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FUNCTIONAL MOSAICISM OF THE LYMPHOCYTE PLASMA MEMBRANE

CHARACTERIZATION OF MEMBRANE SUBFRACTIONS OBTAINED BY AFFINITY CHROMATOGRAPHY ON CONCANAVALIN A-SEPHAROSE

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Summary

Microsomal membranes (consisting largely of plasma membrane) or purified plasma membranes from calf thymocytes were fractionated by affinity chromatography on concanavalin A-Sepharose. Two fractions were obtained: one, designated MF1, eluted freely from the affinity adsorbent containing two thirds of the membrane protein. A second fraction, designated MF2, containing one third of the membrane protein, adhered to concanavalin A-Sepharose and was recovered after mechanical dissociation. Both fractions were homogeneous as assessed by rechromatography. The following control experiments suggested that the fractions were derived from different areas of an individual cell. (1) Fractionation required the binding of membranes to (Sepharose-bound) concanavalin A. (2) The non-retarded fraction MF1 exhibited more binding sites for concanavalin A, suggesting that both fractions were right side-out. (3) Intact thymocytes could not be fractionated on concanavalin A-Sepharose, which made it likely that the membrane subfractions did not originate from different cells. This was supported by an identical fractionation of plasma membranes isolated from a homogeneous T-lymphocyte tumor line. (4) Several membrane-bound enzymes (γ-glutamyltransferase, adenylate cyclase, Mg^{2+}-ATPase) exhibited similar specific activities in both subfractions as well as in the unseparated membrane. In addition to previous findings that the cholesterol/phospholipid ratio was identical (Brunner, G., Ferber, E. and Resch, K. (1976) Differentiation 5, 161—164), this suggests that MF1 and MF2 consisted predominantly of identical membranes, i.e. the plasma membrane. The affinity for concanavalin A was 2.5 times higher in MF2 as compared to MF1. Both fractions were distinct in plasma membrane-bound enzymes: ($K^+ + Na^+$)-ATPase exhibited high specific activity in MF2 compared to MF1 or the unseparated membrane. Similarly, acyl-CoA:lysophosphatidylcholine acyltrans-

Abbreviation: HEPES, 2,4-(2-hydroxyethyl)-1-piperazinylethane sulfonic acid.

ferase was also enriched in MF2. Alkaline *p*-nitrophenyl phosphatase showed also higher activity in this fraction.

The data suggest a close association of receptors with high affinity for concanavalin A and certain membrane-bound enzymes. As activation of lymphocytes was initiated when 14—24% of the available binding sites interacted with concanavalin A, the data suggest a functional mosaicism in the vicinity of activating receptors.

Introduction

It is well established that lymphocytes start to grow and divide upon binding of antigens or mitogens to plasma membrane receptors [1,2]. The proliferative response and the expression of function are initiated when only a certain fraction, between 5 and 25%, of binding sites interacts with ligands such as the mitogen concanavalin A [3—5]. As aggregation of receptors appears to be a necessary step for the activation of lymphocytes [6], this may merely reflect conditions which favor the appropriate organization of receptors required for the induction of cell activation. Recently, however, membrane glycoproteins which bind to concanavalin A were found to be heterogeneous with respect to their binding affinity [7]. Moreover, binding sites which interact with concanavalin A in situations leading to mitosis (i.e. at mitogenic concentrations) exhibited higher affinities than with high concanavalin A concentrations where all binding sites were saturated [4]. This strongly suggested a distinction between lectin receptors, i.e. binding sites responsible for cell activation and biologically inert binding sites.

Recently Zachowski and Paraf [8] and Walsh et al. [9] reported a fractionation of purified lymphocyte plasma membranes by adsorption to concanavalin A-Sepharose. Both groups came to the conclusion that inside-out oriented membrane fragments were separated from right side-out fragments. Independently, we also developed a fractionation method for lymphocyte plasma membranes based on affinity chromatography on concanavalin A-Sepharose [10]. The membranes were isolated after nitrogen cavitation of thymocytes, a pure T-cell population [11]. Here we report that the plasma membrane subfractions obtained were most probably right side-out and derived from different areas of an individual cell. One subfraction (MF2) carried receptors for concanavalin A with higher binding affinity. This fraction was enriched for distinct membrane-bound enzymes, suggesting a functional heterogeneity in the lymphocyte plasma membrane.

Materials and Methods

Cells. (a) Thymocytes: Calf thymus was obtained within 20 min of killing from the local slaughterhouse and placed in phosphate-buffered saline. The thymus was cut into small pieces and macerated gently in a loosely fitting glass tissue grinder to release the thymocytes. Tissue remnants were removed by filtering the cells through a small column of nylon wool (Leukopak, Fenwall Laboratories, Morton Grove, Ill., U.S.A.). The cells were washed in phosphate-

buffered saline and finally suspended in medium or cell disruption buffer. All steps were carried out at room temperature. Under such conditions cell viability was 80—90%.

(b) EL4 lymphoma cells: The T-lymphoma EL-4 was kept by weekly serial passage in C57BL mice. One mouse was inoculated intraperitoneally with 10^6 cells and killed 1 week later. The ascites was collected, the cells washed in phosphate-buffered saline and resuspended in cell disruption buffer.

Cell cultures. (a) Incorporation of [^{3}H]uridine into RNA: 10^7 thymus lymphocytes were cultured in 1 ml Dulbecco's modified Eagle's medium supplemented with 2.5 mg/ml defatted albumin (Sigma Chemical Co., München, G.F.R.) in polypropylene plastic tubes (17 × 100 Falcon No. 2059, Becton and Dickinson, Heidelberg, G.F.R.) in a humidified CO_2/air atmosphere to maintain a pH of 7.3. After 20 h of culture at 37°C, 0.25 μCi [^{3}H]uridine (specific activity 50 Ci/mol, made by mixing [^{3}H]uridine (Amersham-Buchler, Braunschweig, G.F.R.) and uridine (Boehringer, Mannheim, G.F.R.)) was added and the cultures were further incubated for 4 h. The cultures were terminated by the addition of trichloroacetic acid to give a final concentration of 5%.

