CELL CYCLE-DEPENDENT CHANGES IN THE SURFACE MEMBRANE AS DETECTED WITH \[^3H\]CONCANAVALIN A

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A number of attempts have been made recently to study cell surface changes which occur during the cell cycle of tissue culture cells. A number of laboratories have reported that the expression of a variety of cell surface markers (e.g., lectin receptor sites and antigens) change during the cell cycle (1–3). This laboratory has previously demonstrated that normal mouse embryo fibroblasts bind more fluorescent wheat germ agglutinin during mitosis than during any other part of the cell cycle (1). Recently Shoham and Sachs have demonstrated an increase in fluorescent concanavalin A binding at mitosis (4). Cikes and Friberg (5) have demonstrated that a cell surface-localized transplantation antigen is expressed more in S than at any other time in the cell cycle. We have recently demonstrated that the levels of cyclic adenosine monophosphate are lower in mouse embryo fibroblasts during mitosis than at any other time in the cell cycle (6). All of this work suggests that cyclic changes in the expression of a number of cellular processes may be important in the maintenance of a normal cell cycle.

We have recently developed a \[^3H\]concanavalin A binding assay which demonstrates small but reproducible differences in binding between normal and transformed cells (7). This binding assay has been used to demonstrate an approximately threefold increase in \[^3H\]concanavalin A binding to mouse embryo fibroblasts at mitosis (7). In this paper we extend the study of concanavalin A binding at mitosis to study the various cellular processes required for the increased binding observed at mitosis.

MATERIALS AND METHODS

Sources

Concanavalin A was prepared from jack bean (Sigma Chemical Co., St. Louis, Mo.) according to the procedures of Agrawal and Goldstein (8). All tissue culture materials were purchased from Gibco Cultures, Grand Island Biological Co., Grand Island, N. Y. except for the sera which were purchased from Baltimore Biological Laboratories, Baltimore, Md. Tissue culture plates were obtained from Falcon plastics, Division of B-D Laboratories, Inc., Los Angeles, Calif. \[^3H\]acetic anhydride (specific activity 12 Ci/mmol) and \[^3H\]thymidine (specific activity 20 G/mmole) were purchased from Amer sham/Searle Corp., Arlington Heights, Ill. All chemicals and laboratory reagents were purchased...
from either Sigma or Fisher Scientific Co., Spring-
field, N. J.

Cell Line

The maintenance of the 3T3, mouse embryo fibroblast, cell line has been described elsewhere (7).

Agglutinin Binding

\[^{3}H\text{]concanavalin A}\] was prepared according to the techniques described by Frankael-Conrat and Colloms (9) using \[^{3}H\text{]acetic anhydride}. \[^{3}H\text{]acetyl concanavalin A}\] behaves exactly like native concanavalin A on DEAE-cellulose and in the standard agglutination assay and therefore throughout this paper the term \[^{3}H\text{]concanavalin A}\] is used instead of \[^{3}H\text{]acetyl concanavalin A}. A much more complete analysis of \[^{3}H\text{]concanavalin A}\] binding is presented elsewhere (7). Briefly, the 3T3 cell were grown to the desired density in Dulbecco modified Eagle's medium referred to Dulbecco modified Eagle's medium plus 50% calf serum and antibiotics. With either method of synchronization, mitosis occurred approximately 30 h after replating.

DNA Synthesis

The method for determining the S phase of the cell cycle has been previously described (15).

Simian Virus 40 (SV40) Infection

The method used for SV40 infection of the 3T3 cells is described elsewhere (12).

Inhibitors

In order to inhibit protein synthesis, cells were incubated with \(2 \times 10^{-5}\text{ M cycloheximide}\) or Dulbecco modified Eagle's medium minus histidine (13). The "histidine minus" medium has the advantage of inhibiting 80% of protein synthesis within 15 min after its addition to the culture and being 95% reversible within 15 min after the readdition of whole Dulbecco modified Eagle's medium plus 10% calf serum. Inhibition of protein synthesis was determined as the incorporation of \[^{14}C\text{]leucine}\) into TCA-insoluble material. Histidine-minus medium had no effect on RNA synthesis or transport of non-
metabolizable amino acids (15). The calf serum used in the histidine minus medium was exhaustively dialyzed against double-distilled water before use.

