Journal of Cellular Biochemistry 20:81–93

Binding of Isolated 3T3 Surface Membranes to Growing 3T3 Cells and Their Effect on Cell Growth

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We have quantitated by autoradiography the binding of [^{125}I]labeled 3T3 plasma membrane fragments to 3T3 cells growing on the surface of plastic dishes; ie, the same conditions in which these membranes specifically arrest the growth of 3T3 cells early in the G_1 phase of the cell cycle. We have been able to demonstrate that binding of membranes to cells is coincidental with the expression of the growth inhibitory activity of protein(s) present in the membrane fragments. Treatments that reduce binding (heat denaturation of the membranes or culture in the presence of high serum) also reduce growth inhibitory activity. [^{125}I]labeled membranes bound to cells are located primarily on the cell surface (as determined by electron microscope autoradiography) and are exchangeable with unlabeled membranes. We conclude that binding of membranes to cells is necessary but may not be sufficient for the expression of the growth inhibitory activity of these membranes. This approach provides information not only on the average level of binding of membranes to cells, but also provides a quantitative assessment of the variation of the level of membrane to cell binding between different cells in the population.

Key words: 3T3 cells, 3T3 surface membranes, growth inhibition

The mechanisms governing the control of cell growth is a much investigated and controversial area. Based on studies with cultured mammalian cells a number of theories have been proposed to explain the control of the growth of fibroblasts in tissue culture (recently reviewed in [1]). Growth control by cell–cell interactions is one model that has recently been investigated in detail, in particular it has been demonstrated that the addition of cell-surface-derived membrane fragments (and/or more purified membrane components) to growing cells led to cessation of cell growth [2–6]. The evidence indicates that cells are arrested by the addition of membranes in the G_1 portion of the cell cycle in a manner identical to that brought about by high

Received June 21, 1982; accepted July 27, 1982.

0730-2312/82/2001-0081$04.00 **© 1982 Alan R. Liss, Inc.**

cell density, and that membranes do not act by altering components present in the growth medium, or by preventing access of mitogenic hormones to the cell surface [1,7].

It has been assumed that membranes bind to the cells in order to elicit this effect, but no data have been published that demonstrate this point. In a variety of systems, methods have been published that document the specific binding of membranes to cells in suspension (for review see [7]); these methods were unsuitable for our purpose since we wished to measure the binding of membranes to cells growing on the surface of plastic dishes.

This communication presents data indicating that membrane to cell binding is necessary for growth inhibitory activity, as treatments that reduce the growth inhibitory activity of the membranes (such as heating or raising the medium serum concentration) also reduce membrane binding. In particular, [^{125}I]labeled membranes have been shown to bind to the cell surface as determined by electron microscope autoradiography and by displacement by nonradioactive membranes.

MATERIALS AND METHODS

Cells and Their Culture

Swiss 3T3 cells (originally obtained from H. Green, MIT) were grown as described previously [2]. Calf plasma (GIBCO, New York) was further processed to reduce growth promoting activity by chromatography on CM-Sephadex [8]. All other cell culture materials were obtained from K.C. Biologicals, Flow Laboratories, or Falcon Plastic Products. Cell culture medium (Dulbeccos Modified Eagles, DME) was prepared by the Washington University Basic Cancer Center.

Membrane Preparation

Membranes were prepared from confluent cells (typically 1–2 $\times$ 10^8 cells per preparation) as described [2] except that bovine serum albumin (BSA) was omitted from all of the buffers. Only the material designated previously as Band I (bouyant on 9% Ficoll) was utilized in these experiments. This fraction is eight- to twelve-fold enriched for the plasma membrane marker alkaline phosphodiesterase (PDE) as compared to the crude homogenate. Membranes were stored frozen in Tris-buffered saline (TBS; 10 mM Tris, pH 7.4, 140 mM NaCl) until use.

Labeling of Membranes With ^{125}I

Membranes were iodinated according to the following protocol. Membranes were thawed, diluted, and washed by centrifugation with phosphate buffered saline (PBS; 1.18 gm Na_3HPO_4, 0.226 gm KH_2PO_4, 8 gm NaCl, pH 7.4, per liter). The membrane pellet was resuspended in 1.0 ml PBS (typically between 1.0–4.0 mg of membrane protein) and the following were then added in sequence indicated: [^{125}I]Iodine (20 μl, 2.0 mCi, New England Nuclear or Amersham), Lactoperoxidase (10 IU, Calbiochem), glucose oxidase (1.2 units, Sigma Type II from Aspergillus niger), and glucose (5 mM final concentration) in a final volume of 1.06 ml. After 30 min at room temperature the reaction was stopped by the addition of 10 ml of phosphate-buffered iodide (PBI gm/l distilled water: 20.5 NaI; 0.2 KCl; 1.15 Na_2HPO_4; 0.2 KH_2PO_4, pH 7.4) and pelleted at 20,000 g for 30 min (Sorval RC5 or

RC2-B Centrifuge). The iodinated membranes were washed twice more with PBI, once with DME, and finally resuspended in 1.0 ml DME. The range of specific activities obtained was 2–9 × 10^5 cpm/PDE [2]. The specific activities of the membrane preparations varied between 180–500 PDE units/mg protein. Seventy to ninety percent of the iodinated material could be precipitated with 5% trichloroacetic acid. Membranes were immediately diluted and added to the cells as described previously [3].