After 2 h in the cold the precipitated material was sedimented at 1000 × *g* for 10 min and washed twice with 5% trichloroacetic acid. The sediment was then dehydrated with methanol, dissolved in 0.5 ml Soluene (Packard Instruments, Frankfurt, G.F.R.), and measured by liquid scintillation (Mark III, Searle Nuclear Chicago, Häusenstamm, Germany) with 10 ml scintillation mixture containing 5 g 2,5-diphenyloxazole and 0.3 g 1,4-bis (2-(4-methyl-5-phenyloxazolyl)-benzene in 1 l toluene.

(b) Incorporation of [^{14}C]oleate into phosphatidylcholine: 10^7 thymus lymphocytes were incubated in 1 ml Dulbecco's modified Eagle's medium supplemented with 2.5 mg bovine serum albumin/ml in Falcon plastic tubes with 10 nmol [^{14}C]oleate (specific activity 2 Ci/mol, made by mixing [^{14}C]oleate (Amersham-Buchler) and oleate (Sigma Chemicals)). The oleate was stored dissolved in chloroform/methanol (2 : 1, v/v). Before use it was evaporated to dryness and sonicated in medium three times for 20 s at 50 W. After culturing for 4 h, the lipids were extracted and separated on thin-layer chromatography as described previously [12]. Incorporation was measured in a fraction containing phosphatidylcholine and also minor contaminants of phosphatidylinositol and phosphatidylserine.

Isolation of microsomal membranes. The cells were suspended in 0.14 M KCl/0.01 M HEPES/0.0005 M $MgCl_2$, pH 7.0 (cell disruption buffer), and disrupted using the nitrogen cavitation method as described earlier [11]. Briefly, the cell suspensions were equilibrated (at 4°C, if not stated otherwise) for 20 min with 30 atm of N_2 in an Artisan pressure homogenizer (Artisan Metal Products, Waltham, Mass., U.S.A.) with gentle stirring, and then released dropwise. Shortly after the release EDTA (ethylenediaminotetraacetate) was added to a final concentration of 0.001 M.

Nuclei and a "large granule fraction" (containing mitochondria, lysosomes and aggregated microsomal membranes) were pelleted at 18000 × *g* for 20 min in a Beckman J21 centrifuge (Beckman, München, G.F.R.). The microsomes were sedimented at 150000 × *g* for 90 min at 4°C (Beckman Spinco L5 centrifuge, Rotor 60 Ti), washed once with 0.01 M HEPES, pH 7.0, and finally resus-

pended in 0.14 M KCl/0.01 M HEPES, pH 7.0 (microsomal buffer).

Preparation of highly purified plasma membranes. 1 ml of microsomal membranes was layered on top of 3.5 ml 26% CsCl in 0.01 M HEPES, pH 7.0. The gradient was centrifuged for 16 h at 4°C, in a swing out rotor at 240000 × *g* (Beckman Spinco L5 centrifuge, Rotor SW 60 Ti). The plasma membrane was recovered as a band at a buoyant density of 1.10—1.17. It contained 85—90% of the protein applied to the gradient. A second fraction banded at a buoyant density of about 1.25. The cholesterol/phospholipid ratio, as well as the distribution of alkaline phosphatase and acyl CoA:lysosphosphatidylcholine acyltransferase, indicated that the plasma membrane fraction was highly purified, whereas the second fraction contained also internal membranes [13,16]. The details of the purification of thymocyte plasma membranes by CsCl gradient centrifugation will be published elsewhere (Brunner, G., Ferber, E., Golecki, J., Huber, A., Knüfermann, H. and Resch, K., in preparation).

Before the membranes were used, CsCl was dialyzed out using a linear gradient of 10% CsCl and microsomal buffer. Finally, the plasma membranes were dialyzed overnight against microsomal buffer. This slow removal of CsCl was essential to avoid irreversible aggregation.

Fractionation of microsomal membranes (or plasma membranes) on concanavalin A-Sepharose or lens culinaris lectin-Sepharose. Microsomal membranes or highly purified plasma membranes were fractionated by affinity chromatography on concanavalin A-Sepharose as described [10], with slight modifications. Briefly, concanavalin A covalently bound to Sepharose 4B (10 mg concanavalin A per ml gel, Pharmacia, Freiburg, G.F.R.) was washed and suspended in a buffer containing 0.14 M KCl/0.01 M HEPES/0.001 M $CaCl_2$/0.001 M $MgCl_2$/0.001 M $MnCl_2$, pH 6.8—7.0, (fractionation buffer), to a 50% suspension. 50 ml of the concanavalin A-Sepharose suspension was put into a separation chamber as in Fig. 1 of ref. 10. To this 1—3 mg of membrane protein was added (if not stated otherwise) and stirred for 2 min. After 15 min of incubation, the membranes which did not bind were eluted with the same buffer as above. This fraction was designated as MF1. When the first fraction was eluted completely, the concanavalin A-Sepharose beads were stirred by a motor-driven stirrer. The membranes which were eluted during stirring were collected as a second fraction and designated as MF2. Addition of 0.5 M α-methyl mannoside did not remove further membrane material. All separation steps were performed at room temperature. The protein content of the effluent was recorded with an ISCO UA-5 absorbance monitor (ISCO, Colora, Darmstadt, G.F.R.). The membrane preparations were then kept at 4°C before use.

In some experiments membranes were chromatographed on lens culinaris lectin-Sepharose as described for concanavalin A-Sepharose. Lens culinaris lectin-Sepharose (2.3 mg of the lectin bound covalently to 1 ml Sepharose 4B) was a gift of Dr. Bessler, Tübingen, G.F.R.

Fractionation of intact thymus lymphocytes on concanavalin A-Sepharose. Intact cells were fractionated in a similar way to membranes with the following modifications. The cells were suspended in phosphate-buffered saline which was also used during chromatography and $3 \cdot 10^8$ thymus lymphocytes were applied to 10 ml gel. To avoid cell damage, the two fractions were released dropwise instead of pumping out. The cell count of the collected fractions was measured in a hemocytometer.

Protein determination. Protein content of the membranes was measured by native fluorescence as described [14], using a Perkin-Elmer MPF-44 fluorescence spectrophotometer (Perkin-Elmer, Friedrichshafen, G.F.R.).

Binding of ^{125}I-labeled concanavalin A. (a) Preparation of ^{125}I-labeled concanavalin A: Concanavalin A (Pharmacia) was labeled with ^{125}I (NEN, Dreieichenhain, G.F.R.) essentially by the method of McConahy and Dixon [15], as described [16].