3T3 cells were blocked at metaphase in mitosis by incubation with 0.2 mM colchicine.

RESULTS

Fig. 1 demonstrates that \[^{3}H\text{]concanavalin A}\] binding to 3T3 cells increases immediately after subculturing due to the effect of proteases on the cell surface and increases again at mitosis. This increase in \[^{3}H\text{]concanavalin A}\] binding at mitosis coincides with the increase in mitotic index determined as the percentage of cells in metaphase at a given time. At the point of maximal synchronization (approximately 20% of the cells in metaphase), 1.5 times more \[^{3}H\text{]concanavalin A}\] is bound to the total cell culture. It can therefore be calculated that each cell binds approximately four times more \[^{3}H\text{]concanavalin A}\] at mitosis than at any other time in the cell cycle. However, since the cells in mitosis are approximately 25% larger than cells at other stages of the cell cycle, the corrected value for the increased binding at mitosis is approximately threefold. It should be noted that this increase in concanavalin A binding is prevented if the lectin is incubated with 1.0 mM
HOURS AFTER REPLATING

FIGURE 1 [3H]concanavalin A binding to 3T3 cells during mitosis. 3T3 cells were synchronized by replating from the monolayer stage to a lower cell density. The mitotic index was determined as the percentage of cells in metaphase at the designated time. The first point (0 h after replating) refers to a culture examined before dissociation with trypsin. O—O, mitotic index; △—△, cpm [3H]concanavalin A bound to $1 \times 10^5$ cells.

α-methyl mannopyranoside before addition to the cells. The same increase in concanavalin A binding at mitosis is seen if the cells at the monolayer stage are synchronized by changing the medium in which they are growing to Dulbecco modified Eagle's medium plus 50% calf serum.

Correlation of Surface and Nuclear Events

One of the interesting questions to be approached with the binding assay is whether surface events may occur irrespective of nuclear events, i.e., whether blocking mitosis at metaphase with colchicine prevents the increase in [3H]concanavalin A binding or blocks the cell surface in a conformation in which [3H]concanavalin A binding remains high rather than decreasing, as it would if the cells could enter G1. Fig. 2 demonstrates that even if metaphase figures are accumulated due to the incubation of the cells with 0.2 mM colchicine, the surface enters its G1 phase while the nucleus remains in mitosis. Return of the interphase surface configuration is not even delayed. This suggests that under certain circumstances surface events may be dissociated from nuclear events.

Requirement for Protein Synthesis

In a similar experiment an attempt was made to determine the protein synthesis requirement for the increased concanavalin A binding during mitosis. In these experiments 0.02 mM cycloheximide was added 27 h after replating (see Fig. 1) and washed out 32 h after replating. We have observed that both the pattern of [3H]concanavalin A binding and the mitotic index were superimposable on the data presented in Fig. 1, suggesting that protein synthesis is not required during mitosis for the surface change detected as increased concanavalin A binding. The concentration of cycloheximide used will inhibit approximately 95% of the cellular protein synthesis within 30 min after addition.

Incubation of a randomly growing population of 3T3 cells with 0.02 mM cycloheximide for 6 h did not result in increased concanavalin A binding.

Protein Synthesis during S

Fig. 3 is an attempt to determine when, during S, protein synthesis is necessary for the observed increase in concanavalin A binding at mitosis.
3T3 cells were synchronized by replating from the monolayer. At 21 h after replating (before the S phase) and every hour thereafter through 30 h (end of G2), duplicate plates of 3T3 cells were incubated for 1 h in Dulbecco modified Eagle's medium minus histidine. Incubation in this deficient medium results in an inhibition of 80±10% of the protein synthesis within 10 min after incubation. A background of approximately 20% protein synthesis remains in the presence of the histidine minus medium. This inhibition is 95% reversible within 15 min after replacing the medium with complete Dulbecco modified Eagle's medium. At 33 h after replating, the time at which the untreated control showed a maximal mitotic index of 20%, [3H]concanavalin A binding was performed on each set of plates which had previously had protein synthesis inhibited at different times in S. Fig. 3 A demonstrates that those cells in which protein synthesis was blocked between 21 and 26 h after replating still display the usual increase in [3H]concanavalin A binding at mitosis (33 h after replating). Inhibition of protein synthesis between 27 and 30 h after replating resulted in a severe reduction in [3H]concanavalin A binding at 33 h after replating. After 30 h, inhibition of protein synthesis had no effect on the increase in concanavalin A binding at mitosis. This suggests that protein synthesis is required late in S and possibly into G2 in order for the increased binding of [3H]concanavalin A to be observed at mitosis.

