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A Structural Change of the Plasma Membrane Induced by Oncogenic Viruses: Quantitative Studies with the Freeze-Fracture Technique

(polyoma virus/temperature-sensitive Rous sarcoma virus/hamster sarcoma virus/ chick embryo fibroblasts/hamster BHK21 cells)

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ABSTRACT **In BHK21 hamster cells a significant increase in density of intramembranous particles occurs in freeze-fractured plasma membranes after transformation by hamster sarcoma and polyoma viruses.**

A similar change has been observed in chick embryo cells infected and transformed by a mutant of Rous sarcoma virus thermosensitive for transformation, at both permissive and nonpermissive temperatures. There is also an increase in particle density in chick cells infected with the Rous-associated avian leukosis virus type 1.

The newly appeared particles may represent the insertion of new proteins in hydrophobic regions of plasma membrane, in response to the action of oncogenic viruses.

The freeze-etching technique reveals features of the hydrophobic region of the plasma membrane, since the cleavage plane of fractured membrane lies between the two phospholipid leaflets (1). Moreover, intramembranous particles (IM particles) seen by this technique are believed to represent intrinsic membrane proteins inserted within the lipid matrix (2). According to the fluid mosaic model of membrane structure (3), these proteins can diffuse in the lipid matrix and thus assume a random distribution over the cell surface. On the basis of studies with concanavalin A, it has been reported that membrane proteins of virus-transformed cells are more free to move than those of normal cells (4), but the validity of this conclusion has been questioned (5).

In two cell systems, we have been unable to find a significant difference in the distribution of IM particles in normal and transformed cells, even following concanavalin A treatment.

However, quantitative studies in *density* of IM particles have demonstrated a significant *increase* in response to viral infection or transformation by some oncogenic viruses of various types.

MATERIALS AND METHODS

Cells. Two classes of cell systems, mammalian and avian, were used.

(*1*) An asparagine-dependent subclone called C13/8 (6) was derived from the BHK21/13 line of hamster fibroblasts (7). This clone has retained the biological characters of the original line (7); between 5×10^4 and 10^5 cells are required to produce tumors in animals. Transformed cells were clones originated from colonies obtained by plating in soft agarose gel containing 10 μg/ml of dextran sulfate (8) C13/8 cells infected either with hamster sarcoma virus, B34 strain (9), or polyoma virus (small-plaque variant).

Two clones of transformed C13/8 cells, isolated from colonies growing in the gel, were used: (*a*) C13/8/HS5, a clone transformed by hamster sarcoma virus, had undergone approximately 50 passages. (*b*) C13/8/SPy2, transformed by polyoma virus (small-plaque variant) had been passed six times.

These transformed clones both have a high tumorigenicity for Syrian hamsters (10) and possess *in vitro* transformation characters, such as an altered morphology compared to the untransformed cells (more pronounced for the clone HS5, which consists of round or epithelioid refractile cells), growth in agar or in agarose containing sulfated polysaccharides (8), and increased fixation of ruthenium red (10).

Control and transformed cells were grown in 250 cm^3 plastic Falcon flasks with modified Eagle's medium (7) supplemented with 10% of tryptose phosphate broth (TPB) and 10% calf serum. Tests for mycoplasma contamination of the three clones were negative. No C-type virus production could be detected either by electron microscopy of thin sections of cells or by banding the concentrated culture supernatant in a density gradient after uridine labelling.

(*2*) Secondary cultures of chick embryo fibroblasts (CEF) were prepared as described earlier (11) from single Brown Leghorn embryos, free of lymphomatosis virus. They were grown in Eagle's minimal essential medium (MEM) with double concentrations of vitamins and with nonessential amino acids and supplemented with 10% tryptose phosphate broth and 5% calf serum.

Viruses. A thermosensitive mutant of the Schmidt–Ruppin strain of Rous sarcoma virus, called Fu19, was used for infecting and transforming CEF (12); this virus fully transforms cells at 37° but is defective in its transforming ability at 41°; other viral functions are normal at 41° so that cells morphologically reverted at this temperature still produce infectious virus normally. Avian leukosis Rous-associated virus, type 1, (RAV1) was also used for infecting CEF. In both cases CEF were infected 1 day after subculturing (multiplicity of infection: 0.15 for Fu19 and 10 for RAV1), subcultured once more after 1 week, and examined 2 days later.