(b) Binding of concanavalin A to thymocytes: Binding of concanavalin A to intact thymocytes was carried out in 12/75 polypropylene plastic tubes (Greiner No. 115301, Nürtingen, G.F.R.). 1 ml of a thymocyte suspension in phosphate-buffered saline (10^7 cells/ml), was incubated with increasing amounts of ^{125}I-labeled concanavalin A. After incubation for 60 min at 37°C, the cells were spun down and washed twice. The radioactivity of the cell pellet was measured in a gamma spectrometer (Searle-Nuclear Chicago, Model 1085). Concanavalin A tends to readily adsorb to surfaces without involving saccharide binding sites (non-specific binding). The non-specific binding was measured in samples receiving 0.05 M α-methyl mannoside which completely suppresses specific binding, i.e. binding by the saccharide binding sites [3]. The specifically-bound concanavalin A represents the amount of bound concanavalin A which was inhibited by α-methyl mannoside.

(c) Binding of ^{125}I-labeled concanavalin A to membranes: Binding studies with membranes were performed in polycarbonate ultracentrifuge tubes (5/8 × 3 inch, Beckman Instruments) in a total volume of 1.0 ml of fractionation buffer. Membranes were added at concentrations between 20 and 200 μg/ml. Concanavalin A (a mixture of ^{125}I-labeled concanavalin A and concanavalin A) was present at concentrations ranging from 0.5 to 50 μg/ml. Non-specific adsorption was measured in tubes receiving 0.05 M α-methyl mannoside. The reaction mixture was incubated for 60 min at 37°C, then the membranes were centrifuged for 45 min at 150000 × *g* (Beckman Spinco L5 centrifuge, Rotor 50 Ti). An aliquot of the supernatant was collected for radioactivity measurement in a gamma spectrometer. The amount of bound concanavalin A was calculated from the differences of concanavalin A added and concanavalin A measured in the supernatant. The specifically-bound concanavalin A represents the amount of concanavalin A which was inhibited by α-methyl mannoside. Control experiments revealed that unspecific absorption (i.e. "binding" in the presence of α-methyl mannoside) was due exclusively to adsorption to the tubes and not to membranes.

Enzyme assays. (a) Adenylate cyclase (EC 4.6.1.1) was measured as described by Salomon et al. [17], with some modifications. The test was done in a total volume of 0.5 ml instead of 0.1 ml. Because of the low protein concentrations, the incubation time was 30 min. (It was found that with lymphocyte membranes the test was linear for at least 1 h.)

(b) ATPases (EC 3.6.1.3) were tested as described in Averdunk and Lauf [18]. Mg^{2+}-ATPase was designated as the enzyme which was not inhibited by 10^{-3} M ouabain. The ouabain-sensitive enzyme was designated as $(K^+ + Na^+)$-ATPase. Phosphate was determined as in ref. 17 or by the method of Fiske and Subba Row [19].

(c) Alkaline *p*-nitrophenyl phosphatase (EC 3.1.3.1) was determined as

described earlier [16], without addition of Triton X-100.

(d) γ-Glutamyltransferase (EC 2.3.2.2) was measured according to Szasz [20] using 1-γ-glutamyl-3-carboxy-4-nitroanilide and glycylglycine (Boehringer) as substrates.

(e) Acyl-CoA:lysophosphatidylcholine acyltransferase (EC 2.3.1.23) was measured as determined earlier [21]. [1-^{14}C]Palmitoyl lysophosphatidylcholine was obtained from General Biochemicals (Biocult, Karlsruhe, G.F.R.), arachidonoyl-CoA from Applied Science (Serva, Heidelberg, G.F.R.). Distribution of acyl-CoA:lysophosphatidylcholine acyltransferase in membrane subfractions was measured as follows. The microsomal membranes were admixed with substrates and incubated for 15 min at 37°C. The enzyme reaction was stopped by mixing the membranes with concanavalin A-Sepharose. Concanavalin A-Sepharose immediately inactivates acyl-CoA:lysophosphatidylcholine acyltransferase. The membranes were then fractionated as described above. Control experiments indicated that more than 90% of the bound phosphatidylcholine was incorporated firmly into the membranes. Enzyme activity of the subfractions was calculated from the amount of radioactive phosphatidylcholine formed.

Results

Fractionation of thymocyte microsomes or plasma membranes. Microsomal membranes were isolated from calf thymocytes, an essentially pure T-lymphocyte population. For disrupting the cells the nitrogen cavitation method was used. This releases small homogeneous vesicles (with a diameter between 50 and 200 nm) from the plasma membrane, which make up the majority of the microsomal fraction of thymocytes (see below). When these membranes were subjected to affinity chromatography on concanavalin A-Sepharose under the conditions that were described in Materials and Methods, two fractions were recovered (Fig. 1). One membrane fraction, designated as MF1, was not retained on concanavalin A-Sepharose and eluted freely. Membrane vesicles which bound to the affinity absorbent could be eluted after mechanical dissociation. This material was collected in a single fraction, designated as MF2. When increasing amounts of membrane protein were applied to concanavalin A-Sepharose, the distribution between the two fractions was constant up to an amount of membranes, which was defined by the "capacity" of the affinity absorbent (Fig. 2a). Above this, the yield of MF2 was constant for a given

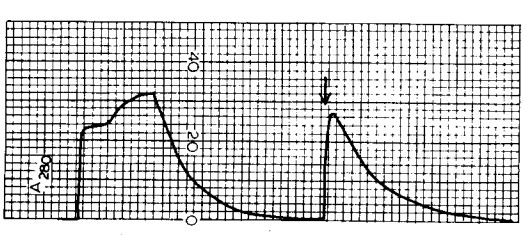

Fig. 1. Fractionation of microsomal membranes on concanavalin A-Sepharose. 3 mg membrane protein was applied to 25 ml concanavalin A-Sepharose gel. The elution profile was monitored in a ISCO UA-5 absorbance monitor. The arrow indicates the beginning of stirring the gel.