Measurement of DNA Synthesis

When [^{125}I]labeled membranes were added to the cells, the previously published procedure [2] for measuring the incorporation of [^{3}H]thymidine into DNA was modified so as to separate the [^{125}I]labeled protein from [^{3}H]labeled nucleic acid. Growing cells on a culture dish were pulsed with [^{3}H]thymidine (5 μCi/ml, 40–50 Ci/mmol, New England Nuclear) in DME/10% dialyzed calf serum for 2 hr at 37°C. The cells were washed once at 4°C with Hanks bovine serum albumin solution and incubated with 5% trichloroacetic acid for 45 min at 4°C. After a further washing with 5% trichloroacetic acid, the cellular protein and nucleic acid was solubilized with 0.30 ml of 0.2 M Na_2CO_3 in 0.1 N NaOH at 37°C for 45 min. The solubilized material was then transferred to microfuge tubes, and BSA (50 μl of a 5.0 mg/ml solution) and trichloroacetic acid (150 μl of a 50% solution) were added, and the tubes were heated at 80°C for 30 min to hydrolyze DNA. The tubes were incubated at 4°C overnight, protein precipitate was collected by centrifugation, and an aliquot of the supernatant was counted to determine [^{3}H] content in DNA. The counts were corrected for the presence of small quantities of [^{125}I]. Cell number was determined in a Coulter counter using duplicate samples [2]. The rate of DNA synthesis was normalized to cell number and the data are expressed as percentage of a control sample to which no membranes were added.

Binding Measurements

Cells were plated in Linbro multiwell dishes as described for measuring DNA synthesis and incubated, under various conditions, with [^{125}I]labeled membranes. For fixation of the cells the media over the cells was removed and the cells were washed once with warm (37°C) Ca^{2+}, Mg^{2+}-free Hanks solution (CMF: consisting in gm/l of 7.46 NaCl, 0.4 KCl, 0.057 Na_2SO_4, 0.048 Na_2HPO_4, 0.06 KH_2PO_4, 0.35 $NaHCO_3$, 4.7 HEPES, 1.0 glucose, pH 7.35). The cells were fixed for 60 min at room temperature by addition of 3% glutaraldehyde in 0.05 M Na cacodylate, pH 7.4 and half-strength Pucks saline G (consisting of gm/l of 0.016 $CaCl_2 \cdot 2H_2O$, 0.40 KCl, 0.15 KH_2PO_4, 0.154 $MgSO_4 \cdot 7H_2O$, 8.00 NaCl, 0.29 $Na_2HPO_4 \cdot 7H_2O$, and 1.10 glucose, pH 7.4). The dishes were then washed twice with CMF, twice with water, and allowed to air-dry. Kodak NTB-2 nuclear emulsion diluted 1:1 with distilled water was then poured directly into the well [2]. After 24- or 48-hr exposure in the dark at 4°C, the emulsion was developed [2], the bottom of each dish punched out and attached to a glass slide with epoxy glue. Random fields were photographed from each slide at × 100 magnification, and individual cells in each field were photographed twice at × 250 magnification—once with phase illumination to determine the cell outline, and again with bright field optics to visualize the silver grains. The cell outline was then traced on a transparency (containing a grid 0.25 cm^2, equivalent to

