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CHARACTERIZATION OF A TEMPERATURE-SENSITIVE MEMBRANE ALTERATION IN CHICK EMBRYO FIBROBLASTS INFECTED WITH A TEMPERATURE-SENSITIVE MUTANT OF ROUS SARCOMA VIRUS

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Summary

The intramembrane particles of freeze-fractured chick embryo fibroblasts transformed with a temperature-sensitive mutant of Rous sarcoma virus (TS68) are distributed differently at the permissive and non-permissive temperatures if, and only if, the cells are treated with glycerol before fixation. Few aggregates of intramembrane particles are present in glycerol-treated cells grown at the permissive temperature for transformation (36°C), while numerous large aggregates of particles are present at the non-permissive temperature (41°C). Changes in the distribution of particles after cells are shifted from 36 to 41°C are observed after 20 min, while a temperature shift from 41 to 36°C causes changes in glycerol-induced redistributions after 1 h. The changes observed in temperature shifts from 36 to 41°C and from 41 to 36°C do not require protein synthesis or RNA synthesis.

Introduction

Using the techniques of freeze-fracture and electron microscopy, we have previously described a difference between normal and Rous sarcoma virus (RSV)-transformed chick embryo fibroblast plasma membranes with respect to the sensitivity of their intramembrane particles to glycerol-induced aggregation [1]. When normal chick embryo fibroblasts (CEF) are exposed to high concentrations of glycerol, the distribution of intramembrane particles is altered from an evenly dispersed pattern, seen in the absence of glycerol, to one in which aggregates of ten to several hundred particles are surrounded by particle-free regions. The intramembrane particles of plasma membranes

from RSV-transformed CEF (RSV-CEF) are less sensitive to glycerol-induced aggregation and are randomly dispersed both in the presence and in the absence of glycerol. In CEF infected with a mutant which is temperature-sensitive for transformation (TS68), the intramembrane particles are insensitive to glycerol-induced aggregation when the cells are grown at 36°C, the permissive temperature for transformation, but are sensitive to glycerol-induced aggregation when the cells are grown at 41°C, the non-permissive temperature.

The intramembrane particles of the plasmalemma visualized by the freeze-fracture technique are integral proteins, some of which may span the plasma membrane [2,3]. Several studies [4—6] have indicated that, in normal cells, mechanisms exist for the interaction of proteins on the internal and external faces of the plasma membrane. Since certain intramembrane particles span the plasma membrane, they may be involved in such transmembrane phenomena. Transformation may alter some of these interactions. Therefore, the different responses of normal and transformed cell plasma membranes to glycerol may be related to differences in the transfer of information across the plasma membrane.

In an attempt to understand the mechanism of the glycerol-induced particle aggregation and the loss of this response in transformed cells, we have studied the kinetics of the acquisition and of the loss of sensitivity to glycerol-induced aggregation of intramembrane particles after temperature shifts of CEF infected with TS68. We have also examined some of the macromolecular requirements for these changes in membrane sensitivity.

Materials and Methods

Eagle's Medium was obtained from GIBCO, Grand Island, NY; fetal bovine serum from Reheis, Chicago, IL; colchicine, cycloheximide and puromycin from Sigma Chemical Co., St. Louis, MO; actinomycin D from Boehringer-Mannheim, Indianapolis, IN; anisomycin from Dr. David Luck, Rockefeller University, New York, NY.

Cell culture. Uninfected chick embryo fibroblasts and chick embryo fibroblasts infected with TS68 [7] (CEF-TS68) were grown on 150-mm plastic petri dishes (Falcon) as described previously [1,8]. In temperature-shift experiments, the cell cultures were changed to media prewarmed to the proper temperature. In those experiments in which cells were exposed to metabolic inhibitors, the cells were preincubated with the drug to be tested for 30 min before the temperature shift. The drugs were dissolved in Eagle's minimum essential medium containing 10% fetal bovine serum. All cells were grown in an atmosphere of CO_2/air (10%/90%).

Preparation of cells for freeze-fracture. Cells to be observed were either fixed directly with 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, or first treated with a graded series of glycerol solutions: 5% glycerol for 5 min, 10% glycerol for 10 min, and 20% glycerol for 20 min. The glycerol was diluted in Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline, as described earlier [1]. In those experiments where cells were incubated with various antimetabolites before exposure to glycerol, the glycerol solutions contained the drug to be tested at the concentration used in the growth medium.

