Inhibition of DNA Synthesis in SV3T3 Cultures by Isolated 3T3 Plasma Membranes

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ABSTRACT 3T3 plasma membranes were added to subconfluent cultures of SV3T3 cells in the presence of fusogens. If this protocol results in the introduction into the SV3T3 cell membrane of 3T3 plasma membrane components responsible for density-dependent inhibition of growth, then the SV3T3 cell cultures would be expected to show decreased rates of DNA synthesis as they approach confluence. Results of these experiments indicate that rates of DNA synthesis in SV3T3 cultures so treated were as much as 63% less than in untreated controls. This effect could not be attributed to the fusogens or to the 3T3 plasma membranes alone. This growth-inhibitory effect is specific for 3T3 membranes and is not observed when SV3T3 plasma membranes are fused with SV3T3 cell cultures. These data support the hypothesis that one aspect of the loss of density-dependent inhibition of growth in SV3T3 cells is a deletion or alteration in plasma membrane components and, further, that density-dependent inhibition of growth can be in part restored to SV3T3 cell cultures by fusing the cells with 3T3 plasma membranes.

Inhibition of DNA synthesis has been observed in many cultured cell lines as those cultures attain monolayer confluence (1–3). Although the mechanism of this density-dependent inhibition of growth has never been fully elucidated, experiments with wounding have indicated that cell-cell contact is one requirement (4, 5). More recently, Whittenberger and Glaser (6) have shown that DNA synthesis can be inhibited in actively growing 3T3 cell cultures by the addition of isolated 3T3 plasma membranes. The effect was specific for 3T3 cells and was not seen when membranes were added to SV3T3 cell cultures. Those authors also showed that a similar inhibition could not be achieved by the addition of plasma membranes isolated from transformed SV3T3 cells.

Subsequent research has shown that the growth-inhibiting effect of 3T3 membranes is probably due to one or more integral membrane proteins (7). Whittenberger et al. (8) and Raben et al. (9) have also reported the partial purification and characterization of a similar 3T3 plasma membrane protein capable of inhibiting growth in subconfluent cultures.

The loss of density-dependent inhibition of growth in SV3T3 cell cultures may be due to a deletion or alteration of the plasma membrane growth-inhibiting protein(s). Using a modification of a fusion technique developed by Poste and Nicholson (10), we have tested this possibility by fusing 3T3 plasma membranes to actively growing subconfluent SV3T3 cell cultures. If this fusion procedure results in the introduction of the normal complement of membrane proteins into the SV3T3 cell membrane, then a restoration of density-dependent inhibition of growth in the transformed cell culture might be expected to occur. We report here the results of such experiments. Up to 63% inhibition of DNA synthesis occurs as the fused SV3T3 cells reach confluence.

These findings suggest that an alteration in membrane proteins contributes to the loss of density-dependent inhibition of cell growth in cultures of SV3T3 cells and that this

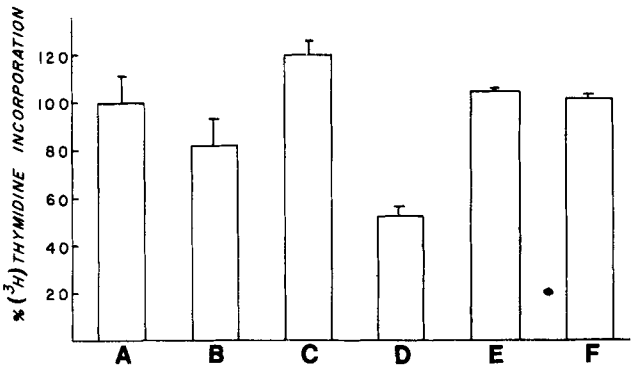

FIGURE 1 Plasma membranes fused to actively growing SV3T3 cultures: effects on [^{3}H]thymidine incorporation rates. 700 μg of membrane protein was fused to cultures containing 3.85 × 10^5 cells. 100% growth in untreated controls represents 10,000 cpm/10^6 cells per hour. (*A*), Untreated controls; (*B*), 3T3 membranes alone; (*C*), fusogens alone; *D*), 3T3 membranes plus fusogens; (*E*), SV3T3 membranes alone; (*F*), SV3T3 membranes plus fusogens.

