Purified Plasma Membranes Inhibit Polypeptide Growth Factor-induced DNA Synthesis in Subconfluent 3T3 Cells

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ABSTRACT Plasma membranes derived from NR-6 cells, a varient line of Swiss mouse 3T3 cells that does not have cell surface receptors for epidermal growth factor (EGF), inhibited EGF-induced stimulation of DNA synthesis by 50% in serum-starved, subconfluent 3T3 cells. Membranes derived from SV3T3 cells were much less effective in inhibiting EGF-induced DNA synthesis. This inhibition on DNA synthesis by NR-6 membranes was not a direct effect of membranes on EGF, nor could it be overcome by high concentrations of EGF. NR-6 membranes were most effective when added 3 h before EGF addition and had little effect when added 2 h or more after EGF. NR-6 membranes also reduced the stimulation of DNA synthesis induced by platelet-derived growth factor or fibroblast growth factor in serum-starved 3T3 cells. These findings indicate that membrane-membrane interactions between nontransformed cells may diminish their ability to proliferate in response to serum polypeptide growth factors.

The growth in culture of untransformed fibroblasts such as murine 3T3 cells, is dependent on a variety of factors in serum as well as on cell density. Numerous theories have been proposed to explain the observations that such cells achieve maximal densities when cultured under a defined set of conditions (1). The most frequently cited of these theories explain the regulation of maximal cell density through constraints that have their origin in direct cell-to-cell contact (2, 3) or limitations in the concentration and/or accessibility of cells to essential growth factors in the serum (4, 5). Whittenberger and Glaser (6) reported a provocative series of experiments in which the addition of 3T3 cell surface membranes to subconfluent 3T3 cells inhibited the ability of these cells to enter the S phase (DNA synthesis) of the cell cycle, suggesting that membrane-membrane interactions play an important role in growth regulation. Recently, a protein has been extracted and partially purified from these membranes that demonstrates the same growth regulatory effects when added to 3T3 cells (7).

Since serum contains a variety of polypeptide factors that are essential for the propagation and maintenance of mammalian cells in culture, we investigated whether plasma membranes alter the proliferative response of 3T3 cells to purified growth factors under serum-deprived conditions. Epidermal growth factor (EGF)[1] (8), fibroblast growth factor (FGF) (9), and platelet-derived growth factor (PDGF) (10) are all potent mitogens for 3T3 cells. In this report, we document that plasma membranes when added to subconfluent 3T3 cells limit the ability of these cells to proliferate in response to these mitogenic factors.

MATERIALS AND METHODS

Cell Culture: Swiss mouse 3T3 cells (clone 42 from G. Todaro, National Institutes of Health), a SV40-transformed variant of these cells (SV3T3), and NR-6 cells were grown in Dulbecco's modified Eagle's medium (DME) plus 10% fetal calf serum as previously described (11). NR-6 is a variant cell line obtained from clone 42 3T3 cells that has no detectable EGF-binding activity (12).

Serum-starved 3T3 cells were prepared in the following manner. Swiss 3T3 cells were plated in a 2-cm^2 Costar wells at a density of ~2×10^3 cells/cm^2 in DME plus 5% fetal calf serum (FCS). 24 h later the medium was changed to DME containing 0.5% FCS, and the cells were incubated for an additional 24 h. Cultures that were 30–40% confluent at the time of membrane addition were used in these experiments.

Plasma Membrane Preparation: NR-6 or SV3T3 cells were grown to confluency on 100-mm Falcon dishes (Falcon Labware, Oxnard, CA) in DME plus 10% fetal calf serum. Plasma membranes were prepared from these cells essentially as described by Whittenberger and Glaser (6). Membrane concentrations are expressed in units of phosphodiesterase (PDE) activity (1 unit of phosphodiesterase activity hydrolyzes 10 nmol of substrate per hour)

[1] *Abbreviations used in this paper:* DME, Dulbecco's modified Eagle's medium; EGF, epidermal growth factor; FCS, fetal calf serum; FGF, fibroblast growth factor; PDE, phosphodiesterase; PDGF, platelet-derived growth factor; SV3T3, a SV40-transformed variant of Swiss mouse 3T3 cells.

