Cyclic Membrane Changes in Animal Cells: Transformed Cells Permanently Display a Surface Architecture Detected in Normal Cells Only during Mitosis

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ABSTRACT A membrane site exposed only during mitosis in normal 3T3 cells, but exposed in all polyoma-virus transformed 3T3 (Py3T3) cells, has been detected. The site was identified by the specific binding of a wheat-germ agglutinin conjugated with fluorescein isothiocyanate.

The receptor site has been studied previously. It binds wheat-germ agglutinin, causing the selective agglutination of Py3T3 cells, but not normal 3T3 cells. A short treatment with low concentrations of trypsin exposes cryptic sites in 3T3 cell membranes; this permits agglutination of these cells. The experiments reported here indicate that these sites normally are exposed during a brief period of the cell cycle for 3T3 cells. Specific hapten inhibitors of agglutination also inhibit the binding of the conjugated agglutinin and prevent fluorescence.

Transformed mammalian cells agglutinate in the presence of certain glycoproteins of plant origin, whereas normal cells do not agglutinate (1, 2). When the normal cells are treated briefly with low concentrations of trypsin, cryptic membrane sites are uncovered and the cells then agglutinate (3).

Agglutination only occurs when most of the cells in a population have exposed binding sites. Therefore, a population having only a few cells with exposed binding sites will not agglutinate. Accordingly, we wished to know if the sites on normal cells were exposed during a small part of the regular cell cycle. Individual cells with exposed binding sites fluoresce when exposed to fluorescein-tagged agglutinin. Using fluorescence binding, we find that agglutinin-binding sites are exposed during mitosis in normal 3T3 cells.

PROCEDURES
Synchronization of 3T3 cells
Synchronized 3T3 cells were prepared from confluent 3-day-old monolayers. The cells were removed from flasks with trypsin and plated at lower densities on cover slips. The maximum percentage of mitotic cells was roughly as expected from reported data (4). The same fluorescence results were obtained when cells were synchronized by the addition of 50% calf serum to confluent monolayers or by the use of a thymi-dine block. Therefore, the fluorescence results obtained with mitotic cells appeared to be independent of the method used to obtain synchrony.

Exposure of cells to fluorescent agglutinin
Fluorescein-tagged wheat-germ agglutinin (FITC-agglutinin) was prepared by reacting the agglutinin (2) with fluorescein isothiocyanate on celite. Details of the procedure will be described elsewhere (Burger, et al., in preparation).

Cells were prepared in three ways as required for different types of experiments. Inhibition of FITC-agglutinin binding by specific haptons was observed with native, unfixed cells. The cells were immersed for 2–10 min at 37°C in FITC-agglutinin that had been incubated with the hapten.

Fixation of cells with ethanol was required for parallel observations of fluorescent and mitotic indices. The cells were fixed after exposure to the FITC-agglutinin. Cells were then counterstained with Evans blue. As with native cells, this procedure gave highly specific membrane fluorescence, with very little nonspecific uptake into cytoplasm. The fluorescent index was determined with UV illumination; the mitotic index was determined with bright-field phase microscopy.

For visualization of mitotic figures in photographs of fluorescence, some nonspecific, cytoplasmic fluorescence was required to illuminate counterstained nuclei. This was accomplished by fixing the cells with ethanol before exposure to FITC-agglutinin, and then counterstaining the nuclei with Evans blue. Under these conditions the fluorescence remained mostly specific, as indicated by the disappearance of dye in the presence of hapten inhibitors. The duration of exposure to FITC-agglutinin was kept short to avoid too much nonspecific fluorescence.

Fluorescence of Py3T3 and mitotic 3T3 cells
FITC-agglutinin specifically labeled all Py3T3 cells (3T3 mouse fibroblasts transformed by polyoma virus) and normal 3T3 cells in mitosis. Interphase 3T3 cells did not fluoresce (Fig. 1).

When 3T3 cells were synchronized, the mitotic and fluorescent indices correlated extremely well (Fig. 2). This quantitative correlation agreed with our qualitative observations of synchronous and nonsynchronous cultures. A slight shift to a later phase was found, however, because prophase cells did
FIG. 1. Fields with a mitotic and interphase cells. These cells were fixed with ethanol, exposed to FITC-agglutinin, and counterstained with Evans blue (Fisher Scientific Co.). The cells were mounted in Elvanol (3), a gift from Dr. R. E. Pollack.
246  Microbiology: Fox et al.

TABLE 1. Specificity of FITC-agglutinin binding

<table>
<thead>
<tr>
<th>FITC-agglutinin</th>
<th>Concentrations (M)</th>
<th>Inhibition of agglutination</th>
<th>Mitotic cells fluorescing (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>+ None</td>
<td>...</td>
<td>...</td>
<td>100</td>
</tr>
<tr>
<td>+ N-Acetylglucosamine</td>
<td>$5 \times 10^{-3}$</td>
<td>Yes</td>
<td>0-20</td>
</tr>
<tr>
<td>+ Di-N-Acetyllchito-bose</td>
<td>$1 \times 10^{-3}$</td>
<td>Yes</td>
<td>0-20</td>
</tr>
<tr>
<td>+ Glucose</td>
<td>$2 \times 10^{-3}$</td>
<td>No</td>
<td>100</td>
</tr>
<tr>
<td>+ N-Acetylgalactosamine</td>
<td>$2 \times 10^{-3}$</td>
<td>No</td>
<td>80-100</td>
</tr>
<tr>
<td>- Bovine serum albumin, FITC-conjugated</td>
<td>400 g/ml</td>
<td>...</td>
<td>0</td>
</tr>
</tbody>
</table>

not fluoresce, and a similar number of late telophase and early G-1 cells did fluoresce.

