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A SIMPLE AND RAPID METHOD FOR THE PREPARATION OF PLASMA MEMBRANES

TOYOZO MAEDA *, KRISHNA BALAKRISHNAN ** and S. QASIM MEHDI ***

Stauffer Laboratory for Physical Chemistry, Stanford University, Stanford, CA 94305 (U.S.A.)

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A simple and rapid method for preparing plasma membranes from isolated cells or tissues is described. The membranes were characterised (a) biochemically by an analysis of specific marker enzymes, (b) by quantitation of cell surface receptors, and (c) immunologically by their ability to elicit specific allogeneic responses from cytotoxic T cells in secondary in vitro stimulations. Based on both biochemical and immunologic criteria, plasma membranes prepared by the method described here are of equal or greater 'purity' compared to those prepared by two other methods that are most widely used to date and the yields are several-fold higher.

Introduction

An understanding of the structure and function of cell membranes is one of the central themes of contemporary cell biology. Although some properties of the cell surface can be studied on intact cells, the analysis of membrane constituents in relation to their function often requires their separation and isolation from the cell cytoplasm and other sub-cellular organelles. Several techniques are available for the preparation of plasma membranes, but they have usually been tailored for the preparations of membranes from particular cell types or for the study of specific membrane functions. (For reviews see Warner and Glick [1] and Crupton and Snary [2]). The development of a simple procedure for membrane isolation that can be used for a wide range of cells and tissues would be useful.

Essentially, membrane preparation involves cell disruption (or tissue homogenization) by various means, followed by several steps of differential and/or density gradient centrifugation. Cell disruption is usually achieved by gentle methods such as Dounce homogenization, or by more vigorous methods such as nitrogen cavitation or Polytron shearing. Each of these methods has its advantages and shortcomings. For example, Dounce homogenization is satisfactory for the preparation of membranes or ghosts from small quantities of isolated cells (such as red blood cells). However, its use with large numbers of cells is cumbersome. Yields are extremely low when membranes are prepared even from micro-thin slices of whole tissues or organs (e.g. muscle, thyroid, etc.). Methods that involve several steps of centrifugation give poor yields of plasma membranes because of losses at each step.

Plasma membrane preparations can be characterised by a variety of methods. In general, the methods used have been tedious and often inaccurate [3]. Many have relied on electron and phase contrast microscopy and on the analysis of specific marker enzymes. The use of specific membrane

* Deceased.
** Present address: DNAX Research Institute for Molecular and Cellular Biology, Palo Alto, CA 94304, U.S.A.
*** To whom correspondence should be addressed.

receptors as general markers for monitoring the recovery of plasma membranes [3,4] enabled us to devise a rapid and simple procedure for the purification of plasma membranes. This method is applicable to a wide variety of cells and tissues. The purity of membranes prepared by the present method is equal to or greater than that achieved by two of the most widely used methods and the yields are several-fold higher.

Materials and Methods

Cells and tissues

EL4 thymoma (H-2^b haplotype) and P815 mastocytoma (H-2^d haplotype) were grown in the ascites fluid of C57BL/6 and DBA/2 mice respectively. Human thyroids were obtained at operation and kept on ice until they were processed within 30 min of removal.

Plasma membranes

Plasma membranes were prepared by three different methods.

Method 1 (the Neville technique [5]). This method has been modified for application to different cell-types and tissues [6]. Briefly, cells or finely minced pieces of tissue in 1 mM $NaHCO_3$, pH 7.5, were homogenized using 30–40 strokes in a Dounce homogenizer. The homogenate was kept on ice for several minutes to allow cell lysis. Following filtration through two fine (No. 120) and then four coarse (No. 40) layers of cheese cloth, the homogenate was centrifuged at $1860 \times g$ for 25 min and the supernatant discarded. The pellet was resuspended, homogenized again in a loose fitting Dounce homogenizer using a few strokes, and centrifuged at $1860 \times g$ for 15 min. The pellet was then homogenized gently using 5 strokes in a very loose fitting Dounce homogenizer and combined with 63% sucrose in 1 mM $NaHCO_3$, pH 7.5, to provide a final concentration of 48% sucrose.