Freeze-fracture electron microscopy. Samples for freeze fracture and electron microscopy were prepared exactly as described by Gilula et al. [1]. The quantitation of particle aggregation has also been described previously [1]. Briefly, 50—100 inner plasma membrane fracture faces were examined per sample, and the distribution of particles in each face was classified as: (i) dispersed—no detectable particle aggregation; (ii) intermediate—aggregates of 10 or fewer particles; (iii) aggregated—distinct aggregates of 10 or more particles. In all analyses, only the cytoplasmic or P face of plasma membranes was examined. The extracellular or E faces were not analyzed.

Results

Kinetics of change in sensitivity to glycerol-induced aggregation

When CEF infected with TS68 (Fig. 1a) are grown at 41°C, the intramembrane particles in the fracture faces of the plasma membranes are evenly distributed. At 41°C, the plasma membranes of TS68-CEF cells appear identical to the plasma membranes of CEF after freeze-fracture. Therefore, we have illustrated only the freeze-fracture replicas from TS68-CEF cells [1]. When the same cells grown at this temperature are treated with increasing concentration of glycerol before freeze-fracture, a striking modification occurs in the arrangement of the intramembrane particles (Fig. 1b) [1]. The particles are now found in large aggregates separated by particle-free regions.

When CEF or TS68-CEF are grown at 36°C and freeze-fractured without prior glycerol treatment, intramembrane particles are randomly distributed (Fig. 1c and d), as in plasma membranes of the same cells grown at 41°C. If CEF grown at 36°C are exposed to glycerol before freeze-fracture, the intramembrane particles form aggregates similar to those observed at 41°C after exposure to glycerol (cf. Fig. 1b and e). When TS68-CEF are grown at 36°C, exposed to glycerol, and freeze-fractured, the intramembrane particles do not aggregate and are found randomly dispersed in the plane of the membrane (Fig. 1f). This relative insensitivity to glycerol is similar to that seen when the plasma membranes of RSV-CEF are examined [1]. Thus, the sensitivity to glycerol-induced aggregation of intramembrane particles in TS68-CEF at 41°C resembles that seen for uninfected cells, while the relative insensitivity to glycerol-induced aggregation of intramembrane particles in the same cells grown at 36°C is like that found in RSV-CEF.

Following a temperature shift from 41 to 36°C, the shift of intramembrane particles from the glycerol-sensitive to the glycerol-insensitive state is extremely rapid in TS68-CEF (Table IA). There is a noticeable increase in the proportion of fracture faces with evenly distributed intramembrane particles within 1 h after a temperature shift from 41 to 36°C (Table IA). 3 h after a temperature shift, the loss of sensitivity to glycerol-induced aggregation is complete, and the proportion of fracture faces with evenly distributed intramembrane particles has reached 65%. No further increase in the proportion of fracture faces with evenly distributed particles is observed over the next 9 h.

The acquisition of the glycerol-sensitive state after TS68-CEF are shifted from 36 to 41°C is also rapid. A small change in the proportion of fracture faces with evenly dispersed and aggregated particles is evident within 20 min,

