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LATERAL DIFFUSION OF HUMAN HISTOCOMPATIBILITY ANTIGENS IN ISOLATED PLASMA MEMBRANES

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We have prepared large (5–10 μm) plasma membrane fragments by lysis of VA-2, human, cells adherent to Sephadex beads. The membrane fragments may be removed from beads by sonication and stained with fluorescent antibodies to human histocompatibility antigens, HLA antigens. Lateral diffusion of labelled antigens is followed by the method of fluorescence photobleaching recovery (FPR). HLA antigens of isolated membranes diffuse at the same rate, approx. $(2–4)\cdot 10^{-10}$ $cm^2\cdot s^{-1}$ as they do in intact cells. This rate may be modified by incubating membranes in a variety of media. Buffers of slightly acid pH (6.5 or less) enhance lateral diffusion, while the presence of divalent ions slightly reduces diffusion rates. Our major finding is that incubation of 37° in 0.10 M phosphate buffer increases lateral diffusion 3–5-fold.

Introduction

Lateral diffusion of membrane proteins has been described in many cell types (for review, see Refs. 1–3) since it was first demonstrated in membranes of heterokaryons [4] and in disc membranes [5].

Though the first diffusion coefficient obtained for any membrane protein was about that predicted from theory, D approx. $3\cdot 10^{-9}$ $cm^2\cdot s^{-1}$, many, though by no means all, other diffusion coefficients are an order of magnitude or more slower than expected from the size of a typical membrane protein and from the viscosities measured for bilayer phospholipids. The chief restraint to lateral diffusion of membrane proteins appears to be the cytoskeleton. Evidence for this is found in the effects of cytochalasin B on lateral diffusion [6,7], in the marked increase of lateral diffusion of membrane proteins in red cells genetically lacking cytoskeletal proteins [8] or chemically stripped of these proteins [9], and in the increase in lateral diffusion in membrane blebs which appear to lack F-actin [10,11]. However, there is no clear understanding of the way in which membrane integral proteins interact with the cytoskeleton, though there are extensive observations on the relationships between such proteins (for review, see Ref. 12). Such understanding might be gained if membranes could be stripped of cytoskeletal proteins and reconstituted with known purified components. This approach has been taken with erythrocytes [13,14], but membranes suitable for cytoskeleton reconstitution experiments are hard to prepare from other cell types.

This laboratory recently described a method for making membrane sheets by lysis of cells attached to cell culture beads [15]. The method yields plasma membrane purified approximately 20-fold, and

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these membranes appeared to be large enough to be used for measurement of lateral diffusion by the fluorescence photobleaching recovery, method. Here we describe our first results with such preparations. We show that diffusion coefficients of HLA marker antigens measured in isolated membranes are close to those measured in intact cells. We further show that some treatments of the isolated membranes alter lateral diffusion coefficients of the antigens.

Materials and Methods

We chose to work with transformed human cells, VA-2, originally used as fusion partners for measurement of lateral diffusion of heterokaryons [4]. These cells adhere well to cell culture beads. The cells were grown in plastic petri dishes in Eagle's minimal essential medium (Gibco), 5% in fetal calf serum (Reiheis). Cells were removed from culture dishes with collagenase/trypsin/chicken serum [4], and washed with fresh minimum essential medium (MEM) and allowed to attach to Cytodex (Pharmacia) beads in pH 5 attachment buffer, as described by Gotlib [5]. From $(1-3)\cdot 10^7$ cells were used for each preparation, requiring $(2-6)\cdot 10^5$ beads for efficient cell attachment.

The membrane preparation was made as described by Gotlib [15]. After cells had attached to beads for 1–3 h at 37 °C they were lysed in cold hypotonic Hepes buffer (pH 7.5). The beads, bearing membranes were washed twice in this buffer, sonicated briefly to further remove adherent cytoplasm, and washed again. Membranes were most readily removed from beads by further sonication for 15–20 s on a Branson Sonifier, working at a setting of 1 with a 50% duty cycle. Time and energy of sonication used varied from batch to batch of membranes.

About 15% of leucine aminopeptidase, a plasma membrane marker, was recovered attached to the beads after lysis, washing and first sonication of the membranes. Purification was comparable to that achieved previously for other cell types [15].

Cell membranes were labelled for fluorescence photobleaching with tetramethylrhodamine conjugates of KE-2 monoclonal IgG or Fab. This antibody, which reacts with all HLA class I gene products, was kindly given to us by Dr. Roger Kennett, Department of Genetics, University of Pennsylvania. Details of the conjugation and specificity of the antibody are given elsewhere [16]. Membranes were suspended in a variety of buffers as described in Results. Suspensions were taken up in 50 μm rectangular section capillary tubes (Vitro Dynamics, Millville, NJ) for microscopy.

Lateral diffusion of antibody labelled HLA antigens of VA-2 cells or membranes was measured by fluorescence photobleaching recovery [17,18] using the analytical method of Axelrod et al. [19]. Further details of our instrument are given in a recent paper [20]. The e^{-2} laser beam radius was 1.1 μm.

