Title
Purified plasma membranes inhibit polypeptide growth factor-induced DNA synthesis in subconfluent 3T3 cells.

Permalink
https://escholarship.org/uc/item/9qr2328p

Journal
The Journal of cell biology, 98(3)

ISSN
0021-9525

Authors
Vale, RD
Peterson, SW
Matiuck, NV
et al.

Publication Date
1984-03-01

DOI
10.1083/jcb.98.3.1129

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

RONALD D. VALE, SCOTT W. PETERSON, NICHOLAS V. MATIUCK, and C. FRED FOX
Department of Neurobiology, Stanford University School of Medicine, Stanford, California 94305; Biological Science Center, Boston University, Boston, Massachusetts 02215; and Molecular Biology Institute, University of California, Los Angeles, California 90024

ABSTRACT Plasma membranes derived from NR-6 cells, a variant 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) (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-cm² Costar wells at a density of ~2 × 10⁴ cells/cm² 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).
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 Antoniaides and Scher (15). Purified PDGF was obtained from R. Ross, University of Washington.

Measurements of DNA Synthesis: [3H]Thymidine incorporation into acid-precipitable material was performed on 3T3 cells grown in 2-cm² Costar wells. Cells were washed once with DME plus 0.5% FCS, and 1 µCi (0.55 µCi/µL) [3H]thymidine (New England Nuclear, Boston, MA) was added in 1 ml of DME plus 0.5% undialyzable FCS. After a 1-h incubation at 37°C, cells were washed twice with cold Hank's 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 acid 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-cm² 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 [3H]thymidine labeled nuclei was performed on these cells by treating them with 1 µCi [3H]thymidine (0.55 µCi/µL) in 1 ml of DME plus 0.25% undialyzable FCS. After a 1-h incubation at 37°C, cells were washed twice with cold Hank's salt solution, fixed 45 min in 1 ml of 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) 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 3T3 mouse 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 [3H]thymidine. The addition of NR-6 membranes to the cells decreased the EGFinduced 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 EGFinduced mitogenesis (Fig. 1). Higher concentrations of SV3T3 membranes did not produce a greater inhibition. This observation presumably reflects the 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 [3H]thymidine incorporation is a valid determination of DNA synthesis, autoradiography of nuclei labeled with [3H]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 15 ng/ml of PDGF, the percentage of cells with labeled nuclei was decreased to 29%. This is consistent with the results of previous studies indicating that PDGF inhibits DNA synthesis in 3T3 cells (9). The inhibition of [3H]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 1 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
are expressed as the percent stimulation of \([3H]\) thymidine incorporation of

Stimulation of \([3H]\) thymidine incorporation was measured 20 h later. Values

Values were removed and added to confluent, serum-starved

Subconfluent 3T3 NR-6 membranes (2 PDE units/well) were incubated with serum-starved, 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

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

| Components preincubated with EGF | Stimulation of \([3H]\) 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 in 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 Alto, CA) at 4°C to pellet any membranes were present. Supernatant fluids were removed and added to confluent, serum-starved 3T3 cells. Stimulation of \([3H]\) thymidine incorporation was measured 20 h later. Values are expressed as the percent stimulation of \([3H]\) thymidine incorporation of EGF-treated as compared with untreated cells.

Table II

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

| Time of membrane addition (h) | Stimulation of \([3H]\) thymidine incorporation (% of control) |
|-------------------------------|----------------------------------------------------------|
| 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/well) were added before or after EGF at the indicated times. \([3H]\)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 G0 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 plasma membranes arrest 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.

**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

**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 \([3H]\)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.

**Figure 2**

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.
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.

This work was supported by National Institutes of Health grant AM 25826 and CA09056.

Received for publication 13 January 1983, and in revised form 16 December 1983.

REFERENCES

1. Holley, R. W. 1975. Control of growth of mammalian cells in cell culture. Nature (Lond.) 258:487-490.

2. Dulbecco, R. 1970. Conditions determining initiation of DNA synthesis in 3T3 cells. Proc. Natl. Acad. Sci. USA 66:204-210.

3. Lieberman, M. A., and L. Glaser. 1981. Density-dependent regulation of cell growth: an example of a cell recognition phenomenon. J. Membr. Biol. 63:1-11.

4. Holley, R., and J. Kierman. 1968. "Contact inhibition" of cell division in 3T3 cells. Proc. Natl. Acad. Sci. USA 60:300-304.

5. Holley, R. W. 1972. A unifying hypothesis concerning the nature of malignant growth. Proc. Natl. Acad. Sci. USA 69:2840-2841.

6. Whittenberger, B., and L. Glaser. 1977. Inhibition of DNA synthesis in cultures of 3T3 cells by isolated surface membranes. Proc. Natl. Acad. Sci. USA 74:2251-2255.

7. Whittenberger, B., D. Raben, M. A. Lieberman, and L. Glaser. 1978. Inhibition of growth of 3T3 cells by extract of surface membranes. Proc. Natl. Acad. Sci. USA 75:5457-5461.

8. Serrero, G. R., D. B. McClure, and G. H. Sato. 1979. Growth of mouse 3T3 fibroblasts I: characterization of a fibroblast growth regulatory factor. J. Biol. Chem. 254:2251-2255.

9. Touster, O., N. Aaronson, J. Dunlay, and H. Hendrickson. 1970. Isolation of rat liver plasma membranes. Use of nucleotide pyrophosphatase and phosphodiesterase I as marker enzymes. J. Cell Biol. 47:604-618.

10. Ross, R., and A. Vogel. 1978. The platelet-derived growth factor. Cell 14:203-210.

11. Das, M., T. Miyakawa, C. F. Fox, R. M. Pruss, A. Aharanov, and H. R. Herschman. 1977. Specific radiolabeling of cell surface receptors for epidermal growth factor. Proc. Natl. Acad. Sci. USA 74:2970-2974.

12. Pruss, R. M., and H. R. Herschman. 1977. Variants of 3T3 cells lacking mitogenic response to epidermal growth factor. Proc. Natl. Acad. Sci. USA 74:3918-3921.

13. Touster, O., N. Aaronson, J. Dunlay, and H. Hendrickson. 1970. Isolation of rat liver plasma membranes. Use of nucleotide pyrophosphatase and phosphodiesterase I as marker enzymes. J. Cell Biol. 47:604-618.