Proc. Nat. Acad. Sci. USA
Vol. 73, No. 12, Part I, pp. 3631–3635, December 1973

Changes in Membrane Structure Associated with Cell Contact

(SV40/3T3/freeze-cleavage/electron microscopy/intramembranous particles/ intrinsic membrane proteins/cell proliferation)

ROBERT E. SCOTT, LEO T. FURCHT, AND JOHN H. KERSEY

Department of Laboratory Medicine and Pathology, University of Minnesota Medical Center, Minneapolis, Minn. 55455

Communicated by Robert A. Good, August 6, 1973

ABSTRACT **Ultrastructural analysis of 3T3 fibroblasts by freeze-cleavage has demonstrated significant changes in cell-membrane structure associated with cell-to-cell contact and malignant transformation. These changes consist of a rearrangement and redistribution of intramembranous particles on the membrane fracture faces exposed by freeze-cleavage. The results show that noncontacted 3T3 cells in low density contain randomly distributed intramembranous particles. With the development of cell-to-cell contacts during the logarithmic phase of growth however, a pronounced aggregation of intramembranous particles is seen. A direct correlation between the degree of cell contact and the percentage of cells showing intramembranous-particle aggregation has been established. By contrast, transformed SV3T3 and SP3T3 cells show no evidence of intramembranous-particle aggregation even at cell densities where cell-to-cell contact is extensive. In view of recent reports that intramembranous particles represent foci of interaction between certain intrinsic membrane proteins and lipids, we propose that cell-to-cell contact of nontransformed 3T3 cells may initiate a change in the distribution of intrinsic membrane proteins associated with intramembranous particles and that these changes may influence control of cell proliferation.**

Control mechanisms that regulate division are influenced by molecular events initiated at the cell membrane. The observation that plant lectins conjugated to Sepharose can stimulate mitogenesis in lymphoid cells suggests that the binding of proteins to a cell-membrane receptor can directly influence the rate of cell proliferation (1). Experiments with mixed leukocyte cultures also show that the interaction of antigenically different cells can also stimulate cell proliferation (2).

Regulation of cell growth and division also appears to be influenced by cell-to-cell interaction of other cell types. Studies of mouse 3T3 fibroblasts have shown that at confluence, cell-to-cell interaction signals a cessation of cell division and cells are arrested in the G_1 phase of the cell cycle; this phenomenon is designated contact inhibition of growth (3, 4). Transformation of 3T3 cells by oncogenic viruses, such as simian virus 40 (SV40) or polyoma virus, has been shown to result in the loss of contact inhibition and the development of tumorigenicity (5).

The 3T3/SV3T3 system and similar model systems have served as the basis for extensive investigation concerning the differences in membrane composition and function of normal and malignant cells. Initial studies showed that malignant cells have higher electrophoretic mobilities associated with higher sialic acid content (6) and a greater density of the membrane glycocalyx (7, 8). More recent studies have demonstrated significant abnormalities in the membrane glycolipids (9–11) and membrane glycoproteins (12, 13) of transformed cells. Transformed cells have also been shown to be specifically agglutinated by certain plant lectins (14, 15).

Our studies use the ultrastructural technique of freeze-cleavage to analyze the density and distribution of intramembranous particles of normal and transformed cells. Freeze-cleavage has been shown to fracture biological membranes along their interior hydrophobic regions exposing intramembranous particles (IMP) on the freeze fracture faces (16). Recent experiments (17, 18) have demonstrated that the IMP in human erythrocytes are associated with a specific type of intrinsic membranous protein; Singer has postulated that IMP in most mamallian cells represent a similar intrinsic protein that spans the lipid bilayer (19).

We report here that there are striking changes in the distribution and density of IMP in the membranes of normal cells associated with cell contact and malignant transformation. We propose that contact between normal cells, but not malignant cells, initiates a change in the distribution of IMP-associated intrinsic proteins and that this change may be a primary event in control of cell proliferation.