Results

Unlabelled VA-2 cells attached and spread well on cytodex cell culture beads. However, cells prelabelled with anti-HLA antibody did not attach well. Therefore all membranes were prepared from unlabelled cells, then released from beads by sonication and then labelled with antibody or Fab fragments. Membranes prepared in this way ranged from six to more than 15 μm in diameter. The size

TABLE I

LATERAL DIFFUSION OF HLA ANTIGENS IN VA-2 MEMBRANES AS A FUNCTION OF BUFFER COMPOSITION

All measurements at 30 °C. Number of measurements given in parentheses.

Buffer	D ($cm^2\cdot s^{-1}$) ($\times 10^{10}$)	R ($\times 100$)
Phosphate-buffered saline (25)	2.1 ± 0.2	67 ± 5
Buffer A [a] (67)	1.8 ± 0.2	49 ± 4
large membranes		56 ± 4
small membranes		40 ± 4 [c]
Hanks' Solution (10)	1.4 ± 0.4	62 ± 8
Hanks' solution +5 mM ATP (14)	2.1 ± 0.2	42 ± 3 [d]
Imidazole (pH 6.3) (29)	3.7 ± 0.4 [b]	67 ± 3

[a] Buffer A: 5 mM Tris-HCl (pH 8.0)/0.2 mM $CaCl_2$/0.2 mM ATP/0.5 mM 2-mercaptoethanol.

[b] D is significantly higher than in any of the other groups.

[c,d] Mobile fraction is significantly lower than in the three buffers not containing ATP for membranes of comparable size.

TABLE II

EFFECT OF pH ON LATERAL DIFFUSION OF HLA ANTIGENS IN VA-2 MEMBRANES

Membranes were suspended in the buffers indicated after their removal from beads. Measurements were at 30 °C for about 45 min per group. Number of measurements given in parentheses.

Phosphate buffer (0.1 M)	D ($cm^2 \cdot s^{-1}$) ($\times 10^{10}$)	R ($\times 100$)
pH 8 (6)	2.2 ± 0.4 [a]	24 ± 4
pH 6 (16)	3.7 ± 0.4	28 ± 3
pH 4 (15)	3.6 ± 0.4	32 ± 2
pH 3 (11)	4.0 ± 0.7	20 ± 2

[a] D significantly lower than other values.

of the membranes, which appeared as flat sheets rather than as closed vesicles, depended upon the extent of sonication required to remove them from the beads. Small membranes often formed clumps of 5 to 20 individual sheets. Membranes removed with trypsin formed closed structures which were too small to be used for fluorescence photobleaching recovery.

Labelling was specific and stable. Membranes were not labelled by R-KE-2 in the presence of an excess of unlabelled KE-2 IgG. There was no recovery seen in six measurements of membranes incubated in goat anti-mouse Ig after labelling with Fab.

Values for D varied somewhat from preparation to preparation, but by less than a factor of 2. There was no correlation between D for a particular batch of membranes and the size of the membranes. R, the fraction of label mobile in the plane of the membrane did vary with membrane size. Hence, though D could be compared between preparations, R was compared only for different treatments of a single membrane preparation, or for pooled data (on membranes in buffer A) from preparations of similar size.

We first compared the lateral diffusion of HLA antigens in membranes with diffusion in intact cells. Values for the membranes and cells used in this comparison were almost identical at 19 °C. The average diffusion coefficient of HLA antigens in intact VA-2 cells, $D = (4.0 \pm 1.5) \cdot 10^{-10}$ $cm^2 \cdot s^{-1}$ (18 cells); in isolated membranes $D = (4.0 \pm 0.5) \cdot 10^{-10}$ $cm^2 \cdot s^{-1}$ (57 membranes). R, the fraction of labelled molecules mobile, was much lower in the small membranes used for this comparison than in cells; 64% of label was mobile in cells, but only 24% in membranes. We were unable to measure D in intact cells at 30 °C, the working temperature for all of our other experiments, due to frequent and large membrane flows. Such flows, seen as fluorescence recoveries to greater than 100% of the initial fluorescence value, were never seen in isolated membranes.

TABLE III

EFFECT OF IONIC STRENGTH AND TIME AT 37 °C ON LATERAL DIFFUSION OF HLA ANTIGENS IN VA-2 MEMBRANES

Membranes prepared in buffer A were resuspended in 0.05 or 0.1 M phosphate (P) buffer, pH 6.0 and either held in ice or in a 37 °C water bath for the times indicated. In a second experiment the buffer was 0.1 M phosphate 0.4 M in NaCl. Number of measurements on each sample given in parentheses.

Buffer	Incubation	D ($cm^2 \cdot s^{-1}$) ($\times 10^{10}$)	R ($\times 100$)
0.05 M P	0 °C, 1 h	2.1 ± 0.2 (15)	27 ± 2
	37 °C, 1 h	2.5 ± 0.2 (10)	28 ± 3
0.10 M P	0 °C, 1 h	2.1 ± 0.2 (15)	42 ± 3
	37 °C, 1 h	4.0 ± 0.4 (9) [a]	39 ± 3
	0 °C, 3 h	2.7 ± 0.2 (10)	65 ± 4
	37 °C, 3 h	11.2 ± 2.0 (12) [a]	56 ± 3
0.10 M P+ 0.4 M NaCl	0 °C, 2 h	4.8 ± 0.5 (14) [b]	40 ± 4
0.10 M P+ 0.4 M NaCl	37 °C, 2 h	8.0 ± 1.5 (15) [b]	39 ± 3

[a] D significantly higher than in 0 °C samples.
[b] D significantly higher than 0.1 M phosphate buffer sample.

