11. For the periodate Schiff reaction we used 6 % acrylamide gels, cross-linked with 2.5 % *N,N'*-methylenebisacrylamide. To monitor the ^{125}I-distribution, we sliced the frozen gels into sixty 1 mm slices and counted these in a Packard Autogamma Spectrometer. Protein components are numbered as in ref. 8.

Other procedures. Protein was assayed by the ninhydrin method [12] or fluorimetrically [9], using bovine serum albumin as standard. To determine the protein concentration of glutaraldehyde-treated membranes the protein fluorescence in 0.1 % dodecyl sulfate was compared with the fluorescence of untreated proteins. Cell viability was measured by Trypan Blue exclusion [7, 8].

RESULTS

Iodination of concanavalin A and of concanavalin A-reactive protein. Dodecyl sulfate polyacrylamide gel electrophoresis shows that monomeric concanavalin A-reactive protein, protein 5.1, isolated from Triton X-100 solubilized thymocyte microsomes as described here and in ref. 6, elutes as a single component (mol. wt. of 55 000) from concanavalin A-Sepharose; it does not aggregate when Triton X-100 is removed by gel filtration. 10^{10} thymocytes yield about 8 mg microsomal protein of which 0.3–0.4 mg is protein 5.1, a plasma membrane component [5, 8]. Since the recovery of plasma membrane protein in the microsomal fraction is 50 %, the amount of 5.1 in 10^{10} cells is 0.6–0.8 mg. When we subfractionate microsomes we recover 80 % of 5.1 (and other plasma membrane markers; [5, 8]) in the plasma membranes fraction. This corresponds to a yield of 0.25–0.32 mg per 10^{10} cells. The remaining 20 % is found in the endoplasmic reticulum fraction. Since previous studies show that only 3 % of plasma membrane material is lost into the endoplasmic reticulum fraction [8] we conclude that the amount of 5.1 recovered in the plasma membrane fraction represents the proportion of the protein in the surface of the intact cells. Correcting for recoveries, this amounts to 0.55 mg/10^{10} cells or $6 \cdot 10^5$ molecules/cell.

Our iodination procedure was highly efficient for both concanavalin A and the glycoprotein (55 000 mol. wt.), giving specific activities of $2–4 \cdot 10^3$ Ci/mol and $0.8–1.6 \cdot 10^2$ Ci/mol, respectively (based on mol. wt. of 106 000 and 55 000, respectively). The contribution of self-iodinated lactoperoxidase to the labelled protein is negligible, at mass ratios lactoperoxidase/concanavalin A 55 000 dalton glycoprotein ≤ 0.02. Indeed, we [8] and others [13, 14] have previously shown that the enzyme does not bind significantly to the surfaces of intact cells and that it does not

penetrate into intact cells [8]. Moreover, our electrophoretic analyses of labelled glycoprotein (Figs. 3 and 4), or of labelled concanavalin A, do not show ^{125}I radioactivity in the 80 000 mol. wt. range, the molecular weight of lactoperoxidase.

The ^{125}I-labelled concanavalin A had the same mitogenic activity as unlabelled lectin; i.e. a 20-fold increase of [^3H]thymidine incorporation [13] by thymocytes during a 6-h cultivation period after 24 h exposure of the cells to 5 μg/ml of lectin [5].

Glutaraldehyde treatment of microsomal membranes. The cross-linking experiments with glutaraldehyde were conducted to determine whether the propensity of isolated glycoprotein (mol. wt. 55 000) to dimerize [6] could be simulated in intact membranes by low concentrations of glutaraldehyde. We find that cross-linking of membrane proteins with glutaraldehyde leads to clear modifications of dodecyl sulfate polyacrylamide electrophoretograms, with a replacement of the low molecular