METHODS

BALB/c 3T3 clone A31 mouse-embryo fibroblasts, spontaneously transformed 3T3 variants, and SV40-transformed 3T3 fibroblasts (a gift of Dr. George Todaro), were grown in Dulbecco's modified Eagle's minimal essential medium supplemented with either 10% fetal-calf serum or 10% calf serum with penicillin (100 units/ml) and streptomycin (10 mg/ml) in a humidified incubator at 37° with a 10% CO_2 atmosphere. Routine cell passage was achieved by treatment of cell cultures with 0.05 mM EDTA and 25 mg/100 ml of trypsin. 3T3 and SV3T3 cells were shown to be free of mycoplasma contamination by culture and electron microscopic analysis (Moses, H., personal communication).

For individual experiments, subcultures were routinely plated at densities of between 2×10^2 and 1×10^3 cells per cm^2 in 75-cm^2 Falcon flasks and the media was routinely changed at 3-day intervals. Growth curves were established by counting cells released by trypsin–EDTA with a hemo-

Abbreviations: IMP, intramembranous particles; IFF, inner fracture face; OFF, outer fracture face; 3T3, contact-inhibited BALB/c 3T3 clone A31 fibroblasts; SP3T3, spontaneously transformed BALB/c 3T3 clone A31 fibroblasts; SV40, simian virus 40; SV3T3, SV-40-transformed BALB/c 3T3 clone A31 fibroblasts.