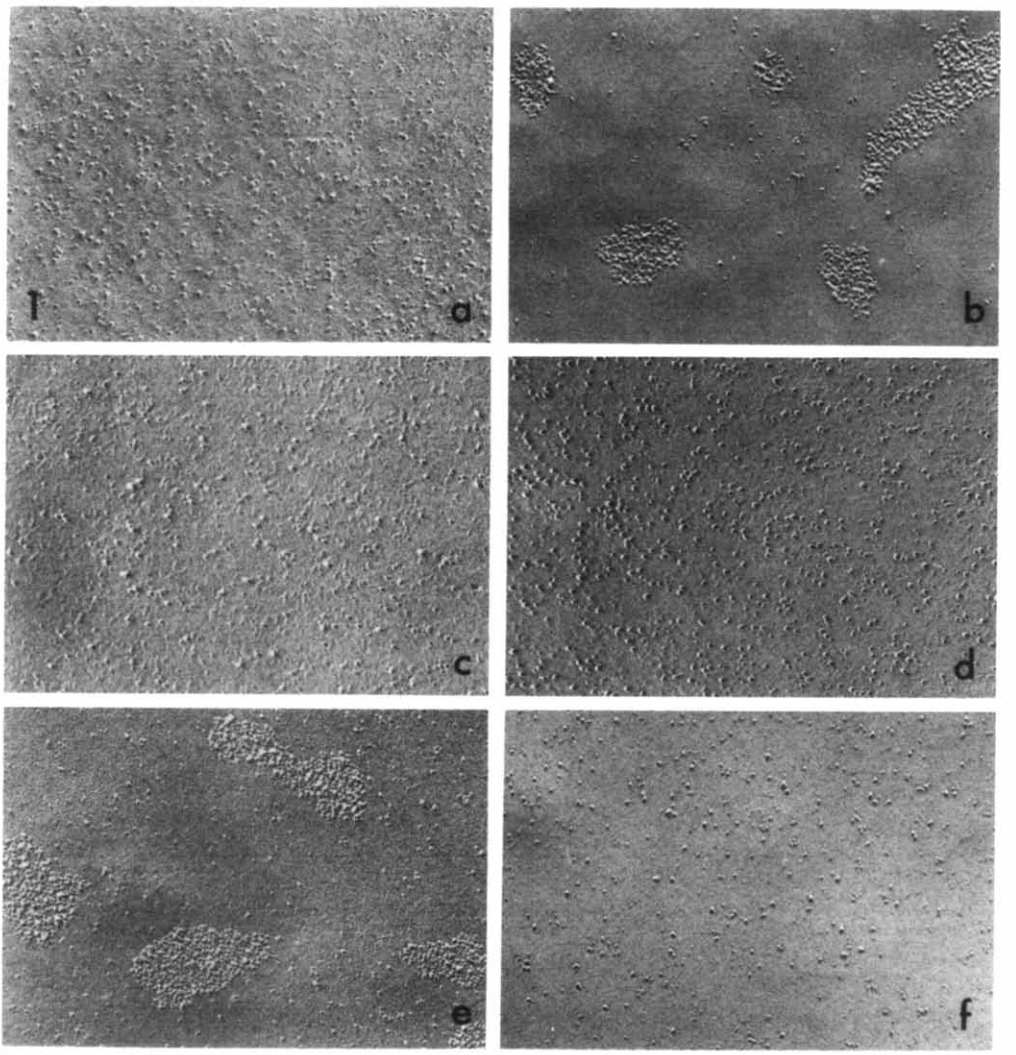

Fig. 1. Distribution of intramembrane particles in CEF and TS68-CEF plasma membranes. (a) TS68-CEF at 41°C; (b) TS68-CEF at 41°C treated with glycerol (c) CEF at 36°C; (d) TS68-CEF at 36°C; (e) CEF at 36°C treated with glycerol; (f) TS68-CEF at 36°C treated with glycerol. Cells were prepared for freeze-fracture and electron microscopy as described previously [1].

and by 1 h after the temperature shift, there is an obvious alteration in the membranes (Table IB). During the next 11 h at 41°C, the proportion of cells containing fracture faces with aggregated particles increases slightly. However, as in the shift from 41 to 36°C, the most significant change in the arrangement of intramembrane particles occurs within the first 3 h after the temperature shift.

Effects of metabolic inhibitors on changes in membrane sensitivity to glycerol

In an effort to characterize further the nature of the membrane alterations which occur after temperature shifts in TS68-CEF, we examined the effects

TABLE I

TIME COURSE OF THE CHANGE IN SENSITIVITY TO THE GLYCEROL-INDUCED REDISTRIBUTION OF INTRAMEMBRANE PARTICLES IN TS68-CEF AFTER TEMPERATURE SHIFT

Cells previously infected with TSNY68 were cultured at either 36 or 41°C for 3 days. At the start of the experiment the cells were shifted to the indicated temperature; (A) cells grown at 41°C then shifted to 36°C, (B) cells grown at 36°C then shifted to 41°C. The cells were fractured as described earlier [1].

Treatment	Arrangement of intramembrane particles (%)		
	Even	Intermediate	Aggregated
(A)			
Time			
0 (control)	16	10	74
1 h	34	9	57
3 h	65	5	30
12 h	58	7	35
(B)			
Time			
0 (control)	58	10	32
20 min	45	7	48
1 h	32	11	57
3 h	30	6	66
12 h	22	8	70

of several metabolic inhibitors on the changes in glycerol-induced membrane sensitivity. When cultures of TS68-CEF are shifted from 36 to 41°C in the presence of inhibitors of DNA-dependent RNA synthesis or protein synthesis, there is no inhibition of the acquisition of sensitivity to glycerol (Table II). It should be noted that one inhibitor of protein synthesis, cycloheximide, does appear to prevent the change from the insensitive to the sensitive state (Table II). However, control experiments demonstrated that this is probably unrelated to the drug's effect on protein synthesis, since two other inhibitors

TABLE II

EFFECTS OF METABOLIC INHIBITORS ON GLYCEROL-INDUCED REDISTRIBUTION OF INTRAMEMBRANE PARTICLES IN TS68-CEF SHIFTED FROM 36 TO 41°C

Cells previously infected with TSNY68 were cultured at 36°C for 3 days. Cells were incubated at 36°C for a further 30 min in the presence or absence of drugs and then shifted to 41°C. At the time of the shift, the medium was removed and replaced with fresh control or drug-containing medium prewarmed to 41°C. The cells were left at 41°C for 1—1.5 h, glycerated, and fixed. The glycerol solutions also contained the drug being tested. Freeze-fracture replicas were prepared as described previously [1].