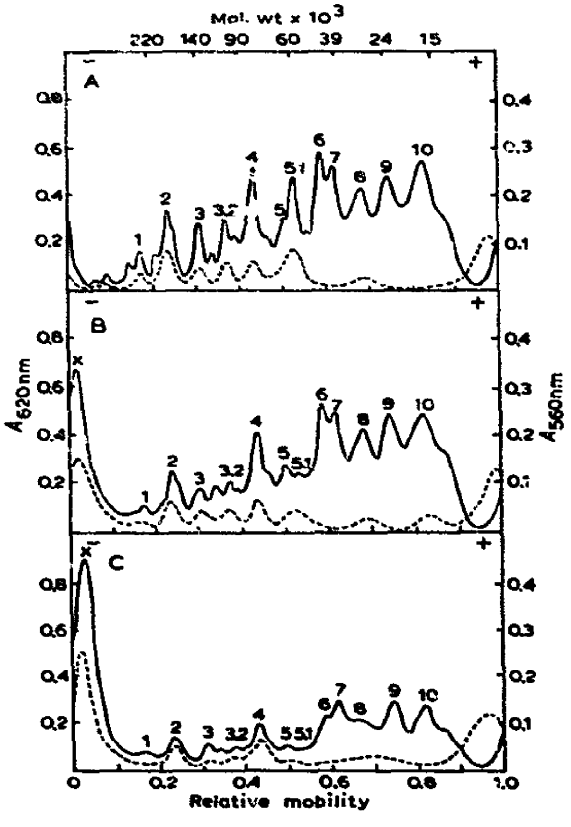

Fig. 1. Dodecyl sulfate polyacrylamide gel electropherograms of rabbit thymocyte microsomes treated with varying concentrations of glutaraldehyde. The abscissa gives the relative mobility and the molecular weights, respectively, the ordinate the absorbance at 620 nm for Coomassie blue and at 560 nm for periodate-Schiff reagent. The proteins and glycoproteins are numbered from 1–10 according to their decreasing molecular weights. The strong band (×) of relative mobility below 0.1 is due to cross-linked membrane proteins. Panel A: untreated microsomal membranes. Panel B: microsomes fixed in 0.0064 M glutaraldehyde for 30 min at 20 °C. Panel C: microsomes fixed in 0.0192 M glutaraldehyde for 30 min at 20 °C.

weight components by multimeric aggregates (Fig. 1). The electropherogram of untreated thymocyte microsomal membranes is illustrated in Fig. 1a. This pattern is modified selectively by exposure of the membranes to increasing glutaraldehyde levels of 0.0016 M–0.025 M (Fig. 1b and c). The low-molecular weight components (6–10), which derive principally from endoplasmic reticulum [8], do not cross-link extensively and then only at high concentrations of glutaraldehyde (i.e. 0.0128–0.0256 M). Even at 0.0192 M glutaraldehyde, components 6–10 can still be clearly identified (Fig. 1c). In contrast, at these high glutaraldehyde levels the high molecular weight proteins and glycoproteins [1–5], derived from plasma membrane fragments [8] are extensively cross-linked.

Use of low-glutaraldehyde levels shows that the 55 000 dalton glycoprotein is unusually susceptible to cross-linking with glutaraldehyde. Thus, with 0.0064 M glutaraldehyde, this protein is reduced by more than 60 % as determined by both Coomassie blue staining and the periodate-Schiff reaction (Fig. 1b). We find a concomitant, but less marked (20–30 %), decrease in components 1 (mol. wt. 220 000), 2.1–2.3 (mol. wt. approx. 160 000), 3 (mol. wt. 130 000), 3.2 (mol. wt. 110 000) and 4 (mol. wt. 75 000) and a large increase in the proportion of staining at the top of the gel (x). Components 6–10 are little affected at this glutaraldehyde concentration.

Uptake of purified 55 000 dalton glycoprotein by intact thymocytes and microsomal membranes isolated therefrom. When intact thymocytes are incubated with glycoprotein (mol. wt. 55 000) for 60 min at 4 °C their viability remains unchanged (> 90 %), indicating that any exchange of this protein into the cells would need to proceed through intact plasma membranes. The binding of the protein to intact cells or isolated membranes (Fig. 2) is complete within less than 30 min limiting at $2.4 \cdot 10^5$ molecules/thymocyte for intact cells and $3.0 \cdot 10^5$ molecules/cell-equivalent in the case of isolated membranes. However, our electrophoretic analyses show no significant enrichment of glycoprotein (mol. wt. 55 000) in the membranes.