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loss of inhibition is partially reversible by the introduction of 3T3 plasma membrane proteins.

MATERIALS AND METHODS

Cell Culture: Swiss 3T3 and SV3T3 cell cultures were obtained from C. F. Fox (University of California, Los Angeles) and D.V. Young (Boston University), respectively. Cultures were grown in a 5% CO_2/95% air atmosphere at 37°C in Dulbecco's Modified Eagle's Medium (DMEM from Gibco Laboratories, Grand Island, NY), 10% fetal bovine serum (FBS from Flow Laboratories, McLean, Virginia), penicillin (5,000 U/ml), streptomycin (5,000 meq/ml), and L-glutamine (2 mM, from Gibco Laboratories).

Plasma Membrane Preparation: A plasma-membrane-enriched fraction was prepared as described by Peterson et al. (11) from 1-d postconfluent cultures of 3T3 and SV3T3 cells. Membranes from postconfluent cultures of 3T3 cells were found to inhibit growth to a greater extent than membranes from subconfluent cultures (data not shown). We would expect, therefore, that these membranes have either a greater quantity or a more active complement of growth-inhibiting factors. These preparations were frozen in 10% glycerol at −70°C for up to 30 d before using. The DNA synthesis-inhibiting capabilities of these membranes were unchanged after freezing.

Radiolabeling of Membranes: 3T3, SV3T3, and erythrocyte plasma membranes were labeled with tritiated sodium borohydride according to the method of Gahmberg and Hakomori (12). Labeled membranes were added to SV3T3 cultures in the presence and absence of fusogens. After washing the cultures three times, cell-associated radioactivity was measured in fused and control cultures.

Fusion: The procedure was modified from that of Poste and Nicolson (10). Cells were plated onto 16-mm culture wells and grown to 90% confluence (~3.75×10^5 cells/well). The experimental cultures were incubated for 30 min at 37°C with membranes suspended in 200 μl of DMEM (10% FBS) to which 50 μg/ml phytohemagglutinin (PHA, Sigma Chemical Co., St. Louis, Mo.) had been added. An equivalent volume of 80% polyethylene glycol (PEG, 6,000 mol wt, Sigma Chemical Co.) in DMEM (10% FBS) was then added. Cultures