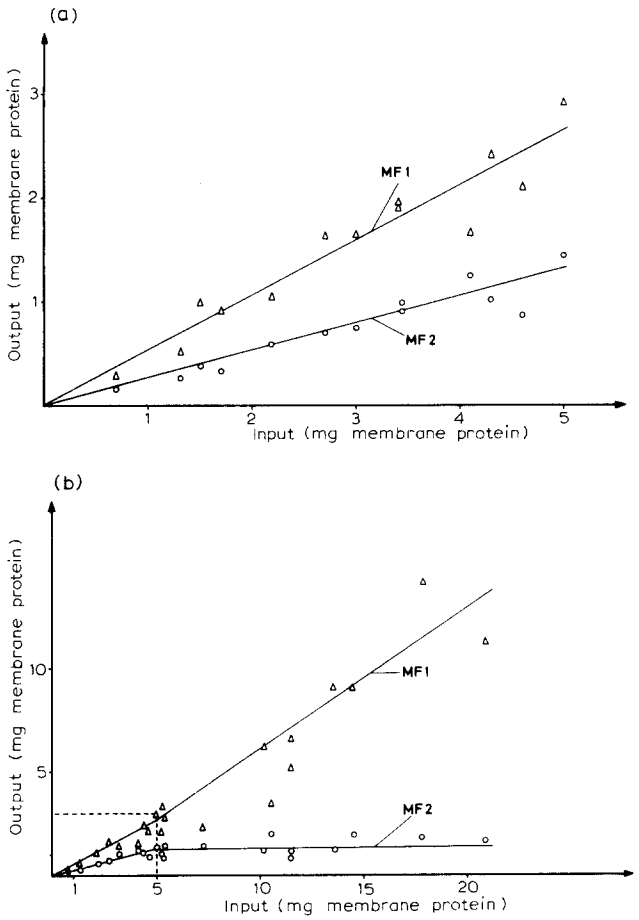

Fig. 2. Fractionation of microsomal membranes on concanavalin A-Sepharose. Microsomes were fractionated with 25 ml concanavalin A-Sepharose gel. (a) 0.6—5 mg membrane protein applied. (b) 0.6—22 mg membrane protein applied.

amount of concanavalin A-Sepharose (Fig. 2b). The capacity of concanavalin A-Sepharose to bind MF2 varied from batch to batch, depending on the number of binding sites that were calculated from the specific binding of [^{14}C]trehalose. The two batches used in this study bound 1.06 or 2.11 nmol [^{14}C]-trehalose per g gel, and exhibited a capacity of 0.442 ± 0.06 or 1.36 ± 0.38 mg membrane protein per 25 ml gel (values are means ± S.D., n = 10 and 18, respectively). In most further experiments, MF1 and MF2 were separated under conditions where the capacity of concanavalin A-Sepharose was not exhausted. In all separation experiments some loss of membrane protein occurred, but the total recovery was relatively high, being 71%. The loss of membrane material appeared to be due to adsorption to the separation chamber, tubing, etc. as it also occurred when membranes were pumped through the separation device without applying Sepharose gel. It should be noted in this respect that during fractionation the membranes were inevitably diluted to low concentrations of 20—80 μg protein/ml. The mean yield of MF1 (calculated as percent of the

TABLE I

FRACTIONATION OF MEMBRANES ON CONCANAVALIN A-SEPHAROSE

	Percent in		Percent recovery
	MF1	MF2	
Microsomes	66.2	33.8 ± 1.6 [a]	71.3 ± 2.8 [a]
Plasma membrane	61.2	38.8 ± 5.1 [b]*	81
Microsomes + α-methyl mannoside [c]	94.2	5.8	100
EL—4 membranes	67.2	32.8 ± 0.3 [d]	82 ± 9 [d]
Erythrocyte membranes	94.1	5.9	95.6

[a] $N = 20$, data given are means ± S.D.
[b] $N = 3$, data given are means ± S.D.
[c] 0.05 M.
[d] $N = 2$, data are means ± ranges.

total recovery) was 66%; the recovery in MF2 was 34% (Table I).

Microsomal membranes isolated from thymocytes are 85—90% plasma membrane as assessed by chemical composition or marker enzymes [13,16]. A minor contamination of 10—15% contains vesicles with higher density (due to a high protein/lipid ratio) which are partly derived from the plasma membrane and partly from internal membranes. To test how these membrane vesicles influenced the separation, highly purified plasma membranes were prepared by CsCl gradient ultracentrifugation. After collecting the plasma membrane fraction, CsCl was dialyzed out slowly as described in Materials and Methods. The purified plasma membrane was subjected to affinity chromatography on concanavalin A-Sepharose. Compared with the fractionation obtained with microsomal membranes, MF2 was slightly higher: 38.8% was recovered in this fraction (Table I).

We have shown in a previous paper that the fractionation of thymocyte membranes was due to the binding of membrane vesicles to matrix-bound concanavalin A. When Sepharose 4B was used, all membrane material eluted in a single non-bound fraction [10]. Similarly, in the presence of the hapten sugar α-methyl mannoside, no or (in some experiments) a minimal adherence of membranes to concanavalin A-Sepharose was observed (Table I). Membranes which were obtained from human erythrocytes (which bind concanavalin A) [22] did not fractionate when mixed with concanavalin A-Sepharose, suggesting that it is the molecular organization of the membrane that is responsible for successful fractionation.

Lens culinaris lectin has the same binding specificity as concanavalin A, but its affinity to lymphocyte membrane receptors is considerably lower [23]. When lymphocyte microsomal membranes were chromatographed on lens culinaris lectin-Sepharose, no separation occurred: all membrane material eluted in a single, non-retarded fraction. This suggests that fractionation of membrane vesicles on concanavalin A-Sepharose was due to the presence of high affinity binding sites for concanavalin A in a fraction of the vesicles (see also below).

Both fractions, MF1 as well as MF2, revealed high homogeneity. In experiments where both fractions were subjected to rechromatography on con-

canavalin A-Sepharose, 89% of MF1 was recovered in the non-bound fraction (i.e. MF1). Similarly, 83% of MF2 was recovered in the bound fraction (MF2).

Fractionation was not due to phase separation at low temperatures. Routinely, the disruption of the thymus lymphocytes was done at 4°C. As mixing of lipids depends on temperature, cooling of cells (and their membranes) below transition temperature could lead to phase separation of lipids which might be responsible for heterogeneity. The transition temperature for mammalian cell membranes is around 16—18°C. Therefore, thymocytes were also disrupted at room temperature (20—25°C). The fractionation of microsomal membranes from cells which were broken at 4°C or at room temperature was identical.

