CELL SURFACE CHANGES AND ROUS SARCOMA VIRUS GENE EXPRESSION IN SYNCHRONIZED CELLS

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ABSTRACT

We have investigated whether cell surface changes associated with growth control and malignant transformation are linked to the cell cycle. Chicken embryo cells synchronized by double thymidine block were examined for cell-cycle-dependent alterations in membrane function (measured by transport of 2-deoxyglucose, uridine, thymidine, and mannitol), in cell surface morphology (examined by scanning electron microscopy), and in the ability of tumor virus gene expression to induce a transformation-specific change in membrane function. We reach the following conclusions: (a) The high rate of 2-deoxyglucose transport seen in transformed cells and the low rates of 2-deoxyglucose and uridine transport characteristic of density-inhibited cells do not occur in normal growing cells as they traverse the cell cycle. (b) Although there are cell cycle-dependent changes in surface morphology, they are not reflected in corresponding changes in membrane function. (c) Tumor virus gene expression can alter cell membrane function at any stage in the cell cycle and without progression through the cell cycle.

It is widely suspected that the cell surface plays a critical role in the regulation of cell growth and metabolism, and the frequency of reports which link cell surface changes to growth control and malignant transformation support this suspicion (1–3). Changes in membrane transport are among the cell surface alterations which seem to be involved in the control of growth. For example, an increased rate of hexose transport is one of the earliest surface changes accompanying malignant transformation, and a decreased transport rate is closely associated with the onset of density-dependent inhibition of growth: exponentially growing cells display three to five times the rate of hexose transport of density-inhibited cells, while transformed cells exhibit a rate which is another three to five times that of exponentially growing cells (4–6). Rates of transport of uridine and thymidine are reduced in cells which are density-inhibited (7–9); however, no difference was noted between exponentially growing and transformed cells (5). These data indicate interrelationships between the transport of small molecules and the growth state of the cell.

Since density-inhibited cells are arrested in the G1 phase of the cell cycle, and since transformed cells and mitotic cells display similarities in binding of lectins to the cell surface (2, 10), one can ask whether cell surface alterations associated with growth control and malignant transformation are intrinsic to the physiological state of the cell or whether they are cell cycle dependent. For example, is the low rate of hexose, uridine, and thymidine uptake seen in density-inhibited cells a result of

of the cells being in the G_1 phase, or is it an intrinsic property of the density-inhibited state? Is the high rate of hexose transport seen in transformed cells also seen in normal cells when they enter mitosis? We have approached these questions by measuring the rates of transport of thymidine, uridine, 2-deoxyglucose (2-DG), and mannitol as functions of the cell cycle. To determine whether these alterations in cell surface function are correlated with changes in cell surface morphology, we have also examined transformed cells and synchronized normal cells by scanning electron microscopy. We found that, although there are morphological similarities between late G_1 and density-inhibited cell surfaces and between mitotic and transformed cell surfaces, the membranes of each of these cell types were functionally distinct, as measured by metabolite transport.

After infection with polyoma virus, cellular DNA synthesis is required for the appearance of enhanced agglutinability by plant lectins (11). On the basis of this result, Burger has suggested a model that requires passage through mitosis in order for cells to undergo permanent surface alterations (2). This model was further supported by the finding that both transformed and mitotic cells have reduced internal pools of cyclic AMP and both apparently bind more lectin on their surfaces (10). We have investigated whether induction of a transformation-specific alteration in cell surface function also is cell cycle dependent. Synchronized cells infected with a temperature-sensitive mutant of Rous sarcoma virus and held at the restrictive temperature were shifted to the permissive temperature at various times during the cell cycle. Expression of the transformed phenotype was monitored by assaying for the transformation-specific increase in 2-DG transport rate. Induction of this manifestation of the transformed phenotype was found not to be cell cycle dependent.