Binding studies with tritiated agglutinin corroborate the results with fluorescence. Bound radioactivity was used to assay binding of agglutinin. Growing, nonsynchronous, normal 3T3 fibroblasts contained about 5% of the number of receptor sites per unit area as Py3T3. Since mitosis takes about 1 hr. out of the total 20-hr cell cycle, agglutinin uptake by mitotic cells in the random culture could fully account for the observed isotope uptake. Lower adsorption of labeled agglutinin by 3T3 cells, as compared to SV40 virus-transformed 3T3 cells, has been reported already for another agglutinin labeled with $^{25}N$i(6).

Specificity of FITC-agglutinin binding

The binding of fluorescent wheat-germ agglutinin in these studies was specific for the membrane sites involved in agglutination. The same hapten inhibitors that prevent agglutination were shown to reduce or prevent fluorescence. Table 1 summarizes the effects of inhibitors on fluorescence. Also shown is the failure of fluorescent bovine-serum albumin to bind specifically to mitotic cells.

We do not believe that the fluorescence of mitotic cells resulted simply from physical concentration of membranes, and high densities of receptor sites, resulting from rounding up and shrinking. Interphase 3T3 cells that became round after detachment by EDTA treatment did not bind FITC-agglutinin. Furthermore, some of the distinctly fluorescent cells in early G-1 phase had returned to an extended form and were of similar sizes as were late G-1 and S cells.

DISCUSSION

This observation of a membrane alteration occurring during a short period of the cell cycle raises new questions about the relationships of cell surface events and nuclear events. Our working hypothesis is that this surface change is a trigger for the processes in the cell cycle leading to the next mitosis. By affecting activities such as RNA or protein synthesis, transport, or (perhaps) concomitant nuclear-membrane changes, the alteration we have observed might be responsible for essential steps in the subsequent S, G-2, or M phases of the cell cycle. We have not, however, eliminated the possibility that this surface change is only one of many cellular responses to mitosis.

Events during DNA synthesis (S) in the preceding cycle might be required for the expression during mitosis of the agglutinin-binding sites. After infection of 3T3 cells with an oncovirus, we have found that permanent exposure of these surface sites required either DNA synthesis (in S phase) or completion of chromosomal replication. Perhaps a similar requirement exists for normal expression of the sites at mitosis.

We propose the existence of positive-feedback loops that relate cyclic functions of the cellular membrane and the nucleus (Fig. 3). Alternating molecular communications between the cell membrane and the cell nucleus might allow a controlled periodic cell cycle. This model depicts the importance of communication between the membrane and the nucleus, and it allows for alterations of the information transmitted from one to the other, as under physiological conditions that alter growth or division. Fig. 3 also suggests several specific experiments that can test the significance of the membrane change. These include identification of the steps in the cell cycle which specifically lead to site exposure, and determination of effects in the subsequent cell cycle after artificial blocking of the binding sites at mitosis.

Using fluorescence microscopy, we have shown that normal cells have specific exposed membrane sites during mitosis which are detected by the same method at all times in transformed cells. This is a new molecular marker of the progression of cells through the cell cycle. These studies extend our investigation into the significance of the cell surface for growth regulation (7).

Fig. 2. Mitotic and fluorescent indices of synchronized 3T3 cells. Method of synchronization in text. Cells were exposed to FITC-agglutinin, fixed with ethanol, stained with Evans blue, and mounted in Elvanol. In control experiments, cells were exposed to FITC-agglutinin and counted without fixing and staining; the fluorescent index was identical to the data reported here. Blind counts of several hundred cells were made by two investigators and were in good agreement.

Fig. 3. Model depicting cyclic communications between the cell membrane and the cell nucleus.
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**Correction.** In the article entitled “Possible Role of Pyrophosphate Linkage in the Active Transport of Sodium Ions” by Jui H. Wang, published in Proc. Nat. Acad. Sci. USA, 67, 59–61 (1970): The word “phosphatidylinositol” in line 4 of page 60 should be replaced by “phosphatidylinositol phosphates.”

**Correction.** In the article “The Time of Appearance of Cold Water Off Somalia,” by Henry Stommel and Roberto Fassetto, which appeared in the July 1968 issue of Proc. Nat. Acad. Sci. USA, 60, 750–751, the authors had thought that the air temperature data at Dante, Ras Hafun, were unpublished. However, the data have appeared in a comprehensive work entitled “Contributo alla Climatologia della Somalia—riassunto dei risultati e tabelle meteorologiche e pluviometriche” (no date of publication), published by the Ministero degli Affari Esteri, Cooperazione Scientifica e Tecnica, a Cura di Amilcare Fantoli. In addition, the authors found that they had transcribed the monthly averages of daily minimum temperature for 1939 incorrectly. The values given in the above reference are (°C): January, 22.8; February, 23.4; March, 23.9; April, 24.1; May, 25.7; June, 23.1; July, 20.2; August, 22.0; and September, 22.2. The authors are indebted to Mr. Byron Kolitz for calling their attention to these facts.

**Correction.** In the paper entitled “Ion Transport Underlying Metabolically Controlled Volume Changes of Isolated Mitochondria,” by S. Izzard and H. Tedeschi, which appeared in the October 1970 issue of Proc. Nat. Acad. Sci. USA, 67, 702–709, the following changes should be made: On p. 707, 4–2 lines from bottom, the sentence “Cation efflux is expressed as the molar ratio of cations lost during shrinkage to cations accumulated during swelling” should be replaced by “Cation efflux is expressed as the ratio of moles of cations lost during shrinkage to the moles of cations accumulated during swelling.” Throughout “absorbance” should be replaced by “optical density.”