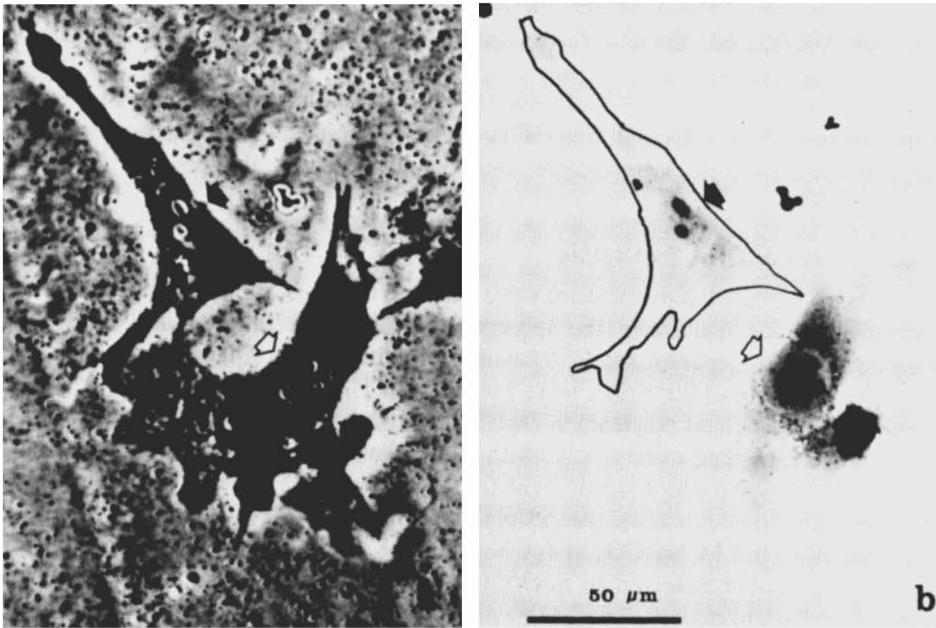

Fig. 1. Example of the method used for identification of cell cultures. The autoradiograms in both a and b are of the same field. In a, the cell outlines are emphasized by phase illumination; in b, the silver grains were emphasized. The cell outline was traced on a transparency from a and overlayed on b, as described in the text and highlighted in the figure by the arrows. The bar represents 50 μm. The grains per unit area of cell or of plastic (background) were then counted. In this example the cells were lightly stained with Giemsa. The solid arrow shows a cell with few grains, while the open arrow indicates a cell with heavy density of grain. The distribution of grains in individual cells is shown under various conditions in Figure 3.

36 μm^2) and overlayed on the picture containing the silver grains. Grains were counted, both over the cell and over the plastic background, and converted to grains/unit area as defined by the grid on the transparency. Grains over at least 20 cells were counted for each sample. Background was obtained by counting grains over an equivalent area of plastic. The pictures in Figure 1 illustrate the method, except that for clarity the grid has been omitted. Note the grains over the cells. Even though the background over plastic appears almost devoid of grains in this particular example, the large area of free plastic on the dish makes a major contribution to the total binding of membranes to dishes (see below) since 70–80% of the area of the dish is free plastic.

Electron Microscope Autoradiography

Cells were grown as described above in 35-mm dishes (5 dishes per sample) and [^{125}I]labeled membranes were added in a final volume of 0.8 ml media. Forty-eight hours later the media were removed, cells were washed one time with CMF, then fixed with 2.5% glutaraldehyde in 0.1 M NA cacodylate, pH 7.4, for 60 min at room temperature. The cells were removed from the dish by scraping with a rubber

TABLE I. Growth Inhibitory Activity of Iodinated Membranes

Sample	[^{3}H]dThd incorporation into DNA (% of control)
Control membranes	
2 PDE	63
5 PDE	32
Iodinated membranes	
2 PDE	50
5 PDE	44

Membranes were iodinated as described in the text. Control membranes were treated in similar fashion except that [^{125}I] was omitted. Cells that received control membranes were assayed for DNA synthesis according to Whittenberger and Glaser [2] and normalized to cell number. Cells that received iodinated membranes were assayed as described in the text. Membranes were incubated with cells for 48 hr. The iodinated membranes contained 90% acid-precipitable (5% trichloroacetic acid) radioactivity with a specific activity of 4.5×10^{5} cpm/PDE unit of membrane [2]. Since these measurements are relatively noisy [2] these data show no significant difference between the two sets of membranes.

policeman into a microfuge tube and allowed to settle for 30 min. They were then pelleted by centifugation and overlayed with 0.1 M Na cacodylate, pH 7.4 containing 5% sucrose, until sectioned [9].

Pellets were postfixed in 2% aqueous osmium tetroxide, dehydrated in an ascending series of alcohols, and embedded in Spurrs Epon. Sections were cut with a diamond knife on a Porter–Blum MT-2 microtome and examined in a Philipps 300 electron microscope at 60 Kv.

The thin sections on copper grids were overlayered with Ilford L-4 emulsion according to the loop technique of Caro [10] and exposed for 5–7 days. Before examination in the electron microscope the grids were stained with lead citrate [11].

Data collection consisted of photographing sequential fields selected from a random position on the grid. Each successive field in which the surface membrane of a cell was visible was photographed with the cell surface centered in the field. The shortest distance of all grains in the field from the surface membrane was then measured from suitably enlarged prints. The shortest distance of at least 400 grains from each sample to plasma membrane on an intact cell was measured, with the use of a Zeiss MOP-30, using opposite polarities for grains inside (+) or outside (−) the cell. The data were then entered into a computer to generate histograms of frequency of grains versus distance from the membrane. Further details of the analyses are given in the legend to Figures 4 and 5.