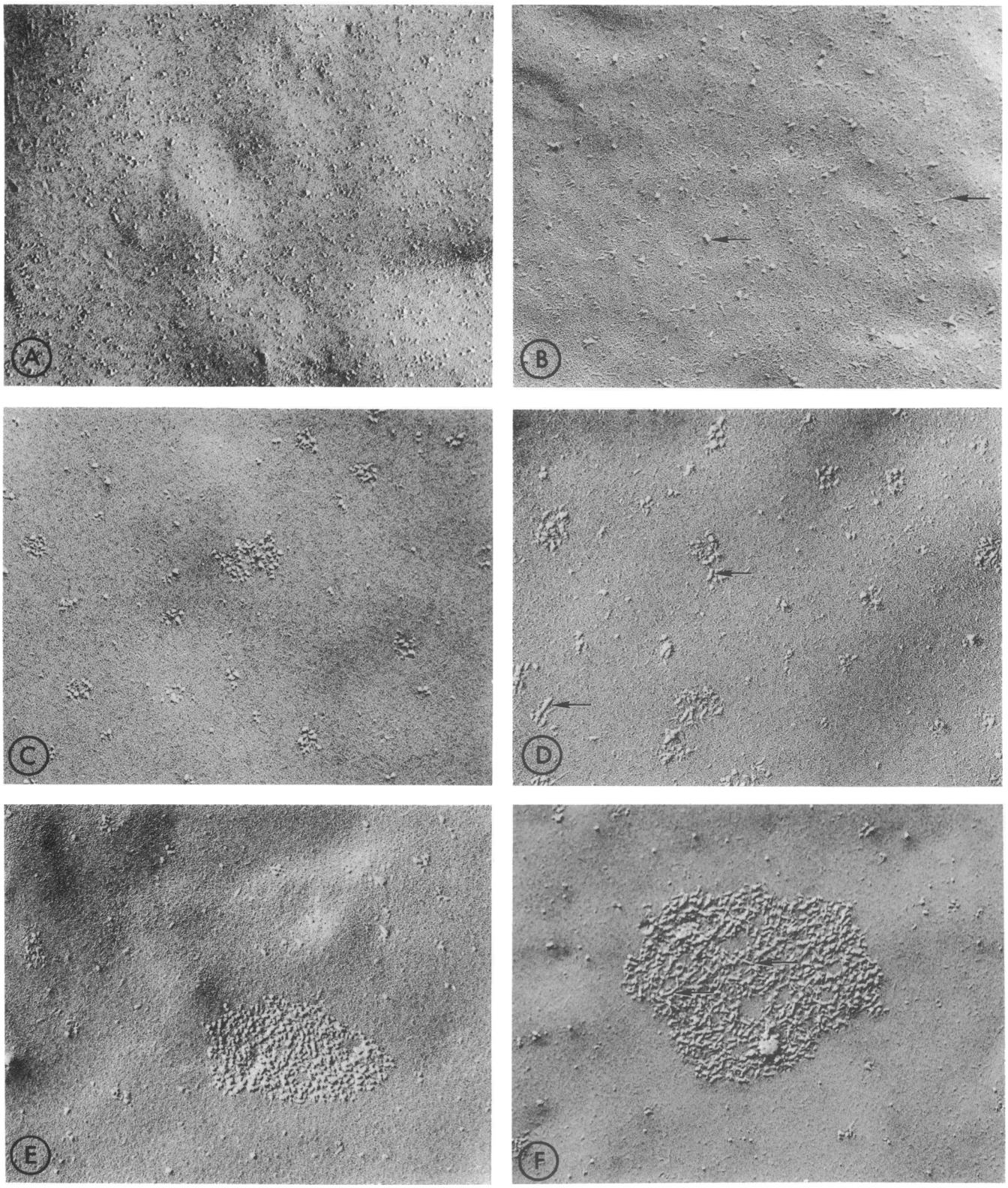

FIG. 1. Freeze-fracture replicas of the inner fracture face (IFF) (*left*) and outer fracture face (OFF) (*right*) of confluent SV3T3 (*A* and *B*) and confluent 3T3 fibroblasts (*C–F*). Intramembranous particles (IMP) are randomly distributed on the fracture faces of the transformed SV3T3 cells, but show varying degrees of aggregation on the fracture faces of contacted 3T3 cells. IMP on the IFF are globular; on the OFF both globular and rod-shaped (*arrow*) particles are present. The fracture faces of noncontacted low-density 3T3 fibroblasts show a random IMP distribution similar to that illustrated on replicas *A* and *B*. ×70,000.

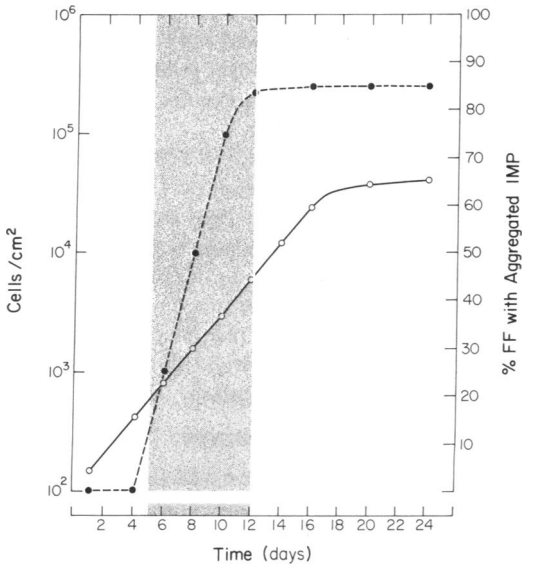

FIG. 2. Growth curve of 3T3 fibroblasts illustrates relationship of cell density (O——O) and percentage of cells that contain aggregated intramembranous particles (●- - -●). Cell-to-cell contacts were established during the interval depicted by the *shaded zone*. *FF*, fracture faces.

cytometer. The degree of cell-to-cell contact was established by phase microscopy, and cell viability was routinely examined by trypan blue dye exclusion.

Samples for freeze-cleavage were prepared as described (20) and with prefixation in glutaraldehyde. Specimens were freeze-cleaved in a Balzars BAE 300 freeze-etch microtome at −100° under 1 × 10^{-6} torr vacuum. Individual freeze-cleaved specimens were immediately shadowed with platinum and carbon, washed repeatedly, dried, and examined in a Philips EM300 electron microscope at 80 kV. IMP distribution was analyzed by examination of low-magnification micrographs taken at ×40,000, which encompassed an average fracture-face surface area of 50–100 μm^2. IMP density was established for each individual cell by counting the particle density on two or more 1μm^2 fracture-face surface areas at a magnification of ×100,000. Cells were designated as showing random distribution of IMP when the entire fracture face was uniformly covered by IMP and when no aggregates containing more than 10 IMP were present. Cells were designated as showing aggregated IMP when clusters of more than 10 IMP were present in association with the presence of large particle-free surfaces. The percentage of cells showing aggregated IMP was established by analysis of approximately equal numbers of inner and outer fracture faces of more than 40 individual cells.