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(13). Whittenberger and Glaser (6) have found that the specific activities of phosphodiesterase in 3T3 and SV3T3 membranes are similar, thus indicating that similar amounts of membrane are being added.

Polypeptide Hormone Preparations: EGF was purified from the submaxillary glands of adult male mice by the method of Savage and Cohen (14). FGF, derived from bovine pituitary glands was a gift from D. Gospodarowicz, University of California, San Francisco. An extract containing PDGF was prepared from outdated human platelets as described by Antonaides and Scher (15). Purified PDGF was obtained from R. Ross, University of Washington.

Measurements of DNA Synthesis: [^{3}H]Thymidine incorporation into acid-precipitable material was performed on 3T3 cells grown in 2-cm^2 Costar wells. Cells were washed once with DME plus 0.5% FCS, and 1 μCi (0.55 μM) [^{3}H]Thymidine (New England Nuclear, Boston, MA) was added in 1 ml of DME plus 0.5% undialyzed FCS. After a 1-h incubation at 37°C, cells were washed twice with cold Hanks' salt solution and incubated with 2 ml of 5% trichloroacetic acid at 4°C for 30 min. Cells were then washed twice with cold 5% trichloroacetic, twice with methanol and solubilized with 0.5 ml of 0.5 N NaOH. This solution was transferred to scintillation vials together with 70 μl of 12 N HCl and 10 ml of scintillation fluid (Amersham, Arlington Heights, IL), and radioactivity was counted in a liquid scintillation counter.

For labeled nuclei experiments, duplicate 2-cm^2 Linbro wells of 3T3 cells were treated with 2 PDE units of SV3T3 or NR-6 membranes and incubated 18 h at 37°C in DME plus 0.5% FCS, or in DME plus 0.5% FCS and 25 ng/ml EGF. Autoradiography of [^{3}H]thymidine labeled nuclei was performed on these cells by treating them with 1 μCi [^{3}H]thymidine (0.55 μM) in 1 ml of DME plus 0.25% undialized FCS. After a 1-h incubation at 37°C, cells were washed twice with cold Hanks' salt solution, fixed 45 min in 1 ml of a 3.7% formaldehyde solution, and rinsed twice with distilled water. The cells were layered with NTB2 nuclear track emulsion (Kodak) and incubated for 5 d at 0°C. The emulsion was developed with D-19 developer (Kodak), fixed in 30% sodium thiosulfate, and the cells stained with 1% toluidine blue (Fisher Scientific Co., Pittsburgh, PA). Percent of labeled nuclei was quantitated by light microscopy.

RESULTS

Whittenberger and Glaser (6) demonstrated that cell surface membranes derived from confluent Swiss mouse 3T3 cells inhibit DNA synthesis by 50% or greater in subconfluent 3T3 cells grown in the presence of 10% FCS. We have obtained identical results when plasma membranes from NR-6, a cell line derived from 3T3 which does not display EGF receptors yet still demonstrates density-dependent growth, were added to subconfluent cultures of 3T3 cells in 10% FCS (data not shown). We subsequently investigated the effects of plasma membranes derived from NR-6 cells on the stimulation of DNA synthesis induced by EGF in serum-starved 3T3 cells. NR-6 membranes were ideal for this study because they do not bind EGF and hence cannot deplete it from the medium.

Fig. 1 describes an experiment in which plasma membranes derived from NR-6 or SV3T3 were tested for their ability to inhibit EGF-induced stimulation of DNA synthesis. Subconfluent 3T3 cells were arrested in the G phase of the cell cycle by serum deprivation. EGF was then added to membrane-treated and untreated cells, and DNA synthesis was determined by measuring the incorporation of [^{3}H]thymidine. The addition of NR-6 membranes to the cells decreased the EGF-induced stimulation of DNA synthesis by >50%. Furthermore, this effect could not be overcome by high concentrations of EGF (50 ng/ml) suggesting that NR-6 membranes and EGF are not in direct competition for a common site. Maximal inhibition was observed at a membrane concentration of 1 PDE unit (data not shown).