RESULTS

We wished to use [^{125}I]autoradiography as a quantitative method to measure the binding of membranes to cells. For this method to be effective, it is necesary that iodination not affect the biological activity of the membranes. The data in Table I show that, within the limitation of the assay of growth inhibitory activity, the iodinated membranes show comparable effect to those of unlabeled controls when assayed by addition to growing 3T3 cells.

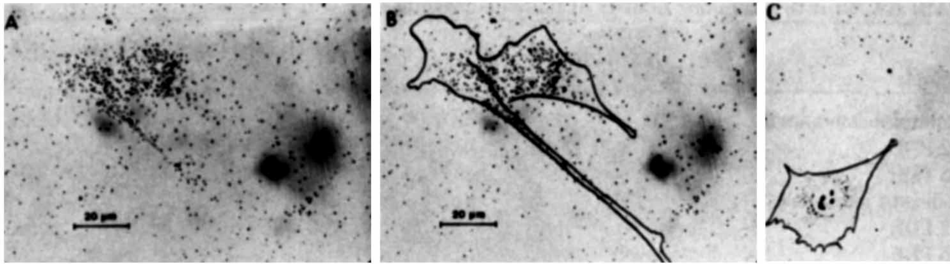

Fig. 2. Displacement of radioactive membranes by nonradioactive membranes. Iodinated membranes were added to cells in 5% serum for 24 hr at which time the medium was changed to 3% serum/3% plasma (panels A and B) or to 3% serum/3% plasma + noniodinated membranes (panel C). Autoradiographs were taken at 48 hours. Panels A and B show the same microscopic field, but in panel B the cell outlines have been indicated by tracing the outlines of the cells from a corresponding picture obtained by phase illumination (see Fig. 1). The same procedure was used for tracing the outline of the cell in Panel C. All panels reproduced at same magnification (bars = 20 μm).

Binding Experiments

Attempts to measure membrane binding to cells in the straightforward way, by adding the radioactive ligand, incubating, washing to remove nonbound ligand, and counting, were unsuccessful. The nonspecific binding of the membranes to the plastic dish (in the absence of cells) was too high (between 80–90% of the total binding as judged by the retention of membranes by empty dishes). The level of [^{125}I]labeled membranes to plastic dishes was very variable, even within one lot of plastic dishes. In the method used here, each well serves as its own control, since binding to background is measured in areas adjacent to the areas occupied by cells. Occasional dishes show such high background that measurements are meaningless, in which case the whole experiment was discarded. In addition, this method provides additional information regarding the distribution of membranes on individual cells, not available by conventional methods.

As clearly shown in Figures 1 and 2, cells incubated with [^{125}I]labeled membranes have associated with them significant amounts of radioactivity, although, as will be detailed, there is significant cell to cell variation. The results of typical binding experiments are shown in Table II. In these experiments, membranes were added to 3T3 cells under different culture conditions designed to alter the level of growth inhibitory activity of the membrane fragments.

Treatments that reduce the growth inhibitory activity of membranes might do so by blocking binding of membranes to cells, or by compensating for the effect of membranes by a different mechanism. We show that heat inactivated membranes which do not inhibit growth do not bind to cells, and that high levels of serum which largely prevent the growth inhibitory activity of membranes also reduce binding (Table II). The observed reduction in growth inhibitory activity, therefore, correlates with a lack of binding activity. Note that the data are presented as net grain counts per unit area, and that background grain density present over plastic is substracted. The data are not significantly altered if the background is not subtracted; ie, if we assume that cells prevent the binding of membranes to plastic underneath them. Typical cells to which [^{125}I]membranes have been added are shown in Figures 1 and 2.

TABLE II. Membrane Binding to Cells Under Various Conditions

Sample	Net grains per unit area on cell surface	$[^3H]$dThd incorporation into DNA (% of control)
Experiment I		
5% serum, +6 PDE membranes	4.89 ± 2.49	41
25% serum, +6 PDE membranes	2.59 ± 1.93	77
5% serum, +6 PDE heated membranes	0.40 ± 0.74	110
Experiment II		
7.5% serum, +4 PDE membranes	2.75 ± 1.39	41
25% serum, +4 PDE membranes	1.25 ± 0.88	87
7.5% serum, +17.5% plasma, +4 PDE membranes	3.16 ± 1.58	67
7.5% serum, +4 PDE heated membranes	0.29 ± 0.39	121

Membranes were iodinated and binding assayed as described in the text. The membranes used for experiment I had 83% radioactivity precipitable with acid, and a specific activity of 1.7×10^5 cpm/PDE unit of membrane. The membranes used in experiment II were 88% acid precipitable with a specific activity of 2.9×10^5 cpm/PDE. These experiments were done four times (with the exception of high plasma, which was done twice) with essentially similar results. The density of grains over plastic (background) was about 0.3–0.5 grains per unit area in different experiments. Between 80–300 grains/cell were present in different experiments. Heated membranes were prepared by heating membranes at 80°C for 20 min in DME before addition to the cells [4].