Membrane subfractions are derived from the same cell. To exclude the possibility that MF1 and MF2 represented membranes which were derived from different cells two experiments were designed.

(a) Attempts to fractionate intact viable thymocytes on concanavalin A-Sepharose failed. Using a wide range of cell numbers applied to the affinity absorbent, all lymphocytes were retained, without exception. In addition, when binding of intact thymocytes was inhibited by increasing concentrations of α-methyl mannoside, the relative fraction which did not bind to concanavalin A-Sepharose (F1) followed saturation kinetics for the inhibition. In Fig. 3 the data are presented in a double reciprocal manner. As can be seen, inhibition of binding follows a straight line, suggesting homogeneity of the cells with respect to binding of concanavalin A. In these experiments, between 81 and 94% of the cells were recovered.

(b) Microsomal membranes were isolated from a homogeneous T-cell tumor line, the EL-4 lymphoma, grown in C57BL mice. Fractionation of these membranes on concanavalin A-Sepharose resulted in a separation which was nearly identical to those obtained with membranes from normal thymocytes (Table I).

Binding of concanavalin A to membranes and membrane subfractions. The binding of concanavalin A was determined by incubating varying amounts of

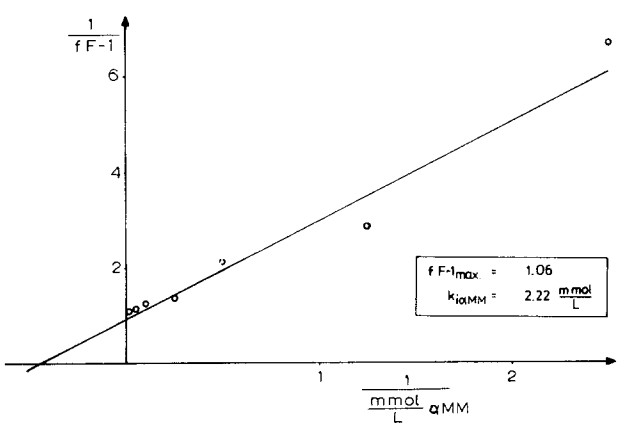

Fig. 3. Fractionation of intact thymocytes on concanavalin A-Sepharose. $3 \cdot 10^8$ thymocytes were admixed to 10 ml concanavalin A-Sepharose gel with varying concentrations of α-methyl mannoside. Double reciprocal plot $fF - 1$, fraction of cells which elutes freely from concanavalin A-Sepharose; $fF - 1_{max}$, fraction of cells which elutes maximally; $K_{i\alpha MM}$, inhibitory constant for α-methyl mannoside.

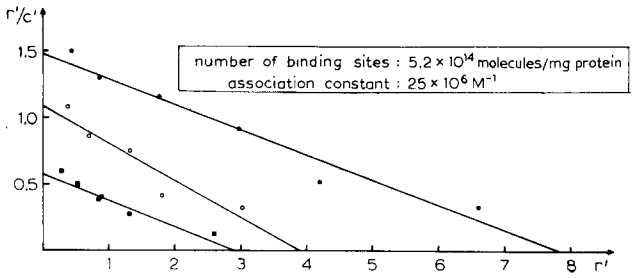

Fig. 4. Binding of concanavalin A to microsomal membranes. Membranes were incubated for 60 min at 37°C with increasing concentrations of concanavalin A. Specific binding was determined as in Materials and Methods. The data were plotted according to Scatchard [24]. The lines were fitted by linear least square regression. r', concanavalin A bound in μg/ml; c', unbound concanavalin A in μg/ml; ■, 25 μg membrane protein; ○, 50 μg membrane protein; ●, 100 μg membrane protein.

^{125}I-labeled concanavalin A with membranes or membrane fractions. After incubation the membranes were spun in an ultracentrifuge. The amount that was bound was calculated after measuring an aliquot of the supernatant, i.e. under equilibrium conditions. In the presence of 0.05 M α-methyl mannoside some "binding" was measured. This amount, however, was independent of the amount of membranes added, being identical also without membranes, indicating that it was due exclusively to adsorption to the centrifuge tubes and not to the membranes.

The amount of concanavalin A that bound specifically (i.e. via its saccharide binding sites) was determined as the amount of concanavalin A that was inhibited by the hapten sugar α-methyl mannoside. When different amounts of microsomal membranes were tested, the specifically-bound concanavalin A increased linearly (Fig. 4). Microsomes bound $5.2 \cdot 10^{14}$ molecules per mg protein, the apparent association constant was $25 \cdot 10^6$ l/mol (M^{-1}).

The results of concanavalin A binding with the membrane subfractions are depicted in Fig. 5. MF1 exhibited more binding sites than MF2. The association constant was 2.5 times higher in MF2 as compared to MF1.

The distribution of membrane-bound enzymes. Several plasma membrane-located enzymes were measured for their distribution in the membrane subfractions (Table II). γ-Glutamyltransferase and adenylate cyclase showed no preferential location, and Mg^{2+}-ATPase was moderately increased in MF1. In contrast, ($K^+ + Na^+$)-ATPase and acyl-CoA:lysophosphatidylcholine acyltransferase were highly enriched in MF2. Alkaline *p*-nitrophenyl phosphatase was also present with higher specific activity in MF2.

Correlation between binding of concanavalin A and the activation of thymus lymphocytes. To permit a direct correlation between binding and cell activation the lymphocytes were culted in serum-free media to avoid any interaction of concanavalin A with serum glycoproteins. In Fig. 6a the specific binding of concanavalin A to intact calf thymocytes is depicted. Optimal binding occurred at concentrations from 30 to 50 μg/ml. The binding sites appeared to be homogeneous, as revealed by a linear Scatchard plot (Fig. 6b) with one lymphocyte exhibiting $1.32 \cdot 10^6$ binding sites. The apparent association constant was 27 l/mol (M^{-1}).