RESULTS

Membrane Ultrastructure of Freeze-Cleaved Contact-Inhibited and Transformed Cells. Fig. 1 illustrates the marked difference in membrane structure of confluent cultures of freeze-cleaved 3T3 and SV40-transformed 3T3 fibroblasts. The fracture faces of 3T3 cells contain large aggregates of IMP (plates *C–F*); the membranes of SV3T3 cells contain randomly distributed IMP (plates *A–B*). The IMP in nontransformed cells are arranged in aggregates of 15–75 particles surrounded by large surfaces that are essentially bare of particles. Fig. 1 also illustrates that both fracture faces of 3T3 and SV3T3 cells contain globular 60–90 Å of IMP, and that, in addition, the outer fracture face (OFF) contains a population of delicate rod-shaped particles measuring about 70 × 200 Å. In confluent nontransformed 3T3 cells, IMP aggregates on the inner fracture face (IFF) consisted of only globular IMP; however, we observed three types of IMP aggregates on the OFF: globular IMP aggregates, rod-shaped IMP aggregates, and aggregates containing both types of IMP.

Analysis of the density of IMP on the fracture faces of confluent 3T3 and SV3T3 cells showed the total number of IMP/μm^2 to be similar (Table 1). The IFF of SV3T3 cells typically contained twice the density of IMP as did the OFF, an observation which has been described in other mammalian cell lines (17, 20, 21). However, as an apparent result of IMP aggregation, the membranes of confluent 3T3 cells showed equal numbers of IMP on the inner and outer fracture faces. This observation suggests that the aggregation of IMP is associated not only with lateral movement of particles in the membrane but also with an apparent reorientation of IMP within different membrane cleavage planes.

The results of experiments to establish the correlation between the extent of IMP aggregation and the degree of contact inhibition are presented in Table 2. 3T3 cells, including A31 subclones 5 and 6, showed a high degree of contact inhibition with a maximal saturation density of 3 × 10^4 cells per cm^2 in 10% fetal-calf serum. About 85% of the fracture faces of these confluent 3T3 cells contained aggregated IMP. Less contact-inhibited cells (3T3 A31) showed a higher saturation density and less IMP aggregation. Spontaneously transformed clones of 3T3 cells were found to contain even less IMP aggregation, especially in clones that grew to saturation densities of more than 3 × 10^5 cells per cm^2. Similarly, there was no significant aggregation of IMP on the fracture faces of viable SV3T3 cells. Experiments did show, however, that cell death, as demonstrated by trypan blue staining, was associated with aggregation of IMP similar to that reported in trypsinized erythrocyte ghosts (17).

The possibility that IMP aggregates represented gap junctions was excluded because aggregates of IMP were present across the entire cell membrane and because IMP aggregates did not show the typical organization of gap junctions.

Effect of Cell Contact on IMP Aggregation. To determine if the aggregation of IMP resulted only from contact between normal contact-inhibited cells, 3T3, SP3T3, and SV3T3 cells were studied by freeze-cleavage throughout the logarithmic

TABLE 1. *Intramembranous particle density on fracture faces of contact-inhibited and transformed 3T3 fibroblasts*

Culture	Density IMP/μm^2* IFF	OFF	Total
Confluent—100% contacted			
3T3 Fibroblasts	300	300	600
SV3T3 Fibroblasts	425	200	625
Low density—0% contacted			
3T3 Fibroblasts	450	175	625
SV3T3 Fibroblasts	425	200	525

* Mean IMP density on fracture faces of 40 or more individual cells.