While heat inactivated membranes essentially do not bind to cells, a significant fraction of the membranes remain associated with cells after incubation in medium containing 25% serum (Table II). There is a rough correlation between the degree of residual binding and the residual inhibition of DNA synthesis. While the residual inhibition of DNA synthesis is in the range of 10–20%, where these measurements become very imprecise, nevertheless the correlation between membrane binding and DNA synthesis is striking with different concentrations of serum. But in the presence of plasma-derived serum this correlation no longer holds (Table II) since, in the presence of high concentrations of plasma, binding of membranes is not affected, and there is a slight decrease in the growth inhibitory activity. Thus we conclude that binding of membranes to cells is required but may not be sufficient for growth inhibition to be expressed.

Distribution of Membrane Fragments Bound to Individual Cells

The binding of $[^{125}I]$labeled membranes to individual cells is not uniform. Figure 3 illustrates the distribution of $[^{125}I]$ over cells after incubation with $[^{125}I]$labeled membranes. The distribution is quite broad (Fig. 3) but the relationship, if any, between the extent of binding to individual cells and the biological activity is not known. For example, are individual cells with high density of bound membranes more likely to be growth arrested than cells with somewhat lower levels of bound membranes? The answer to this question is at present unknown. Treatments that reverse or prevent membrane to cell binding uniformly decrease the density of membranes bound to cells. Figures 3B, C, and D, show the distribution of grains over cells following addition of heated $[^{125}I]$membranes (Fig. 3B), or $[^{125}I]$membranes in the presence of 25% serum (which prevents growth inhibition) (Fig. 3C), or 5% serum–20% plasma-derived serum (Fig. 3D), where the growth inhibitory activity of

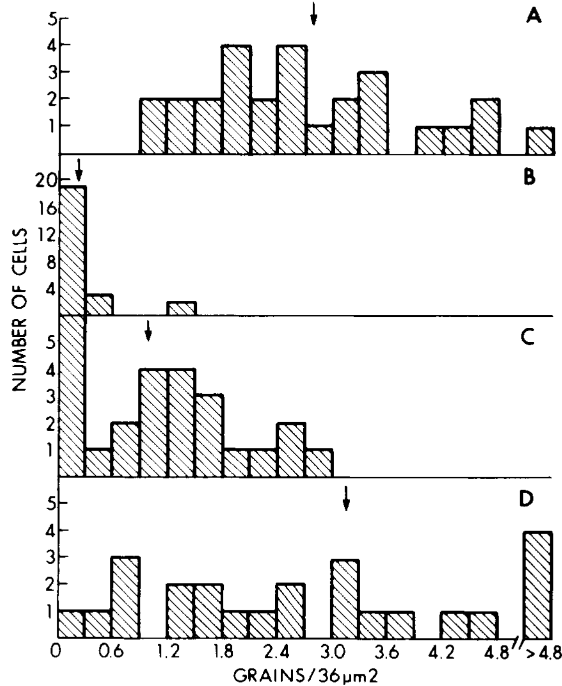

Fig. 3. Distribution of radioactive membranes on cells. The data were obtained as described under Methods. The grain density on individual cells due to the binding of [^{125}I]labeled membranes was assessed under different experimental conditions. Panel A, [^{125}I]membranes added to cells in 7.5% serum. Panel B, heat-inactivated membranes in 7.5% serum. Panel C, [^{125}I]labeled membranes in 25% serum. Panel D, [^{125}I]labeled membranes in 7.5% serum–17.5% plasma-derived serum. The data are from Experiemnt II shown in Table II. The arrows indicate the mean of each set of observations. This method, unlike other methods of assessing binding of membranes to cells, allows an assessment of the heterogeneity of the binding of membranes to cells.

the membranes remains. Note that, under conditions where the membranes are inactive, residual binding is low and appears to be much more uniform than that observed under growth inhibitory conditions.