Next, a sucrose gradient was prepared. On top of 10–12 ml of the membrane suspension in sucrose solution, 7 ml of 45% sucrose, 10 ml of 41% sucrose and 2–3 ml of 37% sucrose (all in 1 mM $NaHCO_3$, pH 7.5), were carefully layered. The tubes were then centrifuged at $63\,600 \times g$ for 120 min. The interfacial band between 37% and 41% sucrose solutions was collected by aspiration with a pipette, diluted 1 : 1 with the $NaHCO_3$ solution and centrifuged at $30\,800 \times g$ for 30 min. The membrane pellet was resuspended in a small volume of the $NaHCO_3$ solution and stored at −20°C, or in liquid nitrogen.

Method 2 (method of Crumpton and Snary [2]). This method has been generally used for the preparation of plasma membranes from cells (lymphocytes) grown in suspension cultures. Cells were washed thoroughly in 10 mM Tris-saline buffer (pH 7.5) and disrupted by nitrogen cavitation. The nuclear particles were removed by centrifugation at $300 \times g$ for 15 min and the mitochondrial particles were removed by centrifugation at $4000 \times g$ for 30 min. The microsomal pellet obtained by spinning the supernatant at $20\,000 \times g$ for 30 min was washed and resuspended in 36% sucrose solution in Tris-buffered saline. It was overlayed with 25% sucrose in the same buffer solution and centrifuged at $100\,000 \times g$ (22 000 rpm in an SW27 swinging bucket rotor) for 18 h. The interfacial band of plasma membranes was carefully aspirated with a Pasteur pipette and washed twice by resuspension followed by sedimentation at $75\,000 \times g$ for 20 min. The purified plasma membrane fragments were stored under liquid nitrogen.

Method 3 (the present method). This is a simple procedure for isolating plasma membranes and is described in detail here.

All plastic or cellulose nitrate tubes used during the isolation of membranes were soaked overnight in 1 mM EDTA, pH 7.0 and rinsed ten times with doubly distilled water. All operations were done in the cold.

The tissue (10–40 g) was minced finely with surgical scissors and kept on ice in a 250 ml round bottom flask. A 3-fold (w/v) excess of homogenisation buffer (10 mM sodium phosphate buffer, pH 7.4, containing 1 mM $MgCl_2$, 30 mM NaCl, 1 mM dithiothreitol, 0.005 mM phenylmethylsulfonyl fluoride, 0.02% NaN_3 and a few micrograms of DNAase) was added and the tissue homogenised using a Polytron homogeniser (Brinkmann) at a power setting of 7.0. (Two to five bursts of 5 s each separated by 1 to 2 min of cooling were sufficient for cell disruption, which was monitored by light microscopy). About 30 ml of the homogenate was layered over 10 ml of a

41% solution of sucrose in the homogenisation buffer and centrifuged at 95 000 × *g* for 1 h in a Beckman SW27 swinging bucket rotor. The white interfacial band of membranes was collected, the sucrose was diluted by adding a 2–3-fold excess of the homogenization buffer and the membranes were pelleted by centrifugation at 95 000 × *g* for 20 min. The membranes were washed twice and stored at −70°C or in liquid nitrogen until they were used. The same procedure was used for preparing membranes from isolated cell suspensions.

For convenience, the three methods of plasma membrane preparation are referred to as Methods 1, 2 and 3, respectively.

Plate binding assay

A simple and sensitive plate binding assay as described by Howard et al. [7] was used to check the ability and specificity of the membrane preparations to bind alloantibody. The anti-H-2^b alloantiserum used in the assay was obtained by hyperimmunising Balb/c mice with $2–3 \cdot 10^6$ EL4 cells.

Cytotoxicity assays

Balb/c mice were immunised intraperitoneally with $3 \cdot 10^7$ EL4 cells. The primed splenocytes ($7.5 \cdot 10^6$ cells/ml, 2 ml volume) were cultured in RPMI 1640 (GIBCO) supplemented with 5% fetal calf serum, antibiotics and $5 \cdot 10^{-5}$ M 2-merceptoethanol at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. 10–60 μg/ml of plasma membranes were used as secondary in vitro immunogens. EL4 cells irradiated with 5000 rads or P815 plasma membranes were also used as immunogens to check the specificity of the cytotoxic response. After 4 days, the induced cytotoxicity was checked on ^{51}Cr-loaded EL4 cells at various effector to target ratios.