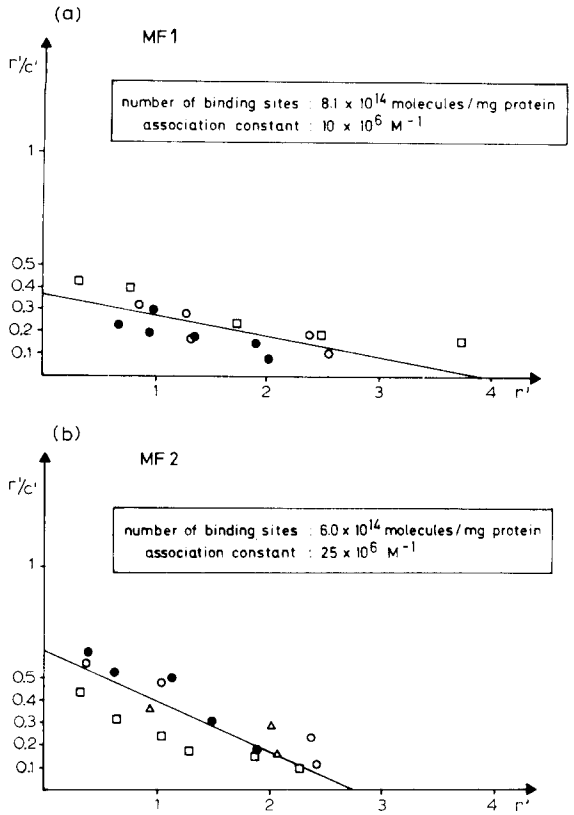

Fig. 5. Binding of concanavalin A to membrane subfractions. 20—40 μg membrane protein was incubated with increasing concentrations of concanavalin A for 60 min at 37°C. Specific binding was determined as in Material and Methods. The data were plotted according to Scatchard [24]. The lines were fitted by linear least square regression. r', concanavalin A in μg/ml; c', unbound concanavalin A in μg/ml. Different symbols represent experiments with different membrane fractions. (a) Specific binding of concanavalin A to MF1. (b) Specific binding of concanavalin A to MF2.

TABLE II

DISTRIBUTION OF MEMBRANE-BOUND ENZYMES IN MEMBRANE SUBFRACTIONS

Data are means of at least four experiments.

	Specific activity (nmol · mg^{-1} protein · min^{-1}, 37°C)		
	Microsomes	MF1	MF2
γ-Glutamyltransferase	12.5	12.5	13.3
Adenylate cyclase *	0.041	0.049	0.057
Mg^{2+}-ATPase	17.2	26.2	19.8
(K^+ + Na^+)-ATPase	5.8	1.8	10.5
Alkaline *p*-nitrophenyl phosphatase	71.8	68.3	134.7
Acyl-CoA:lysophosphatidylcholine acyltransferase **	8.9	5.5	25.2

* Stimulated with 20 mmol/l NaF.

** With arachidonoyl-CoA as substrate.

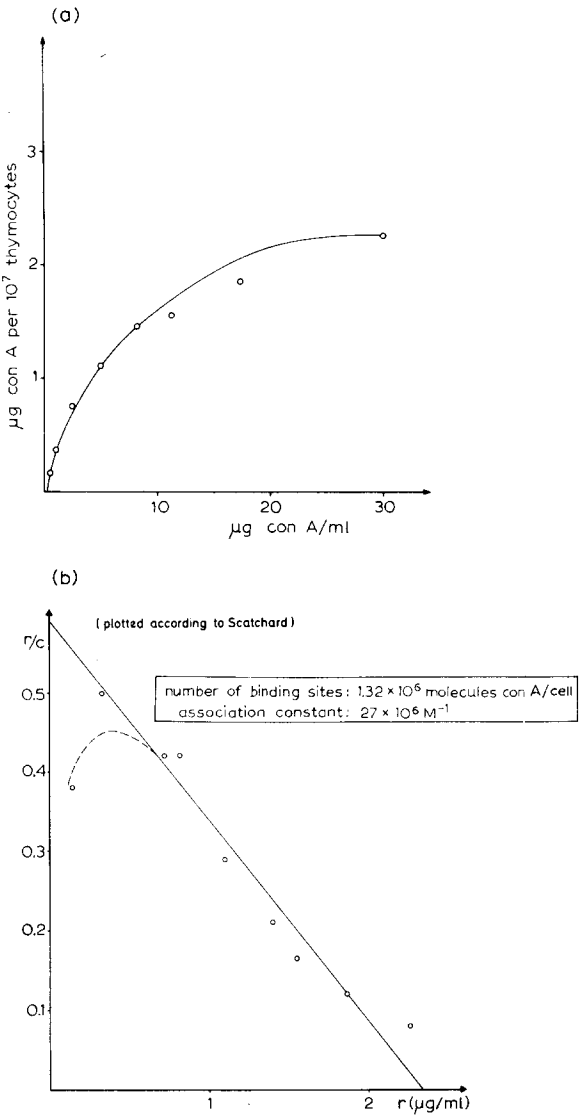

Fig. 6. Specific binding of concanavalin A (con A) to calf thymocytes. 10^7 calf thymocytes in 1 ml phosphate-buffered saline were incubated for 60 min at 37°C with increasing concentrations of concanavalin A. Specific binding was determined as in Material and Methods. The data of b were fitted by linear least square regression. *r*, bound concanavalin A; *c*, unbound concanavalin A. (a) Direct plot of specific binding of concanavalin A. (b) Data plotted according to Scatchard.

The actual number of concanavalin A molecules that are bound to the cell surface, i.e. the equilibrium between binding and dissociation may change during cell culture, but, at least up to 4 h incubation under standard conditions, binding of concanavalin A was constant at various concentrations (data not shown). Addition of 2.5 mg/ml defatted albumin had no influence on the binding of concanavalin A.