MATERIALS AND METHODS

Cell Culture and Transport

Cell culture was as described previously (6), with the exception that tertiary cell cultures were used and exponentially growing cells or cells used for synchronization were plated at 8 x 10^5 cells/35-mm dish. Synchronization was achieved by double thymidine block (12, 13) using 10 mM thymidine. Rates of transport were determined essentially as described (6). Cultures were incubated with the radioactively labeled metabolites for 15 min. [H_2]2-DG was used at 0.5 μg/ml, and uridine and mannitol were used at 1.0 μCi/ml and [H]thymidine at 5 μCi/liter. After incubation at 37°C, cultures were rinsed three times with ice-cold phosphate-buffered saline (6), tilted drained, and the last drop was aspirated off. After 2-DG and mannitol transport, cells were dissolved in 0.5 ml 1 N NaOH. A 0.25-ml aliquot was counted in 10 ml of scintillation fluid containing 2 liters toluene, 1 liter Triton X-100, 300 mg glacial acetic acid, and 12 g Omnifluor (New England Nuclear). Another aliquot was taken for Lowry assays of protein. To measure uridine and thymidine uptake, acid-soluble pools were extracted with cold 5% trichloroacetic acid (TCA) and counted in Triton-toluene scintillation fluid without acetic acid. The cells were rinsed, dissolved in 1 N NaOH, and an aliquot was taken for protein determination. Uptake was linear for the time period used, so all uptake data represent initial rates.

Scanning Electron Microscopy

All scanning electron microscopy was performed on cell cultures fixed in 2% glutaraldehyde in Ca++-Mg++-free, phosphate-buffered saline at room temperature for 6-12 h. Samples were dehydrated through graded ethanol in 0.16 M saline (30, 50, 75, 85, 95, and 100% ethanol) and graded amyl acetate in absolute ethanol. Specimens were dried by the critical point technique and coated with gold-palladium (100-200 Å) in a vacuum evaporator at 5 x 10^-8 torr. Scanning electron microscopy was performed on the JEOL JSM-U3 microscope at 15 kV and a 20° angle from the electron probe.

Assessment of morphological changes was facilitated by viewing 200-300 cells per preparation on a television monitor.

Materials

Thymidine was obtained from Sigma Chemical Co., St. Louis, Mo. and was classified as Sigma Grade. Isotope was obtained from New England Nuclear. Thymidine was obtained from New England Nuclear, Boston, Mass., and Amersham/Searle Corp., Chicago. III. Rous sarcoma virus and Rous sarcoma virus T5 were obtained from Dr. G. S. Martin, Imperial Cancer Research Fund, London, England.

RESULTS

Transport Rate as a Function of the Cell Cycle

To determine whether transport changes associated with growth control and malignant transformation occur normally in cells as they progress through the cell cycle, we have synchronized chicken embryo fibroblasts (CEF) by double thymidine block and, at various times after release from the block, monitored the rate of uptake of
2-DG, uridine, mannitol, and thymidine (Fig. 1). The 2-DG uptake rate seems to drop by approximately 50% during mitosis but is constant during the rest of the cell cycle (Fig. 1a). Neither uridine nor mannitol (Fig. 1b, c) uptake showed significant variation with the cell cycle. Thymidine was taken up extensively only during the S phase (Fig. 1d). DNA content per cell and percent mitosis are given in the bottom panel (Fig. 1e), as indicators of cell synchrony. These data demonstrate that the high rate of 2-DG uptake which is seen in transformed cells (5, 6), and the low rate of 2-DG and uridine uptake seen in density-inhibited cells are not expressions of cell cycle events, but are intrinsic properties of those cell states. However, the low rate of thymidine uptake seen in density-inhibited cells (7, 9) may be considered a cell cycle phenomenon.

One apparent artifact in these studies was the high uptake rate exhibited at time points immediately after release from the thymidine block. This high rate was not noted at equivalent time points during the second S phase, nor was it seen when density-inhibited cells were stimulated to progress through the cell cycle by serum stimulation (data not shown). Thus, this cannot be a genuine cell cycle event. We do not know why release from thymidine block stimulates metabolite uptake.