Electron Microscopy. Hamster cells were harvested 2 days after seeding at the end of their exponential growth, before they attained full confluence. The monolayer was treated with

Abbreviations: IM particles, intramembranous particles; RAV1, Rous-associated virus, type 1; CEF, chick embryo fibroblasts; RSV, Rous sarcoma virus.

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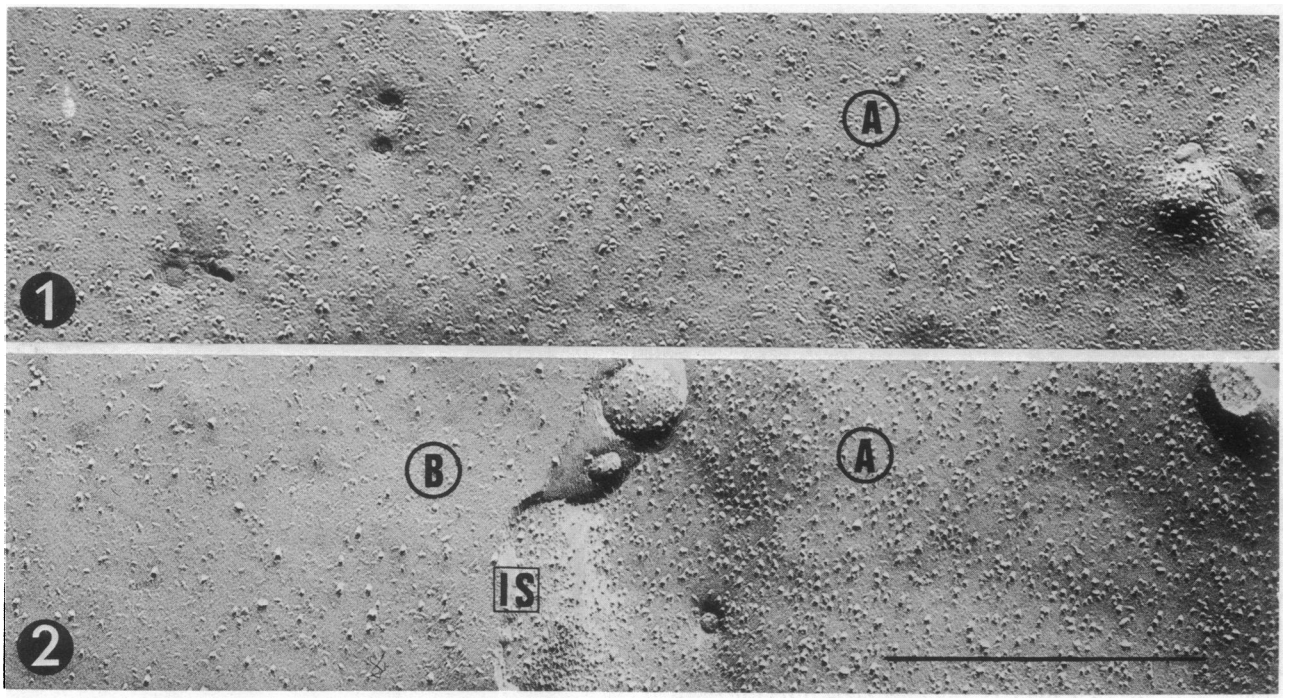

FIGS. 1 and 2. Freeze-etched replicas of plasma membranes. *Fig. 1:* The A fracture face of a BHK21/13/8 cell. *Fig. 2:* The B and A fracture faces of a BHK21/13/8 cell transformed by hamster sarcoma virus (clone HS5). Note the increased number of particles in Fig. 2 and their random distribution. Bar indicates 0.5 μm. IS = intercellular space.

0.2% EDTA in phosphate-buffered saline (pH 7.4) and cells were centrifuged at 600 $\times$ *g* for 5 min. CEF cells were collected in the same way after 2–3 days of growth before confluence was reached. The cell pellets were then fixed in 3% glutaraldehyde in cacodylate buffer (pH 7.2) for 30 min, washed three times, and impregnated with 30% glycerol in distilled water. Freeze-fracturing and replicas were made in a Balzers apparatus at $-150°$.