When we used our established procedures [8] for the separation of microsomal

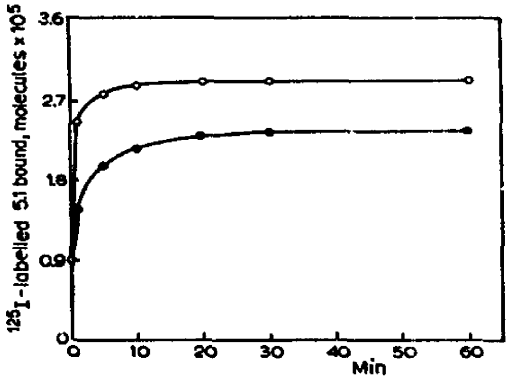

Fig. 2. Time dependent binding of ^{125}I-labelled glycoprotein (mol. wt. 55 000) (5.1) to intact rabbit thymocytes (● — ●) and microsomal membranes (○ — ○) isolated therefrom. The ordinate gives the number of 55 000 dalton glycoprotein molecules bound per cell, the abscissa gives the incubation time in minutes. To calculate the number of molecules bound/cell from isolated membranes we used the value of 1 mg of microsomal protein isolated from $1.25 \cdot 10^9$ thymocytes [8]. The points are mean values of three independent experiments, each determined in duplicate.

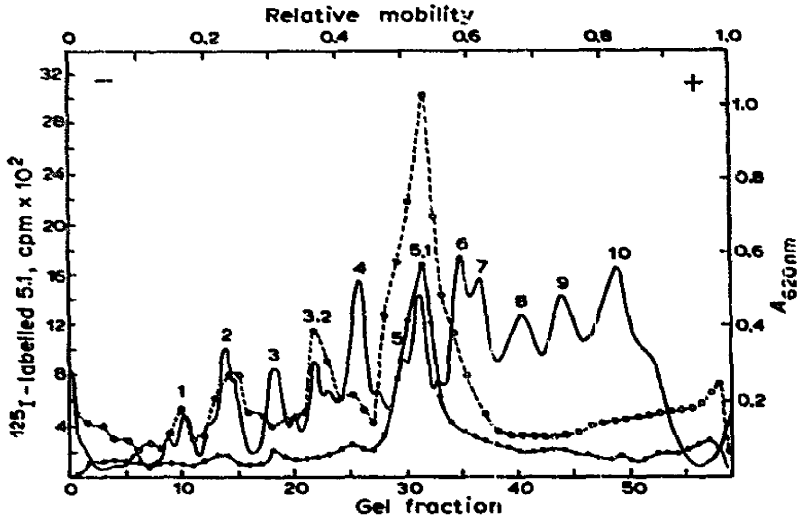

Fig. 3. Dodecyl sulfate polyacrylamide gel electropherogram of rabbit thymocyte microsomes incubated with ^{125}I-labelled glycoprotein (mol. wt. 55 000). The abscissa gives the relative mobility of the proteins and the fractions of the acrylamide gel, respectively, the ordinate the ^{125}I distribution and the Coomassie blue staining for protein at 620 nm. The proteins are numbered from 1–10. Solid line, Coomassie-scan. ●—●, ^{125}I distribution; 1 mg microsomal protein incubated with 12.5 µg ^{125}I-labelled 5.1; ○– – –○, ^{125}I distribution; 1 mg microsomal protein incubated with 62.5 µg ^{125}I-labelled 5.1.

membranes into their plasma membrane and endoplasmic reticulum categories, we found 75–80 % of the incorporated, ^{125}I-labelled glycoprotein (mol. wt. 55 000) in the plasma membrane fraction. It appears, therefore, that the labelled glycoprotein (mol. wt. 55 000) is taken up preferentially by plasma membranes.

With intact cells and isolated membranes saturation levels of ^{125}I-labelled glycoprotein (mol. wt. 55 000) were attained after 25 and 10 min, respectively, without further change for 60 min. Uptake is not altered by the addition of a 10-fold excess of bovine serum albumin in comparison to the glycoprotein (mol. wt. 55 000) concentration.