Treatment	Arrangement of intramembrane particles (%)		
	Even	Intermediate	Aggregated
None (36°C)	56	14	30
Shift 36 to 41°C	19	8	73
plus actinomycin D (1 μg/ml)	31	12	57
plus cycloheximide (1 μg/ml)	58	6	36
plus puromycin (10 μg/ml)	23	17	60
plus anisomycin (10 μg/ml)	19	15	66
plus colchicine ($1 \cdot 10^{-5}$ M)	20	13	67

TABLE III

EFFECTS OF METABOLIC INHIBITORS ON GLYCEROL-INDUCED REDISTRIBUTION OF INTRAMEMBRANE PARTICLES IN TS68-CEF SHIFTED FROM 41 TO 36°C

Cells infected with TSNY68 were cultured at 41°C for at least 3 days. The cells were exposed to drugs, shifted, glycerated, and fixed as described in the legend to Table I.

Treatment	Arrangement of intramembrane particles (%)		
	Even	Intermediate	Aggregated
Control (41°C)	40	10	50
Shift 41 to 36°C	64	10	25
plus actinomycin D (1 μg/ml)	63	14	23
plus cycloheximide (10 μg/ml)	68	12	20
plus puromycin (10 μg/ml)	71	14	15
plus anisomycin (10 μg/ml)	64	7	29
plus colchicine ($1 \cdot 10^{-5}$ M)	56	20	24

of protein synthesis, puromycin and anisomycin, do not prevent the shift to the glycerol-sensitive state.

Conversely, when cells grown at 41°C are shifted to 36°C, the plasma membranes become refractory to glycerol-induced aggregation of the intramembrane particles despite the presence of these drugs, indicating that in this direction, too, the shift is independent of both protein and RNA synthesis. Colchicine, a drug which disrupts microtubules, also does not prevent the change from one state to another after temperature shifts (Tables II and III). Finally, inhibitors of transport and/or oxidative phosphorylation are ineffective in suppressing the membrane changes (data not shown).

Discussion

The observations which we report in this paper illustrate a temperature-sensitive plasma membrane alteration in cells infected with a mutant of Rous sarcoma virus which is temperature-sensitive for transformation. This alteration, namely a change in the glycerol-sensitivity of intramembrane particles, takes place rapidly when cells infected with TS68 are shifted either from the permissive to the restrictive temperature or vice versa. The speed with which the change from the glycerol-insensitive to the glycerol-sensitive state, or vice versa, occurs is equivalent to that observed with several other transformation-associated alterations known to take place rapidly, such as changes in membrane ruffling [9], plasminogen activator levels [10], sugar transport [11] and cytoskeletal organization [12].

The lack of a requirement for either RNA or protein synthesis to change the state of responsiveness of the membranes in cells shifted from one temperature to another seemed surprising at first, since several of the phenotypic changes observed when cells infected with temperature-sensitive transforming viruses are shifted from 41 to 36°C do require RNA and protein synthesis [7,10]. It had been assumed that this requirement for RNA and protein synthesis indicated the necessity for the production at the permissive temperature

of new copies of the protein, src *, coded for by the transforming gene of the virus [13]. Recent experiments, however, indicate that the temperature-sensitive src protein produced by viral mutants such as TS68, can renature after a shift from 41 to 36° C (Ziemiecki, A. and Friis, R., personal communication; Refs. 14—16), allowing certain phenotypic changes such as cytoskeletal and morphological modifications to occur in cells treated with inhibitors of RNA and protein synthesis. This is consistent with our observation that the loss of sensitivity to glycerol-induced intramembrane particle aggregation in TS68-CEF shifted from 41 to 36° C is independent of RNA or protein synthesis.

Conversely, in a shift from 36 to 41° C, new protein and RNA synthesis may not be required because, in the absence of the synthesis of new, active src, the phenotype may shift back to normal simply as a result of the rapid decay or denaturation of pre-existing src protein.

It would be appealing, of course, to attempt to relate these changes in sensivity in glycerol-induced aggregation to those changes in cell phenotype involving processes such as membrane ruffling [9], cytoskeletal organization [12], and transmembrane interactions [17,18]. However, at the present time, we have no information which allows us to do this with any assurance.

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* src, the protein coded for by the gene responsible for transformation in Rous sarcoma virus.