Cell Surface Morphology

To determine whether variations in cell surface function are associated with alterations in cell surface morphology, we have examined the surface morphology of synchronized and transformed cells by the use of scanning electron microscopy (SEM). Cells were synchronized by double thymidine block and, at 1-h intervals after release from the block, were fixed and examined under the scanning electron microscope as described in Materials and Methods. Morphologies characteristic of each phase of the cell cycle were selected and enumerated.

S phase cells have a smooth surface with no microvilli or blebs. These cells are completely flattened out and appear to be fully extended (Fig. 2a). Cells representing the G2 phase (Fig. 2b, c) have extensive microvilli on their surfaces and are less extended. Two examples of the G2 phase cell surface are shown in order to illustrate that variations do occur within the G2 phase. These variations could represent different stages of the G2 phase or simply cell population heterogeneity. Mitotic cells (Fig. 2e, f) display a rounded morphology with extensive coverage by microvilli, but with no substantial bleb formation. Again, two representatives are shown in order to illustrate variations within the mitotic phase.

Cells were brought to G1 either by synchronization with the double thymidine technique (Fig. 2g) or by allowing cells to become density-inhibited (Fig. 2h). The surface morphology of cells brought to G1 after thymidine synchronization (Fig. 2g) is extended relative to mitotic cells and is characterized by a reduced number of microvilli. The surface, however, is not smooth but is covered by large blebs. In contrast, density-inhibited cells have smooth surfaces with no blebs or microvilli (Fig. 2h). Porter et al. (14) explained the differences between the two types of G1 phase cells as reflecting progression through the G1 phase of the cell cycle. This interpretation seems reasonable since it is consistent with the overall morphological progression from mitotic cells, which are rounded and have extensive microvilli, to S phase cells, which are flattened and have no microvilli and few if any blebs. Therefore, we designate the G1 cells with extensive coverage by blebs as early G1 cells and the smooth surfaced density-inhibited cells as late G1 cells. In order to gain some experimental support for this interpretation, we have taken density-inhibited cells and stimulated them to divide by the addition of 10% calf serum or 12.5 \( \mu \text{g/ml} \) of trypsin and monitored the increase in hexose uptake, movement of the cells into S phase by thymidine uptake, and surface morphologies by SEM. We have found that cells stimulated in this way to progress through the cell cycle did not display an "early G1" morphology at any time after stimulation. This is consistent with the notion that the "blebbled" morphology of early G1 cells precedes the smooth morphology of density-inhibited cells in the normal cell cycle progression.

Assignment of these morphological types to particular parts of the cell cycle was confirmed by visually determining the percentage of cells of each morphology present at each stage of the cell cycle (Fig. 3). Between 200 and 300 cells in each preparation were counted for these determinations. Variations in the maximum percentage for each morphological type probably reflect differences in the time span each morphology is displayed.

We have also examined the surface morphology of cells transformed by Rous sarcoma virus (Fig. 2...
FIGURE 1  Rates of uptake of small metabolites as a function of the cell cycle. O-O, Transport; Δ--Δ, cell number. Approximate positions in the cell cycle are given at the top of the figure. The point of 50% cell division is set at 0 h. (a) 2-DG; (b) uridine; (c) mannitol; (d) thymidine; (e) DNA content (measured by the method of Burton [17]) and percent mitosis (determined by phase microscope observation).
FIGURE 2 Scanning electron microscopy of transformed and synchronized normal cells. (a) S phase, × 700, (b) G₂ phase, × 3,200; (c) G₂ phase, × 3,200; (d) Transformed RSV infected cell, × 7,500. (e) mitotic, × 400; (f) mitotic, × 4,000; (g) early G₁, × 3,500; (h) density inhibited, × 500.
FIGURE 3  Enumeration of the various surface morphologies as a function of the cell cycle. $G_2$ (△-△); mitosis (●-●); early $G_1$ (○-○); cell number Δ-Δ.

d) and have found a remarkable resemblance of the transformed cell surface to the mitotic cell surface. This similarity in surface morphology between mitotic and transformed cells is consistent with the suggestion by Burger that mitotic and transformed cells have a similar surface architecture, (2, 10); rounded morphology and extensive coverage by microvilli can be used to describe either cell type.