When microsomal membranes are equilibrated with 1/5 the saturation concentration of ^{125}I-labelled glycoprotein (mol. wt. 55 000), $8 \cdot 10^4$ molecules are bound per cell equivalent. When these membranes are then analyzed by dodecyl sulfate polyacrylamide electrophoresis, only one radioactive peak, corresponding to component 5.1 is observed (Fig. 3). However, when $2.4 \cdot 10^5$ molecules are bound, we observe additional labelled peaks corresponding to components 1, 2, and 3.2 (Fig. 3).

When microsomes containing $3.0 \cdot 10^5$ molecules of ^{125}I-labelled glycoprotein (mol. wt. 55 000) per cell equivalent are treated with 0.0064 M glutaraldehyde, dodecyl sulfate polyacrylamide gel electrophoresis shows that 35 % of the incorporated protein appears in a high-molecular weight aggregate (> 350 000). Concurrently the label in the regions of component 1 and 3.2 disappears, and that in component 2 decreases by 50 % (Fig. 4). These data indicate that the incorporated protein is less susceptible to glutaraldehyde cross-linking than the intrinsic protein.

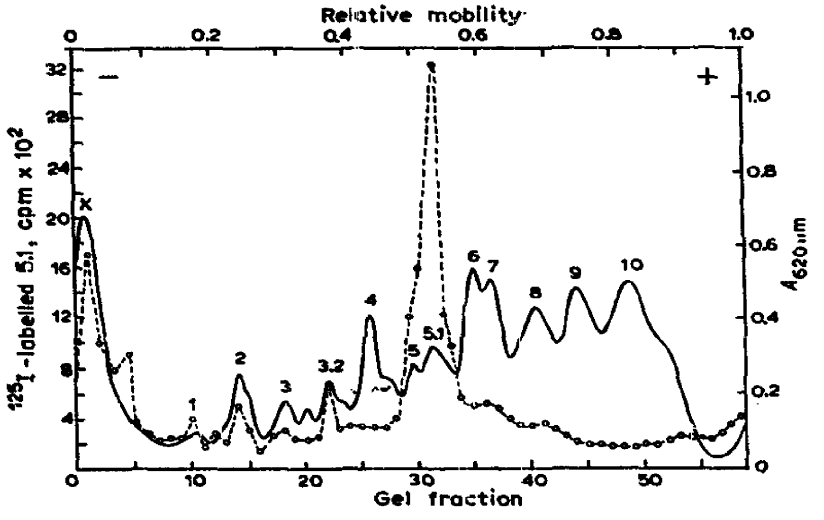

Fig. 4. Dodecyl sulfate polyacrylamide gel electropherogram of rabbit thymocyte microsomes incubated with ^{125}I-labelled glycoprotein (mol. wt. 55 000) (62.5 µg/1 mg membrane protein) fixed with 0.0064-M glutaraldehyde thereafter. The abscissa gives the relative mobility of the proteins and the fractions of the acrylamide gel, respectively, the ordinate, the ^{125}I distribution and the Coomassie blue staining for protein at 620 nm. The proteins are numbered from 1–10. —, Coomassie scan; O- - -O, ^{125}I distribution.

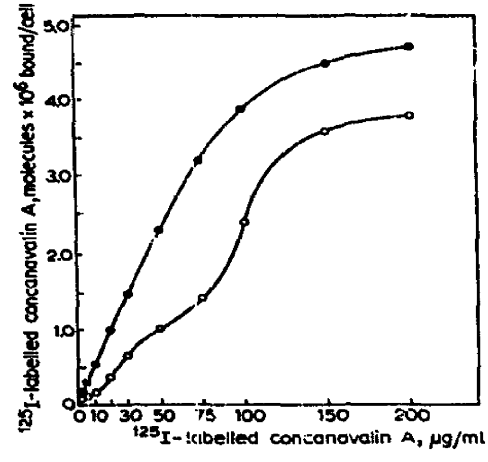

Fig. 5. Concentration dependent binding of concanavalin A to native and glutaraldehyde treated rabbit thymocyte microsomes. The abscissa gives the concentration of concanavalin A, the ordinate the number of molecules of tetrameric ^{125}I-labelled concanavalin A bound per cell (1 mg of microsomal membranes equivalent to approx. $1.25 \cdot 10^9$ thymocytes). ●—●, glutaraldehyde (0.0064 M) treated membranes; O—O, native microsomes. Each measure point is the mean value of four independent binding experiments assayed in duplicate. Range is ±12 % at concanavalin A levels > 100 µg/ml and < 5 % at lower levels.

