SPECIFIC BINDING OF RAT LIVER PLASMA MEMBRANES BY RAT LIVER CELLS

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SUMMARY: A method to determine the binding of radiolabeled membranes to isolated, viable rat hepatocytes is described. Rat liver plasma membranes bind more efficiently than similar membranes from chicken liver; the binding of mitochondrial membranes is negligible. The binding of rat liver plasma membranes is temperature dependent and can be inhibited by preincubation with plasma membranes but not with mitochondrial membranes.

INTRODUCTION

Considerable attention has been focused on cell contact and adhesion and their role in development and disease e.g. neoplasias, but the molecular mechanisms of the adhesion phenomena are still largely unknown. Recently we have introduced a model system consisting of juvenile liver cells for biochemical studies on intercellular adhesion (1-3) and demonstrated that isolated plasma membranes specifically stimulated the aggregation of suspended rat liver cells (1). The recognition and binding of plasma membranes by cells have earlier been described in an embryonic system (4). In this report we present a new method to determine membrane binding and describe some of the characteristics of binding of rat liver plasma membranes to rat liver cells.

MATERIALS AND METHODS

Isolation of cells and membranes. Hepatocytes, plasma membranes (Band 1) and mitochondrial membranes (Band 3) were isolated from livers of male Sprague-Dawley rats (175-250 g) that had been fasted for 15-18 hours as previously described (1, 2). Membranes (Band 1 and Band 3) were prepared in the same way from livers of White Leghorn chickens. All membranes were routinely treated by dialysis against EGTA-medium as described (1,3) before being used in cell binding experiments. Radioactive membranes were prepared by intraperitoneal injection of [^{35}S]sulphate or [^{32}P]orthophosphate (1-2 mCi) 2 hours before sacrificing the animals. Injection of 2 mCi of ^{32}P resulted in approximately 3×10^5 dpm per mg of membrane protein, whereas ^{35}S gave a ten-fold lower specific activity of plasma membranes and a 100-fold lower specific activity of

Abbreviations: EGTA, ethylene glycol bis (β-aminoethyl ether)-N,N'-tetraacetic acid; HEPES, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid.

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mitochondrial membranes. The membranes were stored in Buffer 3 (see below) at -20°C in a number of small portions and were freezed and thawed only once before being used for cell binding experiments.

Incubation of cells and membranes. Cells and membranes were incubated together in Buffer 3 (1,2), which contained 8.0 g NaCl, 0.35 g KCl, 0.16 g $MgSO_4 \cdot 7H_2O$, 0.18 g $CaCl_2 \cdot 2H_2O$, 2.4 g HEPES, H_2O to 1 l, adjusted to pH 7.4 with 1 M NaOH. DNase I (Sigma DN-25) was added to a final concentration of 0.05 mg/ml. Rotary or reciprocal shaking was used. In the rotational shaking, incubation was carried out in plastic dishes with flat-bottomed wells 16 mm in diameter (Linbro Sci. Co., Cat.No. FB16-24TC) while round-bottomed plastic tubes 16 mm in diameter (Falcon Plastics, Cat.No. 07/2037) were used in the reciprocal shaker. All experiments were made in duplicate incubations.

Separation of cell-bound and unbound membranes. Cells and membranes were separated according to buoyant density by centrifugation in isotonic Percoll (a gift from Dr. L. Kågedal, Pharmacia Fine Chemicals, Uppsala, Sweden) with a final salt composition corresponding to Buffer 3. Percoll is a solution of colloidal silica particles coated with polyvinyl pyrrolidone, which forms a self generating density gradient in high centrifugal fields (5). Density was determined with a density column made from organic liquids (5). The buoyant densities determined by isopycnic centrifugation in selfgenerated Percoll gradients were 1.024-1.027 g/ml for Band 1, 1.015-1.019 g/ml for Band 3 and 1.075-1.094 g/ml for viable hepatocytes. After freezing and thawing these densities changed to 1.025-1.032 g/ml for Band 1 and 1.020-1.027 g/ml for Band 3. The small proportion of non-viable cells (≤10%) in the cell preparations had a buoyant density of 1.034 g/ml which was significantly different from the heaviest plasma membranes (Band 1). Thus unbound membranes could be separated from cell-bound membranes by zone centrifugation at low speed on a cushion of Percoll with a density of 1.033 g/ml under which conditions no generation of a density gradient occurred. The incubation mixture (200 µl) was layered on top of 10 ml Percoll (1.033 g/ml) in a conical glass centrifuge tube which was centrifuged at room temperature for 10 min at 2000 rpm in a swinging bucket rotor. Cells and cell-bound membranes sedimented to the bottom while unbound membranes stayed on the top of the cushion. Unbound membranes were removed by aspiration and the pellet was dissolved in 2% Triton X-100 containing 0.3 M NaCl (200 µl). The dissolved pellet was transferred to a scintillation vial and the tube was rinsed with 1% Triton X-100 containing 0.15 M NaCl (500 µl) which was pooled with the dissolved pellet. As a reference another 200 µl of the incubation mixture were transferred directly to a separate scintillation vial. Ten ml of Insta Gel were added to each vial.