Reversibility of Membrane to Cell Binding

Reversal of the binding of membranes to cells provides additional information regarding the attachment of membranes to cells and the surface location of the radioactive membranes. Thus, for example, membrane–cell interactions, which involve covalent bond formation or fusion of the isolated membranes with cell surface, would not be expected to be reversible (unless the binding is highly cooperative [7] as would be the case with a multivalent ligand). We have examined the reversibility of the binding of [^{125}I]membranes to cells in situations where the cells are incubated with membranes for 24 hr (in 3% serum/3% plasma). Excess membranes were removed and new media (either 3% serum/3% plasma, 25% serum, or 3% serum/ 3% plasma plus noniodinated membranes) placed over the cells. After an additional

TABLE III. Displacement of [^{125}I]Labeled Membranes by Nonradioactive Membranes

Sample	Net grains per unit area on cell surface	[^{3}H]dThd incorporation into DNA (% of control)
Cells at 24 hours	3.43 ± 1.43	—
Cells at 48 hours, in fresh medium	3.24 ± 1.67	76
Cells at 48 hours, in fresh medium + nonradioactive membranes	0.11 ± 0.22	57

Iodinated membranes (95% acid precipitable radioactivity, 4.6 × 10^5 cpm/PDE) were added to cells (4 PDE/well) in 3% serum/3% plasma for 24 hr. The membranes were then removed and, without washing, fresh medium (either 3% serum/3% plasma ± 4 PDE of nonradioactive membranes) was added to the cells (0.3 ml per well). After an additional 24 hr at 37°C the cells were assayed for both membrane binding and DNA synthesis. Note that the binding of [^{125}I]labeled membranes to cells did not change in 3% serum/3% plasma between 24 and 48 hr. This experiment has been done twice with similar results.

24 hr at 37°C the cells were assayed for both DNA synthesis and membrane to cell binding. The results are shown in Table III and selected cells are shown in Figures 2A and 2C. Incubation from 24–48 hr in fresh medium containing 3% serum/3% plasma displaced only a small amount of membranes from the cell, whereas incubation during the same time period either in high serum or with noniodinated membranes effectively removed iodinated membranes from the cells. The displacement of bound, [^{125}I]labeled membranes by nonradioactive membranes strongly suggests that most of the membranes are located on the cell surface and not internalized.

An alternative explanation would be that bound membranes are internalized by the cells and degraded, and the nonradioactive membranes simply prevent further binding of radioactive membranes to cells. We could not directly determine whether cells degrade membranes, since significant release of [^{125}I]labeled low molecular weight material was observed when membranes were incubated in growth medium on plastic dishes in the absence of cells. For this reason, additional experiments to ascertain the surface location of the bound membrane vesicles were performed by electron microscope autoradiography, as detailed below. It should be pointed out, however, that removal of excess radioactive membranes from dishes after 24 hr incubation with cells, followed by continued incubation in growth medium for 24 hr, does not decrease the amount of [^{125}I] membranes bound to cells, a result that would not be expected if the cells rapidly internalized and degraded bound membranes fragments, but is consistent with surface location of the [^{125}I]labeled membrane fragments.

ELECTRON MICROSCOPE AUTORADIOGRAPHY OF BOUND, IODINATED MEMBRANES

In order to ascertain if the grains observed over cells with the light microscope are on either the cell surface or internalized, electron microscopic autoradiography of cells to which [^{125}I]labeled membranes had been bound was done. Figure 4 shows electron micrographs obtained utilizing the protocol described under Methods. The figure demonstrates that this method preserves reasonable cytology of the cells and allows a determination of the location of silver grains in relation to the surface of the