Marker enzyme analysis of the plasma membrane fragments

The plasma membrane fragments were tested for the specific activities of the following enzymes: adenylate cyclase [8], ($Na^+ + K^+$)-ATPase [9], 5′-nucleotidase [10], NADPH-cytochrome *c* reductase [11], glucose-6-phosphatase [12] and succinate dehydrogenase [13]. The first three enzymes are markers for the plasma membrane and the others are specific for microsomes, endoplasmic reticulum and mitochondria, respectively.

Iodination of thyrotropin and its binding to membranes

Radioiodination of bovine thyroid-stimulating hormone (thyrotropin; a generous gift from Professor J.G. Pierce) was by a modification of the chloramine T method and specific binding of thyrotropin to plasma membranes was measured as described in detail earlier [4].

Results

Yields

The yields of plasma membranes obtained by the three methods were compared on the basis of their protein content. The present method of membrane preparation (Method 3) yielded approx. 50-fold and 10-fold higher quantities of plasma membranes as compared to Methods 1 and 2, respectively. Starting with 1 g wet weight of tissue, Method 3 reproducibly yielded 1–2 mg of plasma membrane protein, while Method 1 yielded only 0.05 mg. Similarly, when membranes were prepared from $5 \cdot 10^8$ cells in suspension, Methods 1 and 2 gave 0.02 mg and 0.1 mg, respectively, while Method 3 yielded 1.0 mg.

Binding of radioiodinated thyrotropin

Specific binding of ^{125}I-labeled thyrotropin to human thyroid membranes prepared by Methods 1 and 3 was compared. As shown in Fig. 1a, hormone binding per unit of membrane protein was similar in the two preparations.

Antigenic specificity of the purified plasma membranes

The ability of purified plasma membranes from EL4 cells to bind anti-H-2^b alloantibody is shown in Fig. 1b. Membranes from P815 cells (H-2^d) do not bind this alloantibody as they do not share histocompatibility determinants with EL4 (H-2^b). The amount of histocompatibility antigens in the plasma membranes prepared by Methods 2 and 3 is essentially the same as indicated by the level of alloantibody binding.

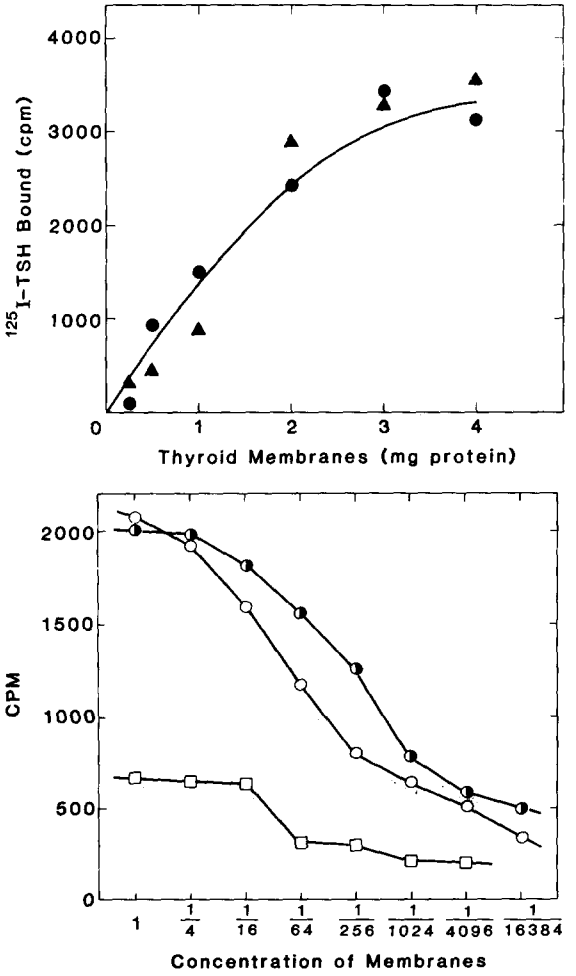
Immunogenic specificity of the plasma membrane fragments

Balb/c (H-2^d) mice were first primed with EL4 (H-2^b) cells. EL4 membrane fragments prepared by Methods 2 and 3 or P815 (H-2^d) membranes

prepared by Method 2 were then used in vitro as stimulators to obtain cytotoxic T cells from the primed Balb/c splenocytes. As shown in Table I, the cytotoxicity elicited by EL4 membranes was specific (since P815 membranes were ineffective in eliciting a secondary allogeneic response). Membranes prepared by both methods were equally immunogenic.