Other methods. Protein was determined according to Lowry et al.(6) with bovine serum albumin as a standard. ^{35}S and ^{32}P were determined by liquid scintillation counting in Insta Gel (Packard Instrument Co.).

RESULTS

Membrane binding is expressed as µg of membrane protein per 10^6 cells assuming an even distribution of radioactivity and protein in the membranes. Under conditions where significant binding of rat liver plasma membranes to rat hepatocytes occurred no differences were observed whether the membranes were labeled with ^{35}S or ^{32}P. The ways of mixing cells and membranes during the incubation influenced the rate of binding as seen in Fig. 1A. Maximal rate of binding was obtained at 130 rpm in the rotary incubation while lower speeds resulted in a slower binding. Rotary mixing proved to be superior to recipro-

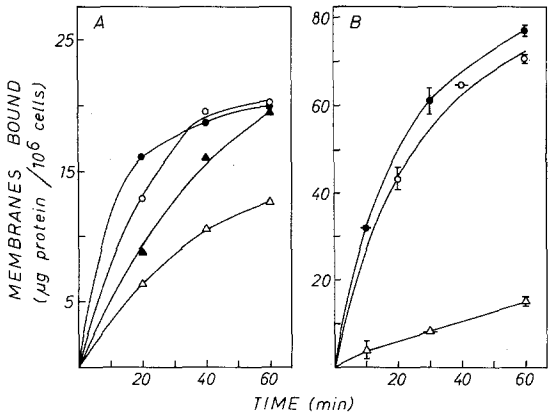
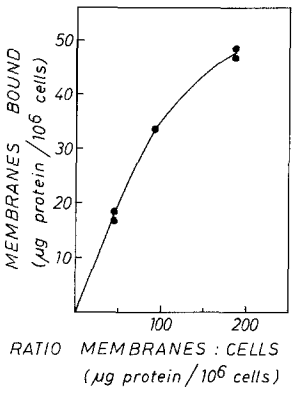


Fig. 1. The effect of rotation speed (A) and temperature (B) on membrane binding. Cells and membranes were incubated in 500 µl of Buffer 3 containing 0.05 mg DNase/ml. The amount of membranes is given as protein. The rotational mixing was carried out with a gyratory shaker (G24, New Brunswick Scientific).
A) $2x10^6$ cells were incubated at 37°C with 93 µg of [^{32}P]rat liver plasma membranes at rotation speeds of 70 (Δ), 100 (▲), 120 (O) and 130 (●) rpm.
B) $1x10^6$ cells were incubated with 200 µg of [^{32}P]rat liver plasma membranes and rotary mixing (130 rpm) at 8°C (Δ), 22°C (O) and 37°C (●). Vertical bars indicate range of duplicate incubations.

cal shaking since the latter caused more physical damage of the cells. The reproducibility of the binding with rotary incubation was good as demonstrated by the use of duplicate measurements (see Fig. 1B). However, it was necessary to use DNase in the incubations since otherwise too high levels of membrane binding, especially of mitochondrial membranes, were obtained, probably due to release of DNA from a small proportion of cells that became damaged.

Other parameters that affected the membrane binding were the temperature and the ratio of membranes to cells. A decrease in the temperature decreased the amounts of bound membranes as shown in Fig. 1B. In Fig. 2 it is demonstrated that an increase in the ratio of membranes to cells in the incubation resulted in an increased amount of bound membranes, but the results indicate that the cells can be saturated with membranes. With all rat liver plasma membrane preparations tested, only around 40% of the membranes could bind to the cells even at high ratios of cells to membranes.

The specificity of the membrane binding was investigated with mitochondrial membranes from rat liver (Band 3) and with Band 1 (presumably plasma membranes (1)) and Band III from chicken liver. As seen in Fig. 3 Band 3-membranes both from rat and chicken only bound to a small extent, whereas Band 1-chicken



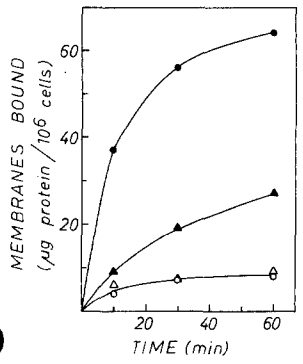


Fig. 2. The effect of varying membrane to cell ratios on membrane binding. $2x10^6$ cells were incubated with varying amounts of [^{32}P]rat liver plasma membranes for 60 min at 20°C and with rotary mixing (130 rpm). For further details see Text and legend to Fig. 1.

Fig. 3. The specificity of membrane binding. $1x10^6$ cells were incubated at 37°C with 200 µg of [^{32}P]rat liver plasma membranes, Band 1 (●), [^{32}P]rat liver mitochondrial membranes, Band 3 (O), [^{32}P]chicken liver membranes, Band 1 (▲) or [^{32}P]chicken liver membranes, Band 3 (Δ). Rotary mixing at 130 rpm. For further details see Text and legend to Fig. 1.

membranes bound significantly although much less than rat liver plasma membranes.