Particle Density Measurements. Micrographs were enlarged on photographic paper covered by a grid in order to eliminate errors due to distortion of the picture upon processing of the photographs and to make the counting of particles per unit area easier. Data corresponding to the counting of at least 1 μm^2 of each membrane picture were statistically analyzed by the Dunett's test for variance analysis ($P \leqslant 0.05$) (13). About 30 pictures chosen at random of each cell preparation were analyzed in this way.

According to current convention of most membrane systems studied with the freeze-fracture technique, the outwardly directed fracture face of the inner leaflet is termed A, while the complementary face is B.

RESULTS

Random distribution of IM particles in control and transformed cells

No significant deviation from random distribution could be observed in membrane fractures of BHK21 cells, either normal ones or those transformed by hamster sarcoma virus (Figs. 1 and 2) or polyoma virus. Rare examples of aggregation were seen in damaged cells. An extensive aggregation was also observed in cell ghosts prepared by the $ZnCl_2$ technique (14).

Using the peroxidase coupling technique to detect concanavalin A fixation on the cell surface, we had previously noted a more heterogeneous distribution of lectin binding sites in some transformed cells (15). However, in freeze-fractured membranes, no significant changes in the original random distribution of IM particles could be detected in either clone of BHK21 cells, transformed or untransformed, upon incubation of cells with various doses of concanavalin A ranging from 1 μg/ml to 100 μg/ml. Trypsination of cells before concanavalin A fixation also did not affect the random distribution of IM particles.

TABLE 1. *Particle distribution on the A and B fracture faces of BHK21 subclone and its transformed derivatives*

Cell designation	Transforming virus	Ref.	A*	B*	A + B	A/B
BHK21/13/8	—	8	552 ± 125	137 ± 67	689	4
C13/8/HS5	HaSV†	8, 17	1673 ± 260	130 ± 50	1803	13
C13/8/5Py2	Polyoma	—	1069 ± 105	168 ± 65	1257	6

* Particles per μm^2 ± SE.
† Hamster sarcoma virus.

TABLE 2. *Particle distribution on the fracture faces of chick embryo cells*

Designation	Transforming or infectious agent	Incubation temperature (°C)	A*	B*	A + B	A/B
CEF	—	37	457 ± 70	56 ± 40	513	8
		41	410 ± 83	48 ± 35	458	8
CEF/Fu19	Fu19†	37	470 ± 68	88 ± 52	558	5
			680 ± 132	173 ± 65	853	4
		41	444 ± 98	88 ± 61	532	5
			606 ± 123	167 ± 69	773	4
CEF§	—	37	754 ± 113	144 ± 64	898	5
CEF§/RAV1	RAV1	37	762 ± 140		935	4
				173 ± 70		
			1046 ± 106		1219	6

* Particles per μm^2 ± SE.

† Temperature-sensitive (ts) mutant (Fu19) of Schmidt–Ruppin RSV (D) defective in transforming capacity at 41°.

§ Derived from an embryo different from that used in Fu19 infection.

Increased density of IM particles in transformed hamster cells

A clear difference, however, in the number of particles per unit area could be detected. Nearly three times more particles could be observed in the clone transformed by hamster sarcoma virus (Figs. 1 and 2 and Table 1) as compared to the control cells. This increase concerns mainly the A face.

This observation was extended to cells of the same origin but transformed by polyoma virus (Table 1).

Similar counts were obtained from mitotic cells isolated by selective detachment from monolayers and from cells brought to the beginning of G1 phase by a 24 hr asparagine-deprivation or to S phase after the addition of asparagine, which permits synchronization of the cell cycle (16).

In addition, some variations in the size of the IM particles (ranging from 70 to 120 Å) were observed, but they could not be related to transformation, since they were present in both control and transformed cells (compare face A Fig. 1 to face B, Fig. 2).