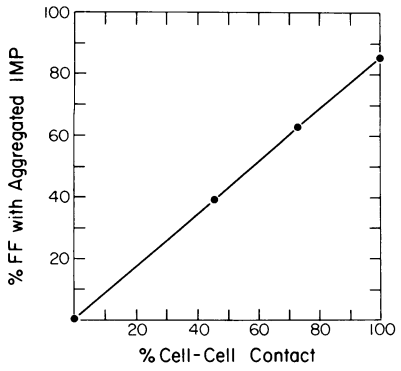

FIG. 3. The linear relationship is illustrated between the percentage of 3T3 fibroblasts showing cell-to-cell contact and the percentage of 3T3 cells that contain aggregated intramembranous particles.

phase of growth and at confluency. Subcultures were plated at densities where no cell-to-cell contact was apparent by phase microscopy and were grown to their respective saturation densities, during which time sequential samples were taken to determine cell count and cell viability and for analysis by freeze-cleavage. Fig. 2 illustrates the relationship between the extent of IMP aggregation and cell density of 3T3 fibroblasts. At densities less than 5×10^2 cells per cm^2, no cell-to-cell contact was apparent in 3T3 cells and the fracture faces of these noncontacted cells show no evidence of IMP aggregation. At a density of 6×10^3 cell per cm^2, all cells were found to be contacted and IMP aggregation was observed on 85% of the fracture faces. Aggregation of IMP reached this maximum percentage on day 12 of culture, 4 days before cessation of the logarthmic phase of growth. These results suggest that changes in membrane structure develop as a result of cell-to-cell contact itself and are not due to changes in

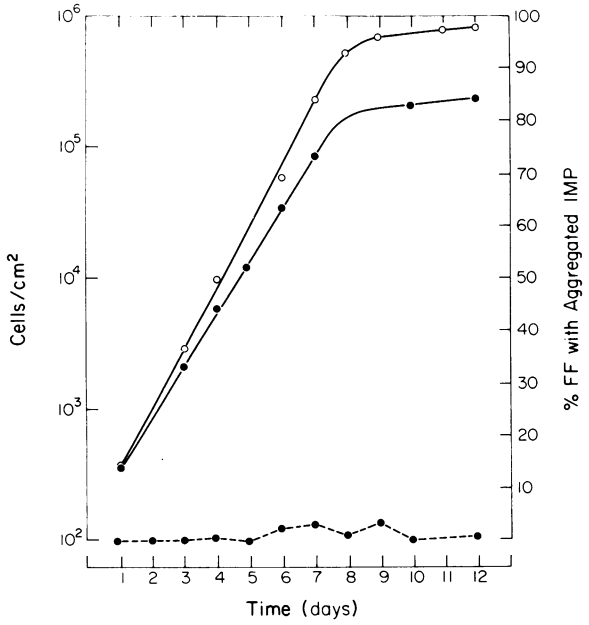

FIG. 4. Growth curve of SV3T3 (O——O) and SP3T3 (●——●) fibroblasts illustrates the lack of significant aggregation of intramembranous particles (●- - -●) in the membranes of transformed cells even at high cell densities.

the cell cycle. Studies to determine in more detail the relationship between IMP aggregation and cell contact are illustrated in Fig. 3, which illustrates the linear relationship between the extent of cell-to-cell contact and the degree of IMP aggregation.

The membranes of SP3T3 and SV3T3 cells failed to show aggregation of IMP after cell contact (Fig. 4). At densities where there are no cell contacts (less than 1×10^3 cells per cm^2), at densities where all cells are contacted (1×10^4 cells per cm^2), and at confluence, no viable transformed cells showed significant IMP aggregation. Preliminary data show that 3T3 cells transformed by polyoma virus and temperature-sensitive SV40 mutants grown at the permissive temperature also fail to show IMP aggregation.