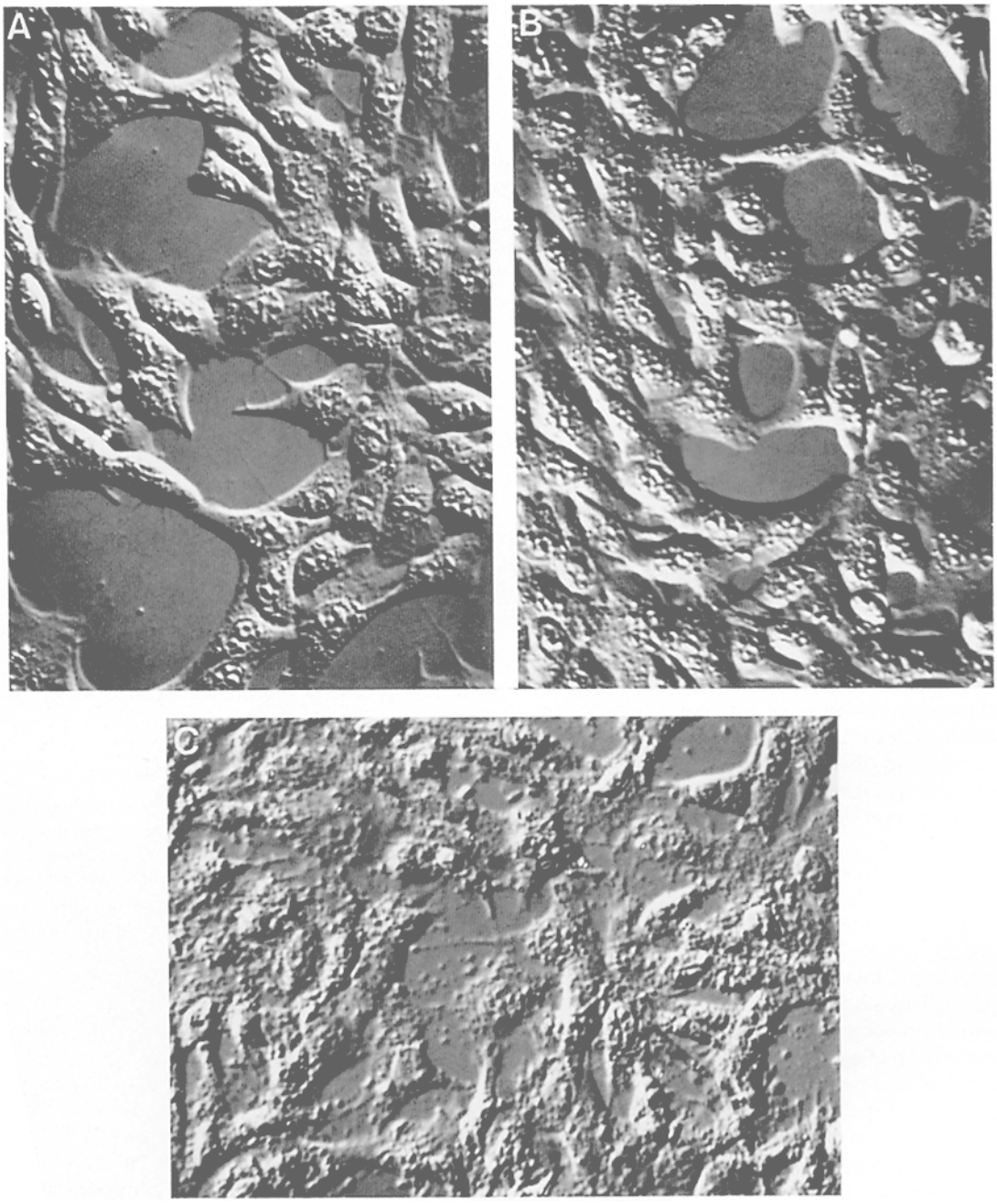

FIGURE 2 Photomicrographs using Nomarski Differential Interference Contrast microscopy illustrating particulation of living cells by plasma membrane vesicles in fused cultures. (*A*), Untreated control; (*B*), 3T3 plasma membranes alone; (*C*), 3T3 plasma membranes plus fusogens. × 1,100.

were incubated for 1 min at room temperature. The solution was then diluted 1:5 with DMEM (10% FBS) and incubated for 9 min at 37°C. Control cultures were treated identically except for the deletion of either membranes or fusogens or both. After treatment, all cultures were washed three times with DMEM and incubated for 18 h at 37°C in DMEM (10% FBS). DNA synthesis was assessed via [^{3}H]thymidine incorporation over a 1-h pulse-labeling period as described by Das and Fox (13). Results are reported as percentage inhibition of growth as compared with untreated controls and are normalized to cell numbers. Periodically, labeled nuclei incorporation assays (6) were done to verify that there was thymidine incorporation into the nucleus.

RESULTS AND DISCUSSION

If loss of density-dependent inhibition of growth in SV3T3 cells is due to a deletion or alteration in a membrane-bound growth-inhibiting protein or its receptor, then introducing these components into the plasma membranes of subconfluent SV3T3 cells might restore density-dependent inhibition of growth in these cultures as they reach confluence. To test this, 3T3 plasma membranes were added to subconfluent cultures of SV3T3 cells in the presence of PHA and PEG to induce fusion. This procedure resulted in up to 63% inhibition of DNA synthesis as the treated cultures reached confluence (Fig. 1). The results with controls, indicated that this effect could not be attributed to the additive effects of membranes and fusogens acting independently. The growth-inhibiting effect was found to be specific to 3T3 plasma membranes. Addition of SV3T3 membranes and fusogens did not inhibit growth, despite the fact that 3T3, SV3T3, and erythrocyte plasma membranes all fused to SV3T3 cells with equal efficiency.