Biochemical characterisation

The results of marker enzyme analysis of whole thyroid homogenates as well as membrane fragments prepared by Methods 1 and 3 are summarised in Table II. The specific activities of marker enzymes for plasma membranes: adenylate cyclase, $(Na^+ + K^+)$-ATPase and 5'-nucleotidase were much enhanced in the purified thyroid membranes as compared with the whole thyroid homogenate. On the other hand, the marker enzymes for the endoplasmic reticulum, mitochondria or microsomal fraction were hardly detected on the purified plasma membranes. The membrane fragments obtained by Method 3 appear to be of equal or greater biochemical 'purity' as compared to those obtained by Method 1.



Discussion

Several techniques are available for the preparation of plasma membrane fragments from cells and tissues. Some of them are applicable to only certain tissues and cell types and yield poor results when applied to other tissues and cell types. In sharp contrast, the present method has broad applications.

As determined by immunological markers, hormone binding to specific membrane receptors and analysis of enzymatic activities (see the Results section), the plasma membranes prepared by

Fig. 1. (a) Binding of ^{125}I-labeled thyrotropin to plasma membrane. Human thyroid membranes prepared by the Neville technique (●) and by the present method (▲) were resuspended in 0.5 ml of 10 mM phosphate buffer, pH 7.4, containing 30 mM NaCl. The concentration of membrane proteins in each duplicate sample is indicated in the figure. Approx. 25000 cpm of ^{125}I-labeled thyrotropin in 0.1 ml of phosphate-NaCl buffer was then added and the mixture incubated at 24°C for 1 h. To correct for nonspecific binding, each sample had a control containing excess (2 IU of Thytropar, Armour Pharmaceutical Co., Phoenix, Arizona, U.S.A.) unlabeled thyrotropin (TSH). The mixture was then centrifuged at 100000 × *g* for 15 min and the supernatant was carefully removed by aspiration. The tip of the tube containing the membrane pellet was then excised and radioactivity measured using a Beckman Autogamma 8000 counter. (b) The ability of purified plasma membrane fragments to bind anti-H-2^b alloantiserum. Microtitre plates were coated with 25 μl of serially diluted membranes in phosphate buffered saline, pH 7.2. After overnight incubation at 4°C, unbound membranes were removed by washing and 25 μl of anti H-2^b antiserum was added. The plates were incubated for 1 h at room temperature, and unbound antibody removed by washing. Then 25 μl of ^{125}I-labeled protein A (20000 cpm) was added and the plates incubated for a further 1 h at room temperature. The plates were washed again and the radioactivity in individual wells was measured using a Beckman Autogamma 8000 counter. ○——○, EL4 (H-2^b) membranes prepared by Method 3; ◑——◑, EL4 membranes prepared by Method 2; and □——□, P815 (H-2^d) membranes prepared by Method 2.

TABLE I

ALLOGENEIC CYTOTOXICITY BY Balb/c LYMPHOCYTES USING PLASMA MEMBRANES AS SECONDARY IN VITRO IMMUNOGENS

Effector cells were obtained as described in Materials and Methods. Cytotoxicity was determined following incubation for 3 h at 37°C with ^{51}Cr-loaded EL4 cells at various effector to target ratios [15]. Values for specific lysis have been corrected for background lysis by Balb/c lymphocytes cultured in medium without any immunogens, and cytotoxicity was calculated as follows.

$$\text{Cytotoxicity} = \frac{\text{Cpm released in the presence of effector cells} - \text{Background}}{\text{Total releasable cpm in target cells} - \text{Background}} \times 100$$

Total releasable cpm was obtained as counts released from the target cells by three cycles of freezing and thawing. The background was determined by incubating the target cells in the presence of Balb/c lymphocytes cultured in medium with no immunogens.