In contrast to the observations with NR-6 membranes, plasma membranes derived from SV3T3 cells only produced a small decrease in EGF-induced mitogenesis (Fig. 1). Higher concentrations of SV3T3 membranes did not produce a greater inhibition. This observation presumably reflects the

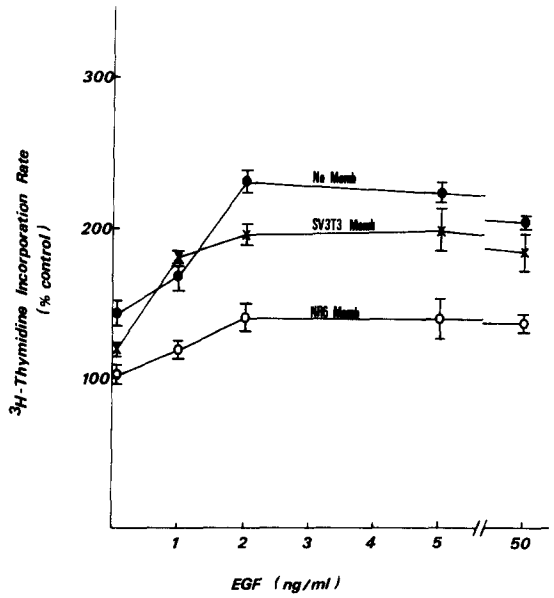

FIGURE 1 Inhibition of EGF-induced DNA synthesis in 3T3 cells by plasma membranes. Serum-starved 3T3 cells were prepared in 2-cm^2 Costar wells as described in Materials and Methods, and incubated for 3 h at 37°C in 0.5 ml of DME plus 0.5% FCS containing no membranes (●) or 2 PDE units of purified membranes from NR-6 (○) or SV3T3 cells (×). Media and membranes not adhering to the cells or dish were then aspirated, and the cells were washed three times with DME plus 0.5% FCS. EGF was added at the concentrations indicated in 1 ml of the same medium, and [^{3}H]thymidine incorporation into DNA was determined 24 h later. All points are the mean values from three independent determinations. The bars indicate the range of experimental variation.

inability of this viral transformed cell line to cease growth even at very high cell densities (16). Similarly, Whittenberger and Glaser (6) reported that SV3T3 membranes failed to substantially inhibit serum-induced DNA synthesis in 3T3 cells.

To confirm that measurement of [^{3}H]thymidine incorporation is a valid determination of DNA synthesis, autoradiography of nuclei labeled with [^{3}H]thymidine was performed. A 24-h incubation with EGF (25 ng/ml) increased the percentage of cells with labeled nuclei from 37% to 52%. However, in cells treated with NR-6 surface membranes (2 PDE units for 3 h), EGF only slightly increased the percentage of labeled nuclei from 34% to 37%. SV3T3 membranes (2 PDE units), on the other hand, did not significantly affect the stimulation by EGF (34% to 49%). Thus, inhibition of [^{3}H]thymidine incorporation by NR-6 membranes is due to an inhibition of DNA synthesis and is not the result of altered membrane transport of thymidine.

Since NR-6 cells cannot bind EGF, it is unlikely that NR-6 membranes inhibit EGF-induced DNA synthesis by removing EGF from the medium or depleting its concentration at the cell surface. Table I additionally shows that NR-6 membranes do not inhibit mitogenesis by inactivating or degrading EGF. In this experiment, NR-6 membranes were incubated with medium containing EGF, and were then removed by centrifugation. No reduction in the ability of this medium to stimulate DNA synthesis was observed as a consequence of previous membrane exposure, indicating that membranes exert their actions on the cells rather than on EGF.