In the transformed cell culture 85% of the cells exhibited the rounded morphology and coverage by microvilli; however, the remaining cells were more extended and had reduced coverage by microvilli. There are three possible explanations for these few morphologically normal cells in the transformed cultures: they may represent a cell cycle-dependent morphology (such as S); they may be cells which contain no active virus; or they may be cells which are infected with virus but have not yet become completely transformed. We cannot at this time distinguish between these possibilities.

**Induction of the Transformed State as a Function of the Cell Cycle**

To determine whether the induction of a transformation-specific membrane alteration by viral oncogenic activity is cell cycle dependent, we have taken advantage of a temperature-conditional mutant of Rous sarcoma virus, RSV-T5 (15, 16). Cells infected with this mutant exhibit a normal phenotype when held at the restrictive temperature (41°C) but rapidly become transformed when shifted to the permissive temperature (36.5°C). High titers of virus are produced at both temperatures. Cells infected with RSV-T5 and held at the restrictive temperature were synchronized, and at various times in the cell cycle cultures were shifted to the permissive temperature. At varying times after the shift, the transformation-specific induction of an increased 2-DG uptake rate was measured. The results demonstrate that induction of this transformation-specific membrane alteration can occur equally well at any stage of the cell cycle. The data in Fig. 4 a demonstrate that the induction of increased 2-DG uptake can occur when progression through the cell cycle is blocked in early S by excess cold thymidine (compare to the asynchronous culture, Fig. 4 j). The concentration of thymidine used completely prevented any increase in DNA in the cultures at 36.5°C (measured by the method of Burton (17)). This confirms earlier results (16) that DNA synthesis is not required for the induction. Induction can also occur in cells which are progressing through S after the removal of the block (Fig. 4 b, c) and in cells arrested in mid-S by removing the block and reapplying it 3 h later (Fig. 4 d).

Cultures which were shifted to the permissive temperature 7 h after removal of the thymidine block were predominantly in $G_2$ and displayed a
normal induction of increased 2-DG transport (Fig. 4 e). Cells shifted to the permissive temperature during the time of mitosis (12 h after removing the block) (Fig. 4 f) also showed a normal ability to increase their rate of 2-DG transport; however, we would not have detected a small inhibition of the induction of 2-DG transport since at any time only a minority of the cells were actually in mitosis (see Fig. 1).

Cells were brought to the G1 phase of the cell cycle in two ways: either by waiting 14 h after removal of the thymidine block (Fig. 4 g) or by allowing a culture to become density inhibited (Fig. 4 h). Cells brought to G1 by either technique
showed a normal ability to increase their rates of 2-DG uptake after the temperature shift.

DISCUSSION

Functional and Morphological Changes in the Cell Surface as a Function of the Cell Cycle

We have examined cell cycle-dependent alterations in cell surface morphology by using scanning electron microscopy, and in cell surface function by measuring uptake rates of 2-DG, uridine, thymidine, and mannitol. The 2-DG uptake rate was constant throughout most of the cell cycle but seemed to drop approximately 50% during mitosis. Because mitosis is such a small percentage of the total generation time, one could argue that with our synchronization method cells from the G2 and G1 phases were present in sufficient numbers to affect the transport rate. Thus, the drop in transport could be due, for example, to cells in late G1. Although this criticism is valid, Bader (18) has shown that cells arrested in mitosis by vinblastine sulfate also transport 2-DG at a reduced rate. The reduced level of transport in mitotic cells is in conflict with the hypothesis that the transformed cell's surface is in a "permanently mitotic" state (2). This hypothesis, if extended to cell surface function, would predict that mitotic cells should have a rate of 2-DG transport as high as that seen in transformed cells. Although mitotic and transformed cells were dissimilar in their ability to transport 2-DG, mitotic cell surfaces displayed a remarkable morphological similarity to the surfaces of Rous sarcoma virus-transformed cells: both cell types were rounded, and covered with microvilli.