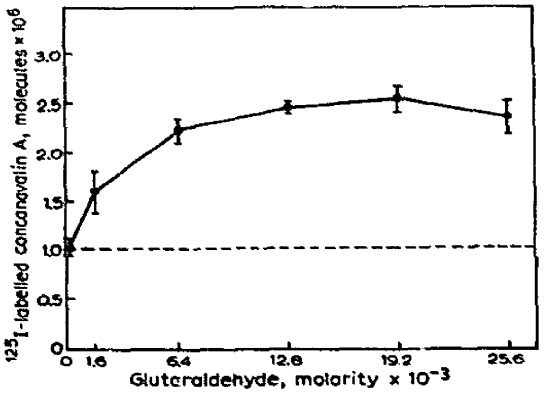

Fig. 6. Binding of concanavalin A to thymocyte microsomes cross-linked with increasing concentration of glutaraldehyde. The abscissa gives the glutaraldehyde concentration at which microsomes are cross-linked for 30 min at 25 °C, the ordinate the number of ^{125}I-labelled concanavalin A molecules bound per cell (1 mg microsomal membranes equivalent to $1.25 \cdot 10^9$ thymocytes). The membrane protein concentration was 0.125 mg/ml. The results are obtained from three independent experiments, each experiment set up in duplicate. The standard deviation is indicated for each point. The ^{125}I-labelled concanavalin A concentration was 50 μg/ml.

Binding of ^{125}I-labelled concanavalin A to native and glutaraldehyde-treated microsomal membranes. Kinetic studies show that the binding of ^{125}I-concanavalin A to native and glutaraldehyde treated microsomal membranes is complete within 15 min at 25 °C. The variation of binding with lectin concentration is illustrated in Fig. 5 for lectin levels between 1–200 μg/ml at membrane concentrations of 0.125 mg protein/ml. The native membranes exhibit a complex binding curve (Fig. 5) with inflection points at 10, 75 and 200 μg concanavalin A/ml. The saturation level is at $4 \cdot 10^6$ tetrameric molecules of lectin per cell equivalent.

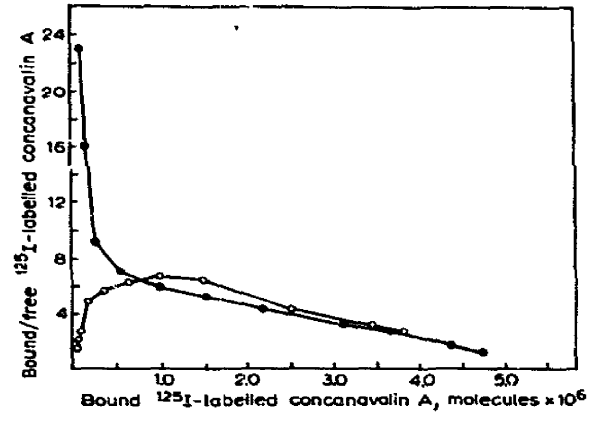

Fig. 7. Scatchard plots of ^{125}I-labelled concanavalin A binding curves to rabbit thymocyte microsomal membranes presented in Fig. 5. The abscissa gives the number of tetrameric ^{125}I-labelled concanavalin A molecules bound per cell, and the ordinate gives the ratio bound/free ^{125}I-labelled concanavalin A. The lines were fitted by the least squares method. (● — ●), plot for glutaraldehyde treated (0.0064 M) membranes; (○ — ○), native membranes.

An entirely different binding is obtained after membranes have been treated with 0.0064 M glutaraldehyde (Fig. 5). The multiphasic character of the curve is eliminated, binding at low lectin levels is markedly increased and saturation occurs at approx. $4.5 \cdot 10^6$ molecules of tetrameric lectin/cell equivalent. As shown in Fig. 6, concanavalin A binding increases with the concentration of glutaraldehyde used for cross-linking up to 0.0192 M; above this level the binding capacity drops.