Secondary immunogen	Concn. (μg/ml)	Cytotoxicity (% ^{51}Cr release), effector-target ratio			
		3	9	27	80
EL4 membrane (Method 3)	10	6.5 ± 1.0	13.3 ± 1.5	35.7 ± 1.6	60.4 ± 1.3
	30	10.9 ± 1.3	27.0 ± 1.8	54.5 ± 1.1	66.5 ± 2.1
	60	15.9 ± 1.0	29.7 ± 1.9	63.6 ± 0.9	73.3 ± 1.4
EL4 membrane (Method 2)	10	12.1 ± 1.3	29.9 ± 1.4	55.4 ± 0.5	75.6 ± 1.2
	30	18.8 ± 1.3	40.3 ± 1.9	65.6 ± 2.0	75.7 ± 2.6
	60	15.1 ± 1.8	33.9 ± 1.1	64.8 ± 0.6	76.6 ± 0.8
P815 membrane (control) (Method 2)	10	1.5 ± 0.9	1.2 ± 0.7	2.3 ± 0.5	0.9 ± 0.2
	30	1.1 ± 1.5	0.3 ± 0.4	1.3 ± 1.5	−0.1 ± 2.9
	60	1.4 ± 1.6	0.9 ± 1.4	1.4 ± 1.8	1.3 ± 0.8

TABLE II

MARKER ENZYME ANALYSIS OF HUMAN THYROID MEMBRANES

Human thyroid tissue was homogenised as described in Materials and Methods. Plasma membranes were prepared by the Neville technique (Method 1) and according to the present method (Method 3). Marker enzymes were analysed in the crude homogenates and in the two membrane preparations as described in the text. n.d., not detected. Figures are presented as mean ± S.E. of triplicates or averages of closely agreeing duplicates.

Enzyme		Enzyme activity in:		
		Thyroid homogenate (10 min at 1000 × g supernatant)	Membranes prepared by	
			Present method	Neville method
Adenylate cyclase [a]	100 mU TSH	0.17	3.77 ± 0.5	4.65 ± 0.6
	10 mM NaF	0.89	12.00 ± 7.0	18.30 ± 2.3
$(Na^+ + K^+)$-ATPase [b]		0.63 ± 0.12	9.55 ± 2.3	7.12 ± 2.2
5′-Nucleotidase [b]		1.39	22.4	19.46
NADPH-cytochrome *c* reductase [b]		0.56	n.d.	0.088
Glucose-6-phosphatase [b]		1.21	0.39	0.21
Succinate dehydrogenase [b]		0.35	n.d.	n.d.

[a] nmol/h per mg protein.
[b] μmol/h per mg protein.

the present method are of equal or greater purity than membranes prepared by the other two commonly used methods. Using this method we have successfully prepared membranes in high yields from several cell types and tissues such as human skeletal muscle, eye muscle, cheek muscle, gluteal muscle, spleen, thymus, liver, adrenal cortex, lymphocytes, chinese hamster ovary cells and rabbit mammary tissue.

Another advantage of the present method is its simplicity and rapidity. It involves a single homogenization step followed by a single centrifugation step. The currently used methods involve tedious techniques of cell disruption followed by repeated differential centrifugation to remove different subcellular organelles and finally (sucrose) density gradient centrifugation for the isolation of plasma membranes. The use of a single 'cushion' of 41% sucrose, instead of a discontinuous sucrose density gradient, stems from a previous observation [4] that membranes (measured by their thyrotropin-binding activity) are unable to penetrate and sediment through this cushion after centrifugation at $100\,000 \times g$ for upto 4 h. Although the activity of enzymes that are labile, e.g., adenylate cyclase may be reduced or destroyed by the shear forces involved in Polytron homogenization, other biochemical activities that could have been lost by lengthy procedures are retained. The presence of phenylmethylsulfonyl fluoride (a protease inhibitor) in the homogenisation buffer and the quick separation of the plasma membrane from the cytoplasm also results in little, if any, damage to the membrane components.

Plasma membranes can be characterized by physical means such as electron microscopy, high resolution phase contrast light microscopy or by biochemical means such as enzymatic analysis or receptor quantification. Of all the above methods, identification and quantitation of specific cell surface receptors is the most convenient and reproducible method for monitoring plasma membrane recovery [3]. In the present work we have used cell surface markers (histocompatibility antigens and hormone receptors) and the activity of specific marker enzymes to assay for the purity and recovery of membranes and the exclusion of other sub-cellular organelles.

The procedure described here reproducibly gives pure preparations of membranes with high yields.

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