Uridine transport showed no variation with the cell cycle. Sander and Pardee (19) have shown that in Chinese hamster ovary cells the uridine transport rate doubles in late G1. This apparent conflict with our results could be due to different cell systems or to an inability of the thymidine block to synchronize the uridine transport system. When using inhibitors such as thymidine to synchronize cell populations, it is important to recognize that all components of the cell which undergo cyclic variations during normal exponential growth may not be entrained by the inhibitor. However, Paterson et al., using HeLa cells synchronized by selective detachment, also find little or no cell cycle variation in uridine uptake (20).

Thymidine transport was utilized primarily to define the S phase. The results indicate that only during S phase does substantial uptake of thymidine occur, as has been shown by others (21, 22). The composite result of the 2-DG and uridine data indicates that the reduced level of transport seen in density-inhibited cells is an intrinsic property of the growth state and is not linked to the cell cycle. However, thymidine transport does show a reduction in both density-inhibited cells and in G1 cells, implying that the reduction seen in density-inhibited cells may be considered a cell cycle-dependent event.

Mannitol differs from the other metabolites used because it is taken up by passive diffusion (6) whereas uridine, thymidine, and 2-DG are taken up by facilitated diffusion. Thus, variation in the rate of mannitol uptake could only occur if there was a gross alteration of membrane composition or structure. Our results showed no variation of mannitol uptake with the cell cycle and are consistent therefore with the absence of major alterations in cell membrane composition or structure during the cell cycle.

Our interpretation of the data presented here rests on the assumption that transport is the rate-limiting step for uptake of these metabolites. This has been shown to be true for 2-DG, uridine, and thymidine uptake (6, 7, 22-24). In the case of mannitol uptake, no metabolism of the hexitol occurs, so the only step measured is nonsaturable uptake, presumably simple diffusion across the cell membrane.

Scanning electron microscopy showed that there are distinct changes in cell surface morphology as a function of the cell cycle as has also been shown by Porter et al. (14). However, the transport data indicated that these cyclic changes in cell surface morphology are not reflected in corresponding changes in membrane function. This observation is particularly relevant to our understanding of the mechanism by which tumor virus infection increases the hexose transport rate. This increased transport rate seems to be associated with an increased \( V_{\text{max}} \) for transport (6, 23, 25, 26). Since the large numbers of microvilli seen on the surfaces of the transformed cells may significantly increase the effective surface area of these cells, one could argue that the increased transport rate is simply a
consequence of increased surface area. The fact that mitotic cells, which also are covered with microvilli, did not display an increased hexose transport rate argues against this hypothesis.

Viral Gene Expression as a Function of the Cell Cycle

To determine whether induction of a transformation-specific membrane alteration was linked to the cell cycle, we used synchronized cells infected with a temperature conditional mutant of Rous sarcoma virus and held at the restrictive temperature. At various times in the cell cycle, cultures were shifted to the permissive temperature and the rate of uptake of 2-DG was assayed. The results demonstrate that the ability of viral gene expression to induce this membrane alteration could occur at any stage of the cell cycle, and did not require progression through the cell cycle or an intervening mitosis. This agrees with previous results showing that DNA synthesis was not required for the induction of increased 2-DG transport either by Rous sarcoma virus or by polyoma virus (16, 27). Since progression through the cell cycle is required for the appearance of enhanced agglutinability (11), it seems likely that the surface alteration which results in agglutinability by lectins is a later manifestation of viral oncogenic activity than is the activation of hexose transport.

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