The fusion technique used in this study was similar to that described by Poste and Nicolson (10), except that the cells were first incubated in PHA and then in PEG. However, this minor change in the protocol did not affect the degree of fusion. When the amount of radiolabeled membranes associated with these SV3T3 cells was compared in the presence and absence of fusogens, it was found that the amount of cell-associated label was increased 10-fold by the presence of fusogens. This value is comparable with that reported by Poste and Nicolson (10). Light micrographs (Fig. 2) show that cells that have had membranes added in the presence of fusogens have particulate material on their surfaces. Cells with either membranes or fusogens alone do not display this particulation. While these data support the contention that fusion has occurred, they do not definitively show that this is the case. Additional investigations using electron microscopic examinations of radiolabeled membrane fusion are presently underway.

A dose-response curve (Fig. 3) shows that ~600 μg of membrane protein/3.73 × 10^5 cells was required for half-maximal inhibition of DNA synthesis via this fusion technique. Using radiolabeled membranes, it was found that 0.77% of the plasma membranes were associated with the cells after fusion. This amount of membrane represents ~1.5 cell equivalents of membranes per fused cell.

A time course of DNA synthesis for 18 h after fusion shows no decrease in synthesis rates due to the fusogens alone up to that time (Fig. 4). The use of PHA and PEG as fusogens was found to cause a mild nonspecific toxicity in exposed cells. However, this effect was transient and SV3T3 cultures treated with fusogens alone returned to optimal growth rates matching those of controls within 6 h. Inhibition of DNA synthesis in cultures fused with 3T3 membranes was initially evident at 11 h after treatment. These results indicate that inhibition

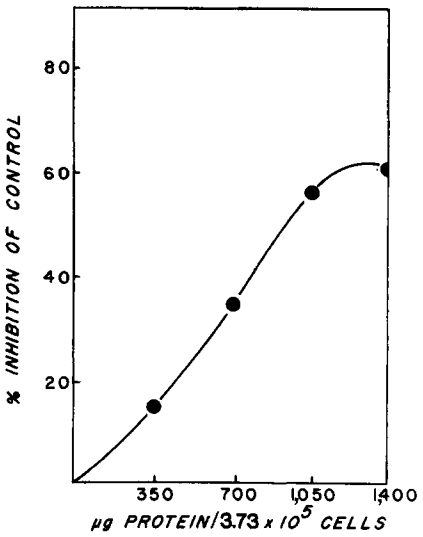

FIGURE 3 3T3 plasma membranes fused to actively growing SV3T3 cultures: effects of membrane concentration on [^{3}H]thymidine incorporation rates. Varying concentrations of 3T3 plasma membranes were fused to SV3T3 cultures containing 3.73 × 10^5 cells per 16-mm well as described in Materials and Methods. DNA synthesis was measured 18 h after treatment.

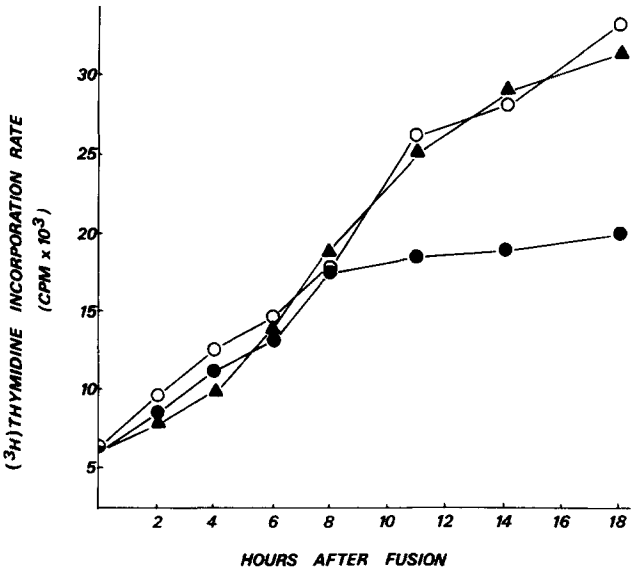

FIGURE 4 DNA synthesis rates in SV3T3 cultures fused with 3T3 plasma membranes. 3.81 × 10^5 cells per 16-mm well were fused with 700 μg of membrane protein as described in Materials and Methods. Incorporation of [^{3}H]thymidine was measured at intervals between 0 and 18 h after fusion. Counts are normalized to cell numbers. O, untreated control; ▲, fusogens alone; ●, fusogens plus 3T3 plasma membranes.

of DNA synthesis is not due to early nonspecific inhibition of growth, or to cell detachment.