In Fig. 7 the binding curves are presented according to Scatchard [14]. The data obtained with native membranes are typical of a positive cooperative response [15]. From a stage of low affinity up to 5 μg lectin/ml, binding passes through a high affinity phase, up to 50 μg concanavalin/ml, and finally reaches an intermediate stage.

In the case of glutaraldehyde-treated membranes, two binding phases are evident with apparent binding constants of $6.8 \cdot 10^5\ M^{-1}$ (below 10 μg lectin/ml) and $1.3 \cdot 10^5\ M^{-1}$ at higher lectin levels. The low affinity binding corresponds to the third phase observed in native membranes. At high lectin levels the binding affinities thus appear identical for native and cross-linked membranes.

The number of 55000 dalton glycoprotein molecules/cell is approx. $6 \cdot 10^5$. This is far below the total number of concanavalin A molecules that can be bound, $4 \cdot 10^6$/cell, but corresponds approximately to the number of positively interacting binding sites.

Taken together the experiments suggest that in native membranes lectin binding induces an enhanced binding affinity that can be simulated by cross-linking of membrane proteins. The binding at lectin levels above 50 μg/ml appears to be of a different category and of relatively low affinity. Indeed, only a small proportion ($< 25\,\%$) of the lectin bound per lymphocyte at saturation can be accounted for by the glycoproteins that are isolated by concanavalin A affinity chromatography.

DISCUSSION

Mitogenic stimulation of thymocytes in vitro with concanavalin A markedly stimulates the turnover of a plasma membrane glycoprotein (mol. wt. 55 000), component 5.1 [5]. This enhanced turnover is due to concomitantly accelerated biosynthesis of 5.1, and its elution into the extracellular space, in a process keeping the proportion of the glycoprotein constant in the membrane [5].

Affinity chromatography of thymocyte membrane proteins using Sepharose-immobilized concanavalin A shows that the concanavalinA reactive protein can occur as a monomer (mol. wt. 55 000), as well as in multimeric form [6]. Analysis, by dodecyl sulfate polyacrylamide gel electrophoresis of the protein eluted by low concentrations (< 0.0016 M) of the "hapten sugar", α-methyl-D-glucopyranoside reveals only the 55 000 mol. wt. species. However, electrophoresis of the material eluted at higher levels (0.01–0.1 M) of "hapten sugar" reveals multimeric species (mol. wt. 110 000; mol. wt. 220 000) in addition to the monomeric form. Moreover, transfer of the isolated monomer to a new column of immobilized concanavalin A followed by elution with high levels of "hapten-sugar" induces the appearance of dimer [6]. Since dodecyl sulfate shifts monomer-multimer equilibria towards the monomer state, the data suggest that it is predominantly the multimeric species that is eluted with high concentrations of hapten sugar and that these partially dissociate

in dodecyl sulfate, giving rise to the monomeric species upon electrophoresis [6]. The elution sequence also indicates that the multimeric species possess a higher affinity for concanavalin A than the monomeric form. Finally, the analyses in ref. 6 indicate that the glycoprotein (mol. wt. 55 000) and its multimers comprise two categories in terms of the antigenic character of the carbohydrate residues. However, these categories behave identically upon dodecyl sulfate polyacrylamide electrophoresis, indicating equivalent peptide moieties, and upon affinity chromatography, indicating equivalent association with concanavalin A [6].

Our present studies with highly purified, solubilized radioiodinated glycoprotein (mol. wt. 55 000) show that this material is readily taken up by viable, unstimulated thymocytes or microsomal membranes isolated therefrom. Moreover, our membrane fractionation studies show that the protein preferentially incorporates into plasma membranes, rather than endoplasmic reticulum. It remains to be established whether the basis of the observed plasma membrane selectivity lies in membrane lipids or other membrane proteins.

Dodecyl sulfate polyacrylamide electropherograms of membranes treated with very low (0.0064 M) levels of glutaraldehyde reveal a selective conversion of the glycoprotein (mol. wt. 55 000) to higher molecular weight species. This suggests that the glycoprotein (mol. wt. 55 000) molecules are either statically or dynamically closer to each other in the membrane than are other membrane proteins. We suspect that glutaraldehyde cross-linking occurs parallel rather than normal to the membrane plane, since glutaraldehyde, a hydrophilic reagent, is expected to couple particularly amino groups at the membrane surface and since glutaraldehyde fixation does not interfere with freeze-fracturing the membrane core parallel to the membrane plane.