Increased density of IM particles in chick embryo cells infected with avian oncogenic viruses

In order to correlate this phenomenon with transformation, another system was used: secondary chick embryo fibroblasts, either normal or transformed by a temperature-sensitive mutant of Rous sarcoma virus defective for transformation at 41° (Fu19). Approximately 60% of cells in the infected cultures were morphologically transformed at 37°, but only a few cells at 41°.

Results of statistical analysis of particle numbers on both faces A and B of fractured plasma membrane are given in Table 2. In uninfected cells at 37° and 41° there was a unimodal distribution of the density of particles. However, in virus-infected cells there were at least two statistically different classes of density, one of which was comparable to that of control uninfected cells. This class may be attributed to the untransformed cells present in the preparation. The other class comprises cells with membranes having a greater number of particles.

The pattern of the two classes present in both faces A and B did not significantly change when the cells were incubated at 41° for 24 hr (Table 2), a treatment which results in complete morphological reversion to normal.

A similar study was made in chick embryo cells infected with RAV1. Such cells also produced a large number of C-type particles (verified by examination of ultrathin sections in the electron microscope). There was again a significant increase in one class of particle density, as compared to the control uninfected cells (Table 2).

DISCUSSION

In both hamster and chick cells investigated a significant increase of intramembranous particles occurs in response to viral transformation and oncornavirus infection.

There is a minor difference in the two systems in the relative partition of particles between A and B faces (see ratio A/B in Tables 1 and 2). In hamster cells, the increase concerns mainly the face A, whereas in chick cells it is evenly distributed in both faces. Possibly the cleavage plane may not be at the same level in avian and mammalian cells.

In contrast to the results of Scott *et al.* (17), we were unable to find in our hamster cells a variation in the density of IM particles either in early G1 and S phases or in mitotic cells. This difference may be attributed to the different cell systems used by these authors. Similarly we could not observe in confluent BHK21 cells or chick embryo fibroblasts reaggregation of IM particles, such as described by Barnett *et al.* (18) in 3T3 contact-inhibited cells.

Two main interpretations can be given for the observed increase: either the increase in particles is related to virus-induced transformation or it is related only to virus production.

The results obtained in avian cells, if taken separately, would suggest that the phenomenon is related to C-type virus production rather than to transformation. In cells transformed by the temperature-sensitive mutant of RSV, the reversion to the fibroblastic phenotype at nonpermissive temperature is not accompanied by a decrease in the particle density, while the production of virus is not affected. Similarly, RAV-infected cells, which are not transformed, demonstrate an increase in particles density to a similar extent.

However, this hypothesis does not fit with results obtained for hamster cells. In neither of the transformed clones studied could C-type virus production be detected. Moreover, there was no difference in the physiological state of the cells that could account for the increase in particle density as compared to the control BHK21 cells. When the cells were synchronized,

no variation in particle density with the cell cycle was observed.

We, therefore, favor the hypothesis that the increase of IM particles density is related to the oncogenic action of viruses; thus, it may be that the insertion of new proteins in the hydrophobic portion of plasma membrane is the primary change induced by these viruses.

It is possible that numerous changes described in the carbohydrate moiety of glycoproteins and glycolipids of the cell surface (19, 20) are caused by a defect in the association of these components to the hydrophobic portion of IM particles within the membrane, that is, that portion modified by the virus. Similarly, morphologic alterations seen in transformed cells, such as loss of fibroblastic shape, may be due to a disturbed binding of the cytoskeleton components to some of the IM particles.

In order to account for the fact that the reversion of the transformed phenotype at 41° of Fu19-infected cells is not accompanied by a decrease in density of IM particles, we may consider the hypothesis that the particles newly incorporated in the plasma membrane contain some virus-coded polypeptides which, despite their thermosensitivity, remain there, but no longer affect other membrane components at high temperature.

A similar supposition may also explain the results obtained with RAV-infected cells. RAV-induced particles may differ from RSV-induced particles in such a way that they are not fully operational in fibroblastic cells, although they can cause transformation of lymphoid cells.

The observation described here may be a general phenomenon in virus-transformed cells. We have recently noted a similar increase of IM particles in plasma membrane of mouse K-Balb/c cells, a line of 3T3 cells transformed by the Kirsten strain of mouse sarcoma virus (21), as compared to untransformed 3T3 cells.

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