The inhibitory actions of NR-6 membranes on EGF-induced DNA synthesis were maximal when membranes were

TABLE I

Retention of Biological Activity of EGF after Incubation with NR-6 Membranes

Components preincubated with EGF	Stimulation of [^{3}H]-thymidine incorporation (% of control)
None	218.3 ± 13.3
NR-6 membranes	210.9 ± 7.2
3T3 cells	236.2 ± 15.4
3T3 cells + NR-6 membranes	234.1 ± 5.4

Samples of EGF (1 ng/ml) were exposed to four different treatments for 3 h at 37°C: *None*, a control experiment in which EGF was incubated in DME/0.5% FCS. *NR-6 membranes*, EGF was incubated in 3 ml of DME/0.5% FCS containing 5 PDE units of NR-6 membranes. *3T3 cells*, EGF in DME/0.5% FCS was incubated with subconfluent 3T3 cells. *3T3 cells plus membranes*, NR-6 membranes (2 PDE units/well) were incubated with serum-starved, subconfluent 3T3 cells for 3 h, excess membranes washed away and EGF added in DME/0.5% FCS. After these treatments, EGF samples were centrifuged at 50,000 *g* for 20 min in a SW50.1 rotor (Beckman Instruments, Inc., Palo Alo, CA) at 4°C to pellet any membranes were present. Supernatant fluids were removed and added to confluent, serum-starved 3T3 cells. Stimulation of [^{3}H]thymidine incorporation was measured 20 h later. Values are expressed as the percent stimulation of [^{3}H]thymidine incorporation of EGF-treated as compared with untreated cells.

TABLE II

Effect of Time of Membrane Addition on the Inhibition of EGF-induced DNA Synthesis

Time of membrane addition	Stimulation of [^{3}H]-thymidine incorporation (% of control)
h	
−3	120.6 ± 15.0
+0.5	162.0 ± 32.8
+2	264.5 ± 32.0
+16	302.5 ± 44.4
+20	295.8 ± 13.1
Control (no membranes)	303.1 ± 6.1

At time −3 h, serum-starved 3T3 cells were washed twice, and 0.5 ml of DME plus 0.5% FCS was added. EGF (5 ng/ml) was added at time 0 h to all samples, and NR-6 membranes (2 PDE units per well) were added before or after EGF at the indicated times. [^{3}H]Thymidine incorporation was determined 24 h later. Values are expressed as the percent stimulation of thymidine incorporation of EGF-treated as compared with untreated cells. Values are the mean and experimental variation of triplicate determinations.

incubated with 3T3 cells before EGF addition (Table II). NR-6 membranes were less effective when added 2 h or more after EGF addition. The results of this experiment suggest that once cells become committed to enter S phase by EGF, membranes are incapable of inhibiting DNA synthesis. Such observations are also consistent with a study by Whittenberger et al. (17) which showed that plasma membranes arrest 3T3 cells in G_0 of the cell cycle. These results also imply that the effects of membranes are specific and not the result of toxic actions upon cells. Further supporting the contention that this effect is not due to membrane-induced toxicity, Whittenberger and Glaser (6) have shown that membrane-treated 3T3 cells have the same replating efficiency as control cells.

Serum contains a complex mixture of polypeptide growth-promoting hormones. It was therefore important to determine if NR-6 plasma membranes inhibit DNA synthesis induced by growth factors other than EGF. FGF and PDGF are polypeptide hormones which are potent mitogens for 3T3 cells (9, 10). Fig. 2 shows that NR-6 membranes reduced the stimulation of DNA synthesis induced by an extract containing PDGF by 50% and inhibited the stimulation mediated by FGF almost completely. We have also obtained similar results