Measurement of concanavalin A-binding to native and glutaraldehyde-treated membranes provides important insights into the role of the glycoprotein (mol. wt. 55 000) in the binding of the lectin to thymocytes. The binding curves of native membranes are complex and reveal three apparent saturation levels, a pattern similar to that reported for intact human lymphocytes [16]. Treated membranes, in contrast, show a simpler saturation curve with comparatively greater binding in particular at low lectin concentrations. Concordantly, the Scatchard plot [14, 15] of binding for native membranes is diagnostic of positive cooperativity at low concanavalin A concentrations and relatively low affinity binding (apparent K_a $1.3 \cdot 10^5$ M^{-1}) at high lectin levels. However, the Scatchard plot for treated membranes reveals two linear segments, suggesting two categories of binding sites or binding processes. The first limb, occurring at low lectin levels, corresponds to a high apparent K_a of $6.8 \cdot 10^6$ M^{-1}. The second limb corresponds to a lower apparent K_a of $1.3 \cdot 10^5$ M^{-1}, equivalent to that found with native membranes at high levels of concanavalin A.

Taken together, the data suggest that concanavalin A induces the formation of dimers (or possibly higher multimers) of the 55 000 dalton glycoprotein through a cooperative process that leads to an increased affinity for the lectin. This cooperativity is not observed in glutaraldehyde-treated membranes in which the binding protein has been artificially brought into its high affinity state by cross-linking.

Direct electrophoretic analyses, as well as the exchange studies, indicate that about $4 \cdot 10^6$ molecules of tetrameric concanavalin A are bound per cell, or membrane cell equivalent, at saturation. 60–75 % of the lectin bound is thus associated

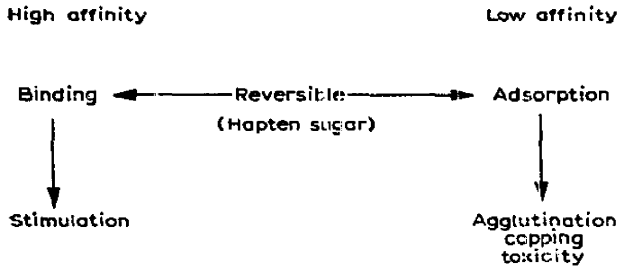

Fig. 8. Binding of concanavalin A to rabbit thymocyte plasma membranes.

with sites other than the glycoprotein (mol. wt. 55 000) and its multimers and those sites account for the low-affinity phase of the binding curves. We suggest that this low-affinity binding does not involve other "concanavalin A receptors", since there are not enough copies of glycoproteins other than the protein (mol. wt. 55 000) to provide the binding sites necessary. Rather, we propose that the low affinity binding represents an adsorption process, that can be reversed by "hapten-sugars" due to a change in the structure of the lectin induced by binding of the "hapten-sugars". This suggestion parallels Cuatrecasas' explanation [18, 19] of the binding of concanavalin A to adipocytes. It also fits the observations of Burger [20] that concanavalin A binds tightly to a variety of plastic surfaces and that this binding can be released by addition of "hapten-sugar".

It has been established that concanavalin A mitogenically stimulates rabbit thymocytes after exposure to $5 \cdot 10^5$ molecules of lectin/cell; higher concentrations of concanavalin A are toxic. Our binding data (Fig. 5) show that under these conditions, only approx. 17 % of the glycoprotein (mol. wt. 55 000) molecules would be occupied, corresponding to approx. 4 % of saturation binding. In view of our present data and our previous demonstration that the turnover of the glycoprotein (mol. wt. 55 000) is augmented by concanavalin A, it is reasonable to propose that this protein is the receptor mediating events leading to mitogenic stimulation (Fig. 8). We suspect, however, that the cell agglutination and various surface transposition phenomena [2, 21, 22] induced by concanavalin A represent adsorbed lectin that is not bound to specific membrane "receptors" (Fig. 8). This suggestion would account for the large, numerically similar concanavalin A binding by diverse cells, including cells that do not respond biologically to this lectin [17, 23–25].

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