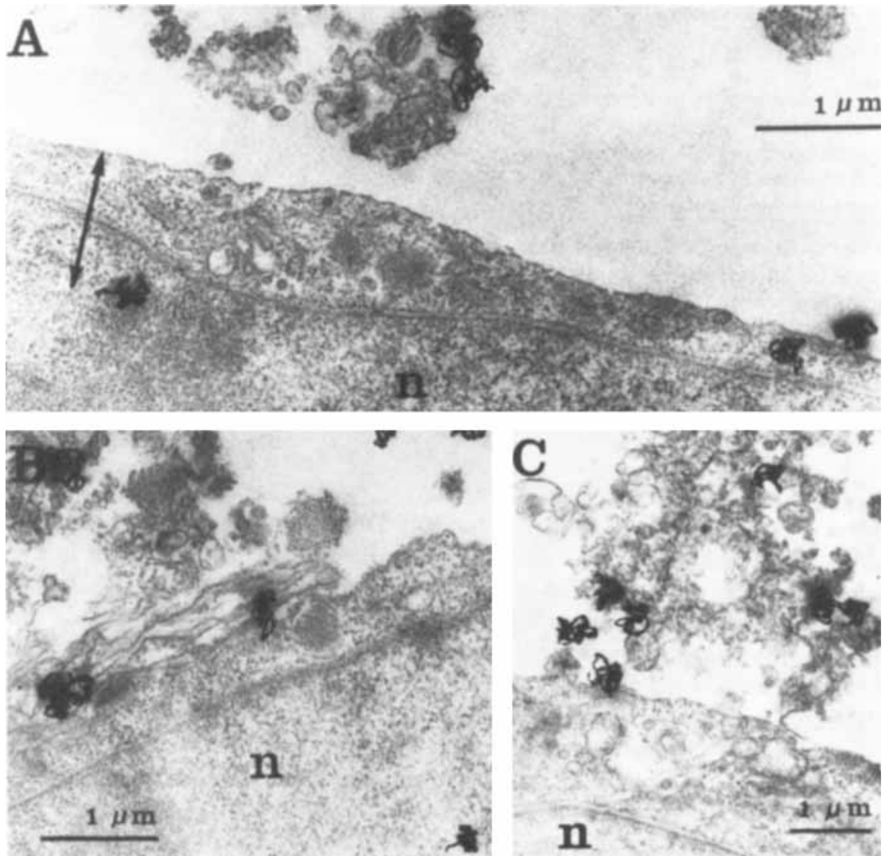

Fig. 4. Autoradiography of iodinated membranes bound to cells. Samples were prepared as described in the text for EM autoradiography. Examples of both internal and external grains are shown. Measurements were made from the center of the grain to the nearest plasma membrane as indicated by the arrow in panel A to generate the histogram shown in Fig. 5 (n = nucleus). Panels A and B show grains near the cell surface, and the figure shows that reasonable cell morphology has been retained. Panel C shows many profiles typical of isolated membrane fragments, which are heavily labeled. Such fragments can also be observed in Panels A and B.

cell. As expected, membrane clumps show a high density of silver grains. Figure 5 shows the histograms of the distribution of distances of the grains from the intact cell surface.

The data in Figure 5A were obtained in the presence of 5% serum (growth inhibitory conditions). The grain distribution peaks at the cell surface and is skewed to the outside as one might expect if membrane vesicles of finite diameter (about 300 nm, M. Lieberman, unpublished observation) are bound to the cell surface*. The precise quantitation of internalized membranes by this procedure is difficult. The most conservative estimate is to assume that grains on the inside of the plasma

*By contrast measurement of the distance of grains from the surface of the nearest membrane vesicle indicates that one-half of the grains are within 170 nm of a vesicle membrane (data not shown).

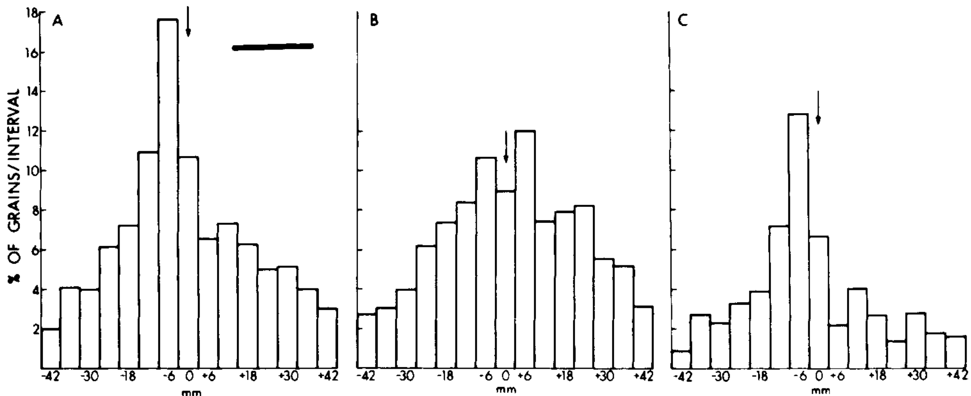

Fig. 5. Location of [^{125}I]labeled membranes bound to cells. The bars show the number of grains with mean distance from the plasma membrane indicated by the number in the ordinate. The distances are given in millimeters measured in photographs (as in Fig. 4) with an overall magnification of × 29,376. Thus, 6.0 mm correspond to 204 nm (the solid bar is 1μm). A, Cells in 5% serum. 702 grains were measured, with the mean = 1.31 mm (a minus sign signifies distance outside the cell; positive sign indicates distance inside the cell), and the 95% confidence levels are 3.62 to 0.98. B, cells in 25% serum. 783 grains were measured, with the mean = -0.117, and the 95% confidence levels -2.39 to 2.16. The membrane fragments added to cells are, on the average, 300 nm in diameter. The source of the radiation, therefore, is not a point source [12] but can come from anywhere on the membrane fragment. The large size of the radiation source can account, in part, for some of the large distances we observe of the grains from the membrane. Panel C shows the differences between the distribution of grain on the cells in 5% serum (panel A) and cells in 25% serum (panel B), where the latter have been scaled to account for the fact that cells in 25% serum have only 45% of the grains per cell than those in 5% serum (see text). Panel C, therefore, illustrates the location of those [^{125}I]labeled membrane fragments whose binding to cells is prevented by 25% serum, and which appear to be primarily located at the cell surface.