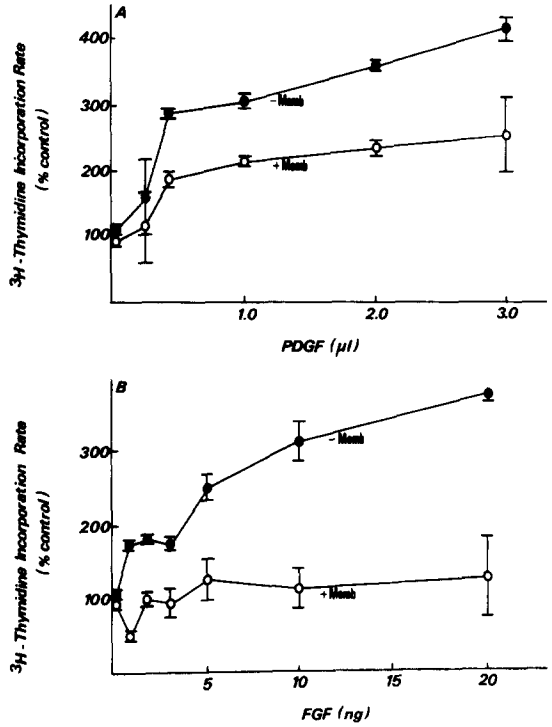

FIGURE 2 Effect of NR-6 plasma membranes on the stimulation of DNA synthesis induced by an extract containing PDGF (*A*) or FGF (*B*). Experimental conditions are those described for Fig. 1. FGF or an extract containing PDGF were added at various concentrations in 1 ml of DME plus 0.5% FCS, and [^{3}H]thymidine incorporation into DNA was measured 24 h later during a 1-h pulse labeling period. All points are the mean values of duplicate determinations, and the bars indicate the range of experimental variation.

using purified PDGF (data not shown). These results indicate that plasma membranes act to inhibit an event that is common to the action of various growth-promoting hormones.

DISCUSSION

As mammalian cells grow in culture, some signal may be generated to impart information on cell density. Such events could lead to a cessation of growth. It has been speculated that membrane-membrane interactions between cells might be the primary event leading to quiescence. In support of such a notion, both the present studies and those by Whittenberger and Glaser (6) have shown that plasma membranes affect the growth of subconfluent 3T3 cells in a way that mimics the density-dependent growth inhibition observed when 3T3 cells approach confluency. Furthermore, Whittenberger et al. (7) have partially purified a protein from detergent solubilized membranes that acts in a fashion similar to that of whole membranes. Natraj and Datta (18, 19) have described a growth-inhibiting factor that is released from confluent BALB/c 3T3 cells by treatment with urea.

Since serum can be replaced by appropriate combinations of polypeptide growth hormones in order to support the growth of mammalian cells in culture (20), it appears as though polypeptide hormones provide the main stimuli for the proliferation of these cells. Our results demonstrate that plasma membranes alter the mitogenic response of 3T3 cells to three important growth hormones, EGF, FGF, and PDGF. A specific membrane interaction is implicated, since SV3T3 cell-surface membranes are incapable of significantly reducing

EGF-induced mitogenesis. Such results indicate that plasma membrane inhibition of DNA synthesis of 3T3 cells grown in 10% serum observed both by us and by Whittenberger and Glaser (6) may be the consequence of an altered cellular response to the many polypeptide growth factors present in serum. We propose that such mechanisms may play an important role in governing the density-dependent growth of nontransformed cells in culture. However, such a model does not necessarily exclude the importance of nutrient uptake (5, 21) and the release of inhibitory molecules (22) in growth regulation.

The mechanism by which plasma membranes alter the mitogenic actions of EGF, FGF, and PDGF is still unknown. Our results suggest that membranes act to inhibit some event that is common to the mitogenic pathways of a number of polypeptide hormones. We have also shown that this mechanism of inhibition does not involve depletion or inactivation of at least one mitogen, EGF, in the medium. Furthermore, Lieberman et al. (23) have reported that 3T3 cell membranes do not alter the binding capacity of 125I-EGF to subconfluent 3T3 cells. It has also been demonstrated that the number of binding sites for EGF even increases as glial cells reach confluency (24). Thus it is likely that the mechanism of membrane-induced growth inhibition involves a step distal to the initial interaction of the mitogen with its receptor. While the biochemical events of the pathway of mitogenesis remain to be elucidated, it has recently been demonstrated that both EGF (25) and PDGF (26) are capable of activating tyrosine-specific protein kinases that may be involved in initiating cell growth. A modification of protein kinase activity as the result of membrane-membrane interaction may be one means of achieving growth regulation.

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