When lymphocytes were cultured with concanavalin A in media supple-

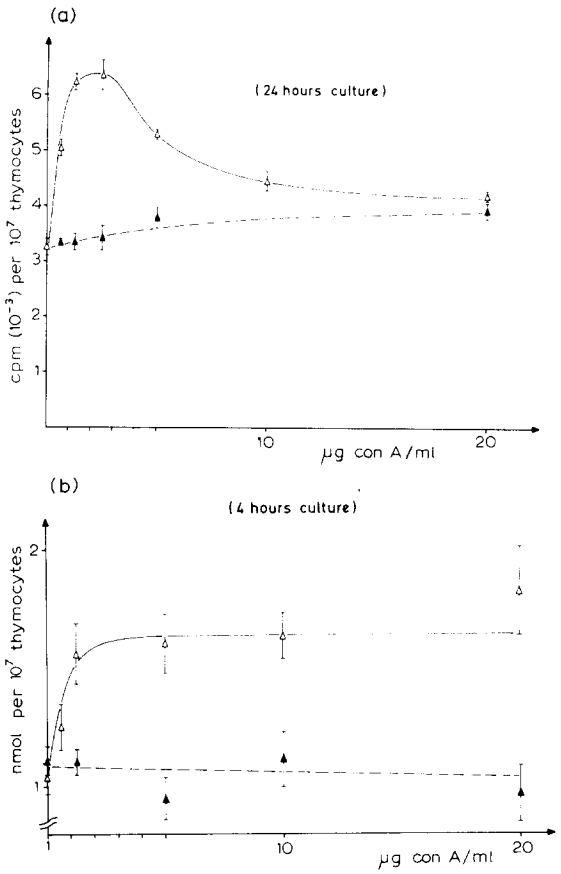

Fig. 7. Activation of calf lymphocytes with concanavalin A (con A). 10^7 calf lymphocytes were cultured in 1 ml Dulbecco's modified Eagle's medium supplemented with 2.5 mg/ml defatted albumin. Open symbols, cultures without α-methyl mannoside; close symbols, cultures that received 0.05 M α-methyl mannoside. (a) Incorporation of [^{3}H]uridine into RNA was measured as in Material and Methods. (b) Incorporation of [^{14}C]oleate into phosphatidylcholine was measured as in Material and Methods.

mented with 2.5 mg/ml albumin, the incorporation of [^{3}H]uridine into RNA after 24 h culture increased dose dependently. Maximal increase occurred at concentrations between 1 and 2 μg/ml concanavalin A. At higher doses the incorporation decreased (Fig. 7a). Similarly, concanavalin A increased the incorporation of [^{14}C]oleate into phosphatidylcholine after 4 h culture. The stimulation was maximal, too, at concentrations between 1 and 2 μg/ml. In contrast the RNA synthesis, activation of the early changes in the membrane phospholipid turnover reached a plateau which was sustained at supraoptimal concentrations of concanavalin A (Fig. 7b). Thus, activation of calf lymphocytes requires the interaction of 14—20% of accessible surface binding sites with concanavalin A, based on the binding curves in Fig. 6.

Discussion

Using affinity chromatography with concanavalin A-Sepharose, microsomal membranes (which contain more than 85—90% plasma membranes) or highly

purified plasma membranes from calf thymus lymphocytes could be fractionated. With the methods applied, two fractions were obtained, MF1 and MF2, which were homogeneous by rechromatography under identical conditions.

MF2, however, was only homogeneous under the conditions chosen for separation. To yield all membrane material that was bound to concanavalin A-Sepharose, MF2 was released with constant stirring of the gel (see Materials and Methods). That the dissociation was complete is supported by the fact that even 0.5 M α-methyl mannoside (and constant stirring) did not release any more membrane material. With graduated forces (i.e. time and vigor of mechanical forces applied together with increasing amounts of α-methyl mannoside) MF2 could be further subfractionated into fractions with increasing affinity for concanavalin A (unpublished results).

Several possibilities exist that might be responsible for the observed fractionation: (*a*) The separation was not due to affinity chromatography, but to unknown physicochemical parameters (e.g. size of fragments). (*b*) The fractions obtained were derived from different membranes (i.e. plasma membrane vs. internal membranes). (*c*) Fractionation was due to the orientation of membranes (i.e. right side-out or inside-out). (*d*) Membranes derived from different cells were separated. (*e*) The membrane fractions originated from different areas of the plasma membrane of individual lymphocytes.

(*a*) When lymphocytes are disrupted by the nitrogen cavitation method [11], the outer membrane fragments into small pieces which form vesicles of 50—200 nm diameter. Assuming a smooth surface of a thymus lymphocyte [25], one lymphocyte with a diameter of about 5—7 μm releases between 1000 and 10000 vesicles. As one calf thymocyte carries $1.32 \cdot 10^6$ binding sites per cell (see Fig. 6) one membrane vesicle exhibits more than 100 binding sites.

Up to a certain limit of membranes applied to concanavalin A-Sepharose the ratio between membranes recovered in MF1 and MF2 was constant (see Fig. 2). When more membranes were added, the amount of MF2 recovered reached a saturation value, the capacity of concanavalin A-Sepharose (Fig. 2b). Each batch of concanavalin A-Sepharose had a characteristic capacity to bind membrane vesicles which roughly corresponded to the number of active concanavalin A molecules bound to the carrier as determined by binding of [^{14}C]-trehalose. Thus, binding of concanavalin A to membrane binding sites appears to be essential in separation of different membrane fractions, indicating that fractionation is due to affinity chromatography. This is supported by our earlier finding that chromatography on Sepharose 4B alone yielded a single non-adherent fraction [10]. In addition, fractionation could be prevented completely by the hapten sugar α-methyl mannoside (ref. 10 and Table I).

(*b*) Affinity chromatography of membranes on concanavalin A-Sepharose is very sensitive to aggregation. As all methods tested to further purify plasma membranes either involve saccharide-containing buffers (which interfere with affinity chromatography) or increase the tendency to aggregate (CsCl, see Materials and Methods) most experiments were done with microsomal membranes. According to various chemical and biochemical criteria, lymphocyte microsomal membranes prepared by the nitrogen cavitation method are not contaminated significantly by internal membranes [11]. When prepared from

thymocytes, microsomes consist of at least up to 85—90% of plasma membranes (refs. 13 and 16, and manuscript in preparation). This is explained by the fact that small lymphocytes do not contain appreciable amounts of endoplasmic reticulum [26]. Accordingly, using plasma membrane vesicles purified by CsCl gradient ultracentrifugation, the relative yield of MF2 only slightly increased over the yield of MF1 (see Table II). We have found previously that the cholesterol/phospholipid ratio is nearly identical in the non-adherent (now designated MF1) or the adherent fraction (MF2) [27] prepared from microsomal membranes. In addition, γ-glutamyltransferase, Mg^{2+}-ATPase and adenylate cyclase had roughly the same specific activity in both fractions as well as in the unseparated membranes. Mg^{2+}-ATPase as well as γ-glutamyltransferase have been shown to be located preferentially in the plasma membrane of calf thymocytes [28,29]. In contrast, adenylate cyclase exhibits its highest specific activities in the nuclear membrane (unpublished results, and ref. 30). Taken together, these data indicate that even the slightly impure microsomal membranes are fractionated. The obtained subfractions consist of predominantly identical membranes, i.e. subfractions of the plasma membrane.