Preincubation of hepatocytes with unlabeled membranes affected the binding of radio-labeled rat liver plasma membranes differently as shown in Fig. 4. Thus, unlabeled mitochondrial membranes hardly affected the binding at all whereas unlabeled rat liver plasma membranes significantly reduced the binding. Unlabeled chicken membranes, Band 1, also reduced the binding, but to a lesser degree than rat liver plasma membranes.

DISCUSSION

Binding of plasma membranes to rat liver cells can conveniently be determined with the present method which is rapid, simple and reproducible. It appears that the results can be interpreted as binding and not merely exchange of labeled components between membranes and cells, since ^{32}P-labeled and ^{35}S-labeled plasma membranes gave exactly the same results. Also, binding of membranes by cells could easily be seen with phase-contrast microscopy. The small variations in the amount of membranes bound between different experiments performed under identical conditions probably is due to that different

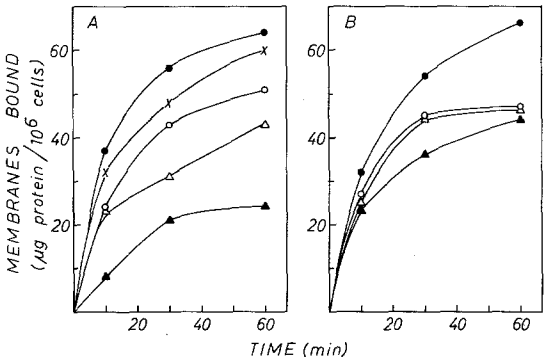


Fig. 4. Inhibition of rat liver plasma membrane binding by preincubation with various membranes. Incubations were carried out with rotary mixing (130 rpm) at 37°C. 1x10^6 cells were preincubated for 10 min with or without unlabeled membranes in a total volume of 450 µl of Buffer 3 containing 0.05 mg DNase/ml. Then 200 µg of [^{32}P]rat liver plasma membranes in 100 µl of Buffer 3 were added and the incubation was continued for the indicated times.
A) Preincubation with no membranes (●), 50 µg (○), 200 µg (△) or 400 µg (▲) of rat liver plasma membranes or with 400 µg of rat liver mitochondrial membranes (x).
B) Preincubation with no membranes (●), 100 µg (○), 200 µg (△) or 400 µg (▲) of chicken liver membranes, Band 1.

cell preparations were used for each experiment. An optimal binding rate requires an efficient mixing of cells and membranes resulting in an efficient collision frequency and at the same time a minimal cell-cell aggregation. At rotation speeds below 130 rpm the cells, but not the membranes, collected in the middle of the wells and were presumably not colliding optimally with membranes but could instead aggregate to a higher extent. This could explain the slower membrane binding when the rotation speed was reduced. The effect of temperature on the binding of plasma membranes is similar to the effect of temperature on cell aggregation and adhesion (1,7).

The fact that only 40% of the plasma membranes could bind to the cells can be explained by several factors. The plasma membrane preparations consist only to 80-90% of pure plasma membranes (8). This means that around 50% of the true plasma membranes cannot bind, which is reasonable since a functional polarity of the liver cell surface has been demonstrated and plasma membrane fragments representing different parts of the surface can be isolated (9). Furthermore, some membrane fragments could exist as inside-out vesicles, and many of the sheet-like junction-containing membrane fragments occurring in our preparations (8) may primarily expose their cytoplasmic sides to the cells, even after treatment with calcium-deficient media which dissociates some of the junctions

(3) and activates the plasma membranes with respect to binding to the cells (1). Similar results have been obtained with embryonic neural retina cells which only bound 20-25% of the plasma membranes isolated from these cells (4).

The binding of membranes to rat hepatocytes showed a reasonable degree of specificity. Thus, mitochondrial membranes bound almost an order of magnitude less than rat plasma membranes and this binding might even be due to a small contamination with plasma membranes. The low but significant binding of chicken liver membranes, Band 1, is interesting in view of the fact that these membranes did not stimulate aggregation of rat hepatocytes (1). However, cross-reactivity in aggregation of liver cells from different species has been reported (10).

The concentration dependent inhibition of rat plasma membrane binding by unlabeled rat plasma membranes further suggests that the binding is saturable. The smaller inhibitory effect by unlabeled chicken membranes, Band 1, and the almost negligible inhibition by unlabeled mitochondrial membranes emphasizes the specificity of the binding. These experiments also demonstrate that the present method to determine binding of plasma membranes to intact cells can be utilized to study the mechanism of cell recognition and as an assay for fractionation of adhesive components from solubilized membranes. We have demonstrated that the specific plasma membrane binding occurs equally well in the presence of 0.01% of the detergent Tween 80, which makes the present method even more suitable for testing the effect of solubilized membrane components.

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