We have earlier suggested that after viral infection the order of DNA synthesis, and in turn the order of synthesis of certain proteins may be different from that observed in normal, uninfected cells (14). Specifically we had envisioned an inversion of the sequence of replication of certain parts of the chromosomal material. An attempt to test this hypothesis was performed in the form of the same experiment as described in the legend of Fig. 3 A but using 3T3 cells infected with SV40. It should be noted (Fig. 3 B) that although DNA synthesis occurs later in the virally infected cells than in the replated normal cells, the curves describing DNA synthesis can be superimposed. Fig. 3 B demonstrates that the protein synthesis necessary for increased concanavalin A binding at mitosis is made in early S in the virally infected cells rather than in late S as in normal cells. Fig. 3 B suggests that this protein is made from the start of S and throughout a large part of S but not in late S, G2, or mitosis.

It should be noted that incubation of a randomly growing population of 3T3 cells with histidine-minus medium for 1 h does not effect [3H]concanavalin A binding. In those cases where the block in protein synthesis results in decreased
DISCUSSION

The binding of \[^{3}H\]concanavalin A increases approximately threefold during mitosis. This increase in binding is independent of the mode of synchronization and is inhibited if \[^{3}H\]concanavalin A is preincubated with 1.0 mM of its specific hapten, \(\alpha\)-methyl mannopyranoside. This suggests that the observed increase in concanavalin A binding is the result of an increase in available receptors for concanavalin A and corroborates our earlier findings that mitotic cells in culture show a dramatic increase in binding of fluorescently labeled wheat germ agglutinin (1). Recently the same result has been obtained with fluorescently labeled concanavalin A (4).

This work also demonstrates that the increased surface alteration during mitosis measured as increased concanavalin A binding is not dependent on protein synthesis during mitosis but rather on the synthesis of proteins during the late S phase of the normal cell. Whether this protein synthesis in late S is primarily responsible for increased agglutinin binding or for the nuclear events following mitosis remains to be determined.
during mitosis is not clear, since neither the nuclear events nor the surface events occur when protein synthesis is blocked in late S.

Completion of the nuclear events in mitosis is apparently not a prerequisite for completion of the surface events in mitosis. In those experiments in which colchicine was used to arrest the cells in metaphase the nuclear events had progressed part of the way through mitosis before being arrested, and therefore we cannot conclude that surface events are independent of nuclear events since prophase may be necessary for the initiation of the observed surface events. The fact that the surface change does continue in the presence of colchicine may explain Stadler and Adelberg's results with cell fusion during mitosis which demonstrate that although cells blocked at metaphase have a higher frequency of Sendai virus-induced fusion, cells which have been kept in colchicine demonstrate a decreasing frequency of fusion with time until they reach a frequency equal to a population of G1 cells (16).

Using the [3H]concanavalin A binding assay we have added some support to a hypothetical model of cellular transformation recently proposed by two of the authors (14). It was suggested that there is a feedback between the surface membrane conformation and control of DNA synthesis. Among the points raised in the model was the suggestion that after viral infection the order of DNA synthesis and in turn the order of synthesis of certain proteins would differ from that of normal, uninfected cells. Such a derangement of protein synthesis might result in the maintenance of the "transformed surface architecture" and a loss of contact inhibition. We have demonstrated that after viral infection there appears to be a rearrangement in the order in which particular segments of the host genome are transcribed. In this particular study it appears that in SV40-infected 3T3 cells the part of the genome which codes for the surface change detected as increased lectin binding is read and translated in early S rather than in late S as in uninfected cells. It has previously been demonstrated that, after infection, satellite DNA synthesis in baby mouse kidney cells is shifted with regard to the time of synthesis in normal, uninfected cells (17).

Thus we have demonstrated a threefold increase in concanavalin A binding to 3T3 cells in mitosis. This increase is at least partially independent of nuclear mitotic events as well as protein synthesis during mitosis. Protein synthesis during S is required for the surface change in mitosis and the timing of protein synthesis is different in cells infected with SV40 virus as compared to the uninfected control.

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