The inhibiting effect induced by 3T3 plasma membranes on SV3T3 cells is reversible (Fig. 5). After the initial period of inhibition, growth rates in treated SV3T3 cultures paralleled those of untreated controls. The time course for reversibility of the inhibitory effect is consistent with known rates of membrane turnover (14). These observations support the contention that the 3T3 membrane components that were introduced into the SV3T3 cells are removed in the course of

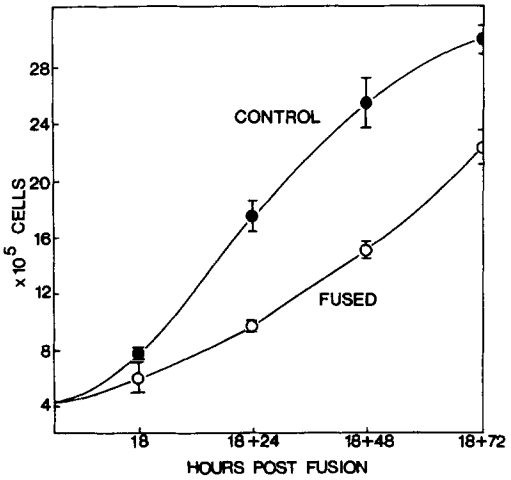

FIGURE 5 Effects of fused 3T3 plasma membranes on continued growth of SV3T3 cultures. Cultures were treated at 0 h and then allowed to grow with daily replacement of growth medium and serum. 18 h corresponds to the time point after fusion at which [^{3}H]thymidine incorporation rates were measured in the original experiment (see Fig. 1). Results are expressed as means and standard deviations from four experiments.

normal membrane processing. This removal would then result in the loss of the induced growth-inhibition as the original protein composition of the SV3T3 plasma membrane was re-established. The observation that neither untreated control SV3T3 cells nor recovered fused SV3T3 cells grow at exponential rates can likely be attributed to the fact that both of these systems were postconfluent and that diffusion of nutrients around the bottom layers of cells was restricted.

When 3T3 membranes were fused to sparse cultures of SV3T3 cells, DNA synthesis was inhibited by 30%. This result apparently contradicts the rest of our data that support the proposed mechanism for inhibition by fusion with membranes. However, there is a high concentration of membranes in the fusion medium (up to 200 cell equivalents). Since only 0.77% of these are fused to the cells, the remainder of the membranes in the medium may be simulating confluent culture conditions for the SV3T3 cells.

Raising the fetal bovine serum concentration to 20% immediately after fusion resulted in a 50% decrease of the inhibitory effect. This finding is consistent with the observation by Raben et al. (9) that membrane-induced inhibition of growth in 3T3 cells can be reduced by increasing the serum concentration.

SV3T3 plasma membranes were fused to near-confluent 3T3 cultures in an attempt to distinguish whether the loss of density-dependent inhibition of growth in transformed cells is due to the deletion of a normal growth-inhibiting protein or, alternatively, to the appearance of a new growth-stimulating factor. It was reasoned that if an aspect of transformation is the appearance of a growth-stimulating membrane component, then fusion of SV3T3 membranes to 3T3 cells should result in growth of these cultures past confluence. The results of this experiment show that 3T3 cells cannot be induced to grow past confluence in this manner, in support of the idea that transformation is due to the deletion or alteration of a 3T3 membrane protein.

The results of these experiments demonstrate that alterations in the plasma membrane are related to the loss of density-dependent inhibition of growth in SV3T3 cell cultures. They also indicate that fusion of membrane components with the plasma membranes of growing cells may be a mechanism for controlling tumor growth in vivo. Experiments are currently underway to investigate this possibility.

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