membrane, in excess of those predicted from a point source on the cell surface (half distance 100 nm), represent internalized membranes. When such an assumption is applied to the data in Figure 5A, then approximately 20–25% of the grains are derived from internalized membrane components. The data in Figure 5B show a similar histogram for grains on cells incubated with membranes in 25% serum (nongrowth inhibitory condition). Since these cells contain 45% of the grain number present on cells incubated in 5% serum (see for example Table II), the distributions in Figures 5A and 5B can be compared by scaling the two observations and subtracting the data in Figure 5B from the data in Figure 5A, this yields the histogram in Figure 5C, which represents the location of those [^{125}I] membranes displaceable from the cell by addition of high serum. The data suggest that the membranes absent from cells incubated in 25% serum are primarily those located on the cell surface, and thus serve to correlate the inhibition of cell growth by membranes with the binding of membrane fragments to the cell surface.

DISCUSSION

The data presented in this communication indicate that membrane binding to cells is correlated with the expression of the growth inhibitory activity. All conditions

examined that block the inhibitory activity of the membranes (with the possible exception of high concentration of plasma-derived serum) also reduced membrane binding. The membrane binding was primarily external (on the cell surface), as shown by the displacement of radioactive membranes by nonlabeled membranes, and by electron microscope autoradiography. Direct membrane to cell contact is thus strongly implicated for the activity of the growth inhibitory proteins in the membranes; membrane to cell binding is a necessary but not sufficient condition for the expression of this growth inhibitory activity.

The assay developed to measure membrane to cell binding is cumbersome and is not satisfactory for determining small differences in membrane binding. Thus, defined mitogens such as epidermal growth factor and platelet derived growth factor, which *partially* reverse the growth inhibitory effect of membranes, do not do so to an extent that would allow us to determine if membrane binding was significantly affected without examining a very large number of cells to determine not only the average membrane density on cells, but also the distribution of membranes on individual cells. In order to obtain reliable differences in membrane binding between cell populations, which show small differences in average growth rate, a correspondingly larger number of cells has to be examined, perhaps by automated methods [13]. Such studies would best be undertaken when the growth inhibitory protein itself has been isolated and binding studies can be performed with the purified protein.

Growth stimulatory agents have been shown to increase endocytosis, yet do not appear to result in detectable increases in the endocytosis of membranes by 3T3 cells as detected by our methods (compare Figs. 5A and 5B) [14–15].

An interesting point that is emphasized by these experiments is that the addition of high concentrations of plasma-derived serum did not reduce binding, whereas serum did have this effect. Since the plasma preparation used partially reversed the growth inhibitory effect, while leaving binding unaffected, this suggests that mechanisms also exist for reversing the growth inhibition activity of membranes without affecting membrane to cell binding. The difference between serum and plasma-derived serum strongly implies that a factor (possibly derived from platelets or a highly cationic factor from serum that is removed by CM-cellulose chromatography) is responsible for blocking membrane to cell binding; the identity of this factor has yet to be established. Since purified platelet-derived growth factor by itself does not show this activity (unpublished observation), other components present in serum but not in plasma-derived serum must be responsible for this effect. The method described in this paper allows quantitative determination of membrane to cell binding in a variety of systems. We recognize the fact that membrane to cell binding may take place via many different ligands, only some of which are related to growth inhibitory action of the membranes; nevertheless the data presented document directly for the first time that membranes do bind to the cell surface under growth inhibition conditions. The fact that the growth inhibitory proteins are essentially as effective after solubilization as compared to the original membrane preparation [5] is suggestive evidence that the growth inhibitory protein(s) is involved in membrane to cell binding. This question can only be answered with certainty when the growth inhibitory proteins are purified and specific antibodies that prevent binding of these proteins to cells become available.

ACKNOWLEDGMENTS

This work was supported by grants GM 18405 from NIH and NSF 77-1572 from NSF to L.G., NS-10244 from NIH to T.W., and a grant BC-371 from the American Cancer Society to M.L. During initial phases of this work, M.L. was supported by grant GM 07157-04 from NIH and subsequently by a Junior Faculty Award (JFRA-47) from the American Cancer Society and supported in part by the fund for teaching and research, Department of Nutrition, Harvard School of Public Health. Tissue culture medium was obtained from the Washington University Cancer Center supported by grant CA 16217. We are extremely grateful to Mrs. Arleen Loewy for assistance in some of these experiments, and to Dr. C. Haudenschild for use of his Zeiss MOP-30.

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