DISCUSSION

The results of this study demonstrate that significant structural changes develop in the membranes of contact-inhibited 3T3 fibroblasts as a result of cell-to-cell contact and malignant transformation. Freeze-cleavage ultrastructural studies show that low-density noncontacted 3T3 fibroblasts have a random distribution of IMP in the cell membrane and, as has been demonstrated in freeze-fracture studies of other mammalian cell lines, including Chinese hamster ovary cells (21), L fibroblasts (21), and human lymphocytes (20), the density of IMP on the IFF of these cells is twice the IMP density on the OFF. Our results show that IMP undergo marked aggregation on the fracture faces of nontransformed 3T3 cells after development of cell-to-cell contact. The aggregation of IMP is also associated with an apparent reorientation of IMP in different planes in the cell membrane so that, after freeze-cleavage, equal densities of IMP are observed on both the IFF and OFF. Sequential analysis of 3T3 cell samples at increasing cell densities during the logarithmic phase of growth demonstrate a linear relationship between the extent of cell-to-cell contact and the percentage of cells with aggregated IMP.

Experiments have been performed to exclude the possibility that the aggregation of IMP distribution might result from inhibition of cell movement associated with cell contact. 3T3 and SV3T3 fibroblasts have been treated with reagents that inhibit cell movement, i.e., cytochalasin B (22) and dibutyryl cAMP (23). The results of these and other experiments show that inhibition of cell movement and membrane ruffling have no direct effect on IMP distribution. In addition, experiments have shown that changes in the cell cycle also have no effect

TABLE 2. *Relationship of intramembranous particle aggregation to saturation density*

Cells	Saturation density (cells/cm^2 in 10% fetal-calf serum)	% Fracture face with aggregated IMP
Contact-inhibited		
3T3 (A31, subclones 5 and 6)	3.5×10^4	85
3T3 (A31)	7×10^4	60
Noncontact-inhibited		
SP3T3 (A31)	3×10^5	5
SV3T3 (A31)	8×10^5	5

on IMP aggregation (Furcht and Scott, manuscript in preparation). These data suggest that cell contact itself induces a change in the structure of the cell membrane of contact-inhibited 3T3 cells.

Transformed SP3T3 and SV3T3 fibroblasts, by contrast, show no evidence of IMP aggregation. The fracture faces of noncontacted and contacted transformed cells consistently show a random distribution of IMP. In addition, the ratio of IMP on the IFF:IMP on the OFF is maintained at 2:1 throughout all stages of growth. These observations are consistent with the hypothesis that changes in the distribution of IMP that develop after cell contact influence control of cell proliferation.

The mechanisms whereby cell-to-cell contact induces aggregation of IMP and how IMP aggregation influences control of cell division are uncertain, as is the biochemical nature of IMP in nucleated mammalian cells. If, however, membrane particles represent intrinsic membrane glycoproteins similar to the association of glycophorin and intramembranous particles described in human erythrocytes (17, 18), certain growth regulatory functions might be altered by their aggregation in the cell membrane. Insight into the functional potential of glycophorins comes from extensive studies on human erythrocytes which show this glycoprotein to be a unique type of intrinsic membrane protein that spans the entire membrane lipid bilayer (18, 24–27). Glycophorin in erythrocytes is orientated in the cell membrane so that the amino-terminal half of the molecule extends outside the lipid bilayer and is covalently linked to about 30 oligosaccharide side chains that contain receptors for MN blood-group substances, phytohemagglutinin, wheat-germ agglutinin, and influenza virus (18, 26). The middle portion of the molecule consists of extremely hydrophobic amino acids that are tightly bound to the membrane lipids (28). It is this segment that spans the lipid bilayer and appears to form the structural core of the IMP. The third region of this glycoprotein and the region representing the carboxy-terminal segment of the molecule extends into the aqueous environment of the cytoplasm where it is thought to interact with cytoplasmic proteins (24, 25). In general, three functions have been proposed for such glycophorins: they may transmit signals across the cell membrane (24), they may function as transport molecules (25, 28), and they may interact with membrane lipids (28) to influence the fluid characteristics of the cell membrane and thereby the activity of certain membrane-bound enzymes (29).