(*c*) Fractionation of lymphocyte membranes by binding to concanavalin A-Sepharose has been reported recently using a murine plasmacytoma [8] or pig lymph node lymphocytes [9]. With the plasmacytoma 50% and with the pig lymphocytes 30—50% of the membranes did not adhere to concanavalin A-Sepharose. Both groups reached the conclusion that they had separated inside-out membranes from right side-out membranes. The critical difference to our experiments is that different cell disruption techniques were applied which indeed may release differently oriented membranes. The nitrogen cavitation method which we applied for cell disruption has been shown to result in membrane vesicles that have preserved their original orientation [31]. In addition, our binding studies support this strongly. The non-adherent fraction MF1 exhibited even more binding sites in a Scatchard analysis than the adherent fraction MF2 or the unseparated membranes. The membrane vesicles obtained with the nitrogen cavitation method are sealed, as their buoyant density depends on the osmotic pressure of the buffer [31]. In our experiments, microsomal membranes floated exclusively on top of a 26% dextran (M_r = 110000) in 0.01 M HEPES, pH 7.0) (ρ = 1.08), whereas in 26% CsCl all membranes were recovered at densities higher than 1.10. This shows that thymocyte microsomal membranes also are semipermeable to solutes in their external environment and most likely impermeable to molecules of the size of concanavalin A. Thus, as binding studies were performed in isotonic buffers, concanavalin A most probably can only reach binding sites at the outer surface of the vesicles. Since plasma membrane carbohydrate is located exclusively on the outside surface [32], the vast majority of the microsomal or plasma membrane vesicles and, concomitantly, both membrane subfractions must be right side-out.

(*d*) Thymus lymphocytes consist nearly exclusively of T-lymphocytes, but within the thymus, cells of different differentiation stages are found [33]. Therefore, the possibility exists that membranes from different cells were fractionated. Two sets of experiments make this highly unlikely:

(1) Intact thymus lymphocytes all adhered to concanavalin A-Sepharose (see

Fig. 3). In addition, when adherence was inhibited by graduated amounts of α-methyl mannoside, the recovery in the unbound cell fraction followed kinetics which, when plotted in a double reciprocal manner, resulted in a straight line. Thus suggests that α-methyl mannoside acted as a competitive inhibitor for cells homogeneous with respect to binding of concanavalin A.

(2) When membranes were prepared from a T-cell line, the mouse T-lymphoma EL-4, and fractionated on concanavalin A-Sepharose, a distribution of subfractions was obtained that was identical to those obtained with calf lymphocytes. Normal mouse thymocytes also fractionated in the same way (data not presented). Thus, at least fractions which closely resemble those of normal cells can be obtained from membranes of homogeneous cell population, suggesting that membrane subfractions of normal thymocytes are also derived from the same cell. In addition, all lymphoid cells tested showed a remarkably similar fractionation of their membranes on concanavalin A-Sepharose including rabbit thymocytes [10] and lymph node cells, and chicken thymus and bursa Fabricii cells (i.e. pure T- and pure B-lymphocytes).

We have shown earlier that rabbit thymus lymphocytes enriched for cells of different maturation stages respond initially in an identical manner to concanavalin A [35], suggesting that all thymus lymphocytes have the capacity to be activated by concanavalin A. This may explain why with respect to concanavalin A receptors thymocytes behave like a homogeneous cell population.

(*e*) The above results thus indicate that both fractions, i.e. MF1 and MF2, were derived most probably from different areas of the plasma membrane of an individual lymphocyte. This is supported by two additional findings.

(1) The binding of concanavalin A: As shown in Fig. 5, the association constant for concanavalin A was 2.5 times higher in the adherent fraction MF2 compared to the unbound fraction MF1. It should be noted that unseparated membranes as well as intact cells (Fig. 6) showed an association constant which was close to that measured in MF2. From the differences measured in the separated fractions one would expect a bend in the Scatchard plot of the binding data of unseparated membranes. Indeed, in some experiments such a bend was observed (data not shown). However, this bend occurs at rather high concentrations of concanavalin A (which can be seen if the binding plots of both separated fractions are superimposed) where the sensitivity of the method for measuring concanavalin A binding is low. The differences in the affinity of concanavalin A in MF1 and MF2 may well be the critical parameter which is responsible for fractionation.

(2) The distribution of membrane-bound enzymes: The activities of several membrane-bound enzymes exhibited a characteristic distribution. Adenylate cyclase and γ-glutamyltransferase were roughly identical in all fractions while Mg^{2+}-ATPase was somewhat increased in the non-bound fraction MF1. In contrast, $(Na^+ + K^+)$-ATPase was located nearly exclusively in MF2, whereas its specific activity in MF1 was barely measurable. It has been shown that addition of soluble concanavalin A to isolated lymphocyte membranes modulates the activity of $(Na^+ + K^+)$-ATPase [34]. However, the total activities recovered in both fractions represented 81% of the original activity in the unseparated membrane, excluding that the apparent dissociation of $(Na^+ + K^+)$-ATPase in MF1 and MF2 was due to binding of concanavalin A. A similar enrichment of

enzyme activity in MF2 was also found for acyl-CoA:lysophosphatidylcholine acyltransferase. As the product of its catalytic activity, i.e. phosphatidylcholine, is incorporated firmly into the membrane, this enzyme assay could be performed before the separation of the membrane subfractions on concanavalin A-Sepharose, excluding any modulation of enzyme activity due to binding of concanavalin A (see Materials and Methods).

Thus, in the lymphocyte plasma membrane receptors with high affinity for the mitogen concanavalin A appear to be closely linked to distinct membrane-bound enzymes. As mitogens must interact only with a certain fraction of their binding sites to trigger differentiation of proliferation, the data suggest that functional areas exist in the plasma membrane of lymphocytes that are associated with the initiation of lymphocyte activation.

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