A variety of buffers affected diffusion and mobility of HLA antigens in membranes (Table I). The presence or absence of Ca^{2+} or Mg^{2+} did not affect D or R, but the inclusion of ATP in the buffer (Buffer A or Hanks' balanced salt solution) significantly reduced the fraction of antigens mobile. Slightly acidic buffer, pH 6.3 imidazole, greatly enhanced D without affecting R. This effect was not due to the imidazole, since acidic phosphate buffers over a pH range of 3 to 6 had the same effect (Table II).

In all of the experiments described above cells were suspended in buffer at 4 °C and held in ice

until measurements were made. During measurement, the cells spent no more than 45 min at 30 °C. Golan and Veatch [9] have shown that incubation of red cell ghosts in buffers at 37 °C for extended times increases lateral diffusion of membrane proteins. With this in mind, we incubated membranes in buffers of low to high ionic strength at 0 °C or 37 °C for periods of 1 to 3 h and measured lateral diffusion (Table III). Increasing incubation time at 37 °C increased lateral diffusion more than 4-fold. The mobile fraction of antigens, R, was also increased, both by increased ionic strength and by time in this medium (compare R for membranes in 0.05 M buffer with R for membranes in 0.10 M buffer at 0 °C) but not by incubation at 37 °C (compare R for membranes in 0.10 phosphate buffer at 0 °C or 37 °C). Still higher ionic strength buffer, 0.1 M phosphate buffer, containing 0.4 M NaCl, increased D after incubation at either 0 °C or 37 °C.

Discussion

A preparation of large plasma membrane fragments previously characterised biochemically [15] is here shown to be useful for the study of lateral diffusion of membrane MHC antigens. Though the preparation has the drawback that it must be labelled by removing purified membranes from the beads to which they adhere, it has the great advantage of allowing manipulation of the cytoplasmic face of the membrane. Hence it offers an approach to the detailed study of the effects of cytoskeletal proteins on lateral diffusion of membrane proteins.

In this initial study we have shown that diffusion coefficients for HLA antigens in isolated membranes are close to those in native cell membranes, though there is less recovery of fluorescence in small membranes than in intact cells or in large membranes. This difference in fluorescence recovery may be due to a number of factors. In particular a significant amount, 6–10% of label is bleached in membranes of 6–8 μm diameter. Wey and co-workers [21] studied lateral diffusion of labelled rhodopsin in disc membranes, approximately the diameter of the small membranes described here. They calculated that fully mobile labelled rhodopsin molecules in disc membranes roughly the size of our membrane fragments would appear to be only 60% mobile due the bleaching of about 10% of the entire labelled population by the laser beam. Variations in fluorescence recovery may also be due to differences in the level of labelling of large versus small membranes, and hence to differences in immobile background fluorescence between the two. Of course the difference could also be due to differential damage to large or small membranes in the course of preparation. In any case, we minimised this artifact by only comparing buffer and other treatments of membranes of a similar size, and when possible only within a single day's preparation.

We have been able to modify diffusion of HLA antigens in isolated membranes by manipulating the pH and ionic strength of incubation buffers. We found that prolonged incubation of membranes at 37 °C can increase lateral difusion coefficients 5-fold over controls, and that the ionic strength of the incubation buffer affects the extent of changes in diffusion coefficient or mobile fraction of labelled antigens. These observations are similar to those reported on lateral diffusion of labelled band 3 molecules in erythrocyte ghosts by Golan and Veatch [9]. They showed that, up to a point, increases in the ionic strength of suspending buffers increased lateral diffusion of band 3; beyond this point further increases in ionic strength reduced lateral diffusion coefficients and fractional mobilities. Our series is not as complete as theirs, and unlike them, we did not incubate membranes in the presence of protease inhibitors, hence the effects of long incubations at elevated temperatures may be due to endogenous proteinase activity. However, despite this we are in good agreement with their qualitative findings. In both series of experiments we also note that D and R may be separately affected by some treatments. This split, between the fraction of molecules free to diffuse and the diffusion coefficient of those that diffuse at all, has also been noted for treatments affecting the lateral diffusion of MHC antigens in intact cells [20].

Koppel and co-workers [8] suggest that lateral diffusion is regulated by the polymer matrix of the cytoskeleton, rather than by interactions between membrane proteins and specific cytoskeletal proteins. If this model, devised for erythrocytes, ap-

plies to nucleated cells as well, then treatments affecting D may alter the polymerization or stability of the cytoskeletal network, while treatments affecting R could act on specific anchoring proteins. Further work with isolated membranes and purified cytoplasmic proteins, for example actin or gelsolin, should give us further insights into the mechanisms for control of lateral diffusion in plasma membranes.

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