It is our hypothesis that IMP are probably associated with glycophorins in 3T3 fibroblasts, that cell-to-cell contact induces a change in the distribution of IMP-associated fibroblast glycophorins, and that this change in glycophorin distribution may influence cellular proliferation by effecting membrane fluidity, membrane transport, or intercellular communication.

We acknowledge technical assistance of Virginia L. Ellis, the scientific advice and support of the members of the Minnesota Membrane Group, and the financial support of the National Institutes of Health, Grant CA-13458 and Contract CP-33357.

1. Greaves, M. F. & Bauminger, S. (1972) *Nature New Biol.* **235,** 67–69.
2. Ling, N. R. (1968) in *Lymphocyte Stimulation* (North Holland Pub. Co., Amsterdam).
3. Nilausen, K. & Green, H. (1965) *Exp. Cell Res.* **40,** 166–168.
4. Todaro, G. J., Lazar, G. K. & Green, H. (1966) *J. Cell Comp. Physiol.* **66,** 325–334.
5. Aaronson, S. A. & Todaro, G. J. (1968) *Science* **162,** 1024–1026.
6. Kraemer, P. M. (1966) *J. Cell. Physiol.* **67,** 23–34.
7. Martinez-Palomo, A., Braislovsky, C. & Bernhard, W. (1969) *Cancer Res.* **29,** 925–937.
8. Martinez-Palomo, A. (1970) *Int. Rev. Cytol.* **29,** 29–75.
9. Hakomori, S. (1970) *Proc. Nat. Acad. Sci. USA* **67,** 1741–1747.
10. Hakomori, S. & Murakami, W. T. (1968) *Proc. Nat. Acad. Sci. USA* **59,** 254–260.
11. Robbins, P. W. & MacPherson, I. A. (1971) *Proc. Roy. Soc. Ser. B* **177,** 49–58.
12. Meezan, E., Wu, H. C., Black, P. H. & Robbins, P. W. (1969) *Biochemistry* **8,** 2518–2524.
13. Buck, C. A., Glick, M. C. & Warren, L. (1971) *Biochemistry* **10,** 2176–2180.
14. Burger, M. M. & Goldberg, A. R. (1967) *Proc. Nat. Acad. Sci. USA* **57,** 359–366.
15. Aub, J. C., Sanford, B. H. & Wang, L. H. (1965) *Proc. Nat. Acad. Sci. USA* **54,** 400–402.
16. Branton, D. (1968) *Proc. Nat. Acad. Sci. USA* **55,** 1048–1056.
17. Tillack, T. W., Scott, R. E. & Marchesi, V. T. (1972) *J. Exp. Med.* **135,** 1209–1227.
18. Marchesi, V. T., Jackson, R., Segrest, J., Tillack, T. W. & Scott, R. E. (1972) *Proc. Nat. Acad. Sci. USA* **69,** 1445–1449.
19. Singer, S. J. & Nicholson, G. L. (1972) *Science* **175,** 720–731.
20. Scott, R. E. & Marchesi, V. T. (1972) *Cell. Immunol.* **3,** 301–317.
21. Scott, R. E., Carter, R. L. & Kidwell, W. R. (1971) *Nature New Biol.* **233,** 219–221.
22. Carter, S. B. (1967) *Nature* **213,** 261.
23. Johnson, G. S., Morgan, W. D. & Pastan, I. (1972) *Nature* **235,** 54–56.
24. Marchesi, V. T. (1973) *Hosp. Prac.* **8,** no. 6, 76–84.
25. Singer, S. J. (1973) *Hosp. Prac.* **8,** no. 5, 81–90.
26. Segrest, J. P., Kahane, I., Jackson, R. L. & Marchesi, V. T. (1973) *Arch. Biochem. Biophys.* **155,** 167–183.
27. Bretscher, M. S. (1971) *Nature New Biol.* **231,** 229–232.
28. Segrest, S. J., Jackson, R. L., Marchesi, V. T., Guyer, R. B. & Terry, W. (1972) *Biochem. Biophys. Res. Comm.* **49,** 964–969.
29. Grischam, C. M. & Barnett, R. E. (1973) *Biochemistry* **14,** 2635–2637.