Blockage of Prereplicative Progression by Cytochalasin D in Serum-Stimulated GC-7 Cells

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ABSTRACT. When growth-arrested GC-7 cells, a cell line from African green monkey kidney, are stimulated with 10% calf serum, they enter S phase 14–15 h later. Cytochalasin D at 0.6 μg/ml blocks the entrance into S phase, and inhibits, though only partially, the increase in protein synthesis after serum stimulation. Since partial inhibition of protein synthesis by cycloheximide interferes with accumulation of labile proteins and thus blocks the entrance of serum-stimulated cells into S phase, the effects of these two inhibitors are compared. Cytochalasin D at lower concentrations reduced the rate of entry into S phase without affecting the length of the prereplicative phase, whereas cycloheximide extended the prereplicative phase dose dependently without affecting the rate of entry into S phase. Cytochalasin D affected neither individual [35S]methionine-labeled spots on two-dimensional polyacrylamide gel nor degradation of cellular proteins. These results indicate that cytochalasin D, though it interferes with protein synthesis, blocks prereplicative progression of serum-stimulated GC-7 cells in a different manner than cycloheximide.

Serum induces proliferation in growth-arrested cells by controlling an ordered sequence of events directed toward S phase. In contrast with the progress of extensive studies on the biochemical events occurring early after growth stimulation, the sequence of events operating at mid/late prereplicative phase still remains unknown. Maness and Walsh (5) have reported that dihydrocytochalasin B blocks initiation of DNA synthesis in Swiss 3T3 cells stimulated with serum. We also reported that when quiescent GC-7 cells were stimulated to proliferate with serum, the entrance into S phase was blocked in the presence of 0.6 μg/ml of cytochalasin D (7). Cytochalasins could be a unique tool for understanding the process after growth stimulation, especially at mid-prereplicative phase, because the progression of the cell cycle in GC-7 cells is sensitive to the drug not throughout the cycle but at the stage 8–10 h after serum stimulation (7).

Cytochalasins D (3) and B (8), when used at higher concentrations, release polysomes from the cytoskeleton and inhibit protein synthesis. The increase in total protein synthesis in serum-stimulated GC-7 cells is partially blocked by 0.6 μg/ml of cytochalasin D, though synthesis is not reduced below the level in unstimulated...
control cells (7). On the other hand, it has been established that the partial inhibition of protein synthesis by cycloheximide blocks G1 phase progression (1, 2, 9, 10), and suggested that an accumulation of some unstable proteins with short half-lives is required for the G1 progression (2, 9). A protein, molecular weight 68,000, synthesized in G1 phase has been supposed to take part in the growth regulation at the restriction point (4).

These observations have prompted us to see whether a partial inhibition of protein synthesis by cytochalasin D is involved in blocking the cell cycle progression. In this paper, we compare the effect of cytochalasin D with that of cycloheximide on protein metabolism and on kinetic patterns of entry into S phase of serum-stimulated GC-7 cells and suggest that cytochalasin D does not block the prereplicative progression of serum-stimulated GC-7 cells through the inhibition of protein synthesis.

MATERIALS AND METHODS

**Cells and culture conditions.** GC-7 cells, originated from African Green monkey kidney, were cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% immobilized calf serum (Irvine Scientific Co.) as previously described (7). The cells were growth-arrested in serum-free DMEM for 3 days. The density of serum-arrested cells was 6.5–8.0 × 10^3/cm² and was 1/4–1/5 of confluent monolayer. Cytochalasin D (Aldrich Chemical Co.) dissolved in dimethylsulfoxide (1 mg/ml) was stored at -20°C and diluted with DMEM before use. Cycloheximide (Nakarai Chemicals Ltd.) was dissolved in phosphate buffered saline (PBS) at a concentration of 2 mg/ml and stored at -20°C. The frequency of cells entering S phase was monitored by autoradiography (7).

**Incorporation of [³H]leucine.** Cells plated in 35-mm plastic dishes were incubated for 1 h in medium deficient in leucine and containing 10% dialyzed serum and [³H]leucine (3 µCi/ml, 5.0 Ci/m mol, New England Nuclear). They were then washed with cold PBS and dissolved in 1 ml of 0.5 N KOH. The lysate was kept overnight at the room temperature and then precipitated by adding 5 ml of cold 5% trichloroacetic acid. tRNA-charged amino acid was liberated during alkali treatment. Acid-precipitable materials were collected on a GF/c filter, washed with 5% trichloroacetic acid, and dried. Radioactivities were measured in a liquid scintillation spectrometer. Since cytochalasin D at the concentration used did not affect the transport of leucine, incorporation of [³H]leucine into acid precipitable materials is used as an index of protein synthesis.

**Two-dimensional gel analysis.** Cells plated in 35-mm dishes were labeled for 1 h in 0.5 ml of Eagle’s minimal essential medium deficient in methionine and containing 100 µCi/ml of [³S]methionine (1250 Ci/m mol, Amersham) and 10% dialyzed serum. They were then washed with cold PBS and solubilized in a solution containing 0.3% sodium lauryl sulfate and 10% 2-mercaptoethanol. DNA was digested by DNase I (80 µg/ml). The lysate was mixed with solid urea (final concentration, 8.5 M) and with an equal volume of a solution containing 8.5 M urea, 5% 2-mercaptoethanol, and 4% Nonidet P-40, and then applied on the first dimension gel containing 0.8% pH 5–7, 0.8% pH 6–8, and 0.4% pH 3–10 Bio Lyes (Bio-Rad). Two-dimensional gel electrophoresis was performed according to the method of O’Farrell (6). After the run, the gel slab was fixed, treated with EN³HANCE (New England Nuclear), dried, and exposed to Kodak X-Omat AR-5 film.

RESULTS

**Inhibition of total protein synthesis.** The increase in protein synthesis following serum stimulation of growth-arrested GC-7 cells was partially inhibited by 0.6 µg/ml
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of cytochalasin D (Fig. 1). Cytochalasin D at higher concentrations up to 1 μg/ml gave similar results (data not shown). The protein synthesis in unstimulated cells was little affected by cytochalasin D. The dose-dependency of the inhibition in protein

Fig. 1. Effect of cytochalasin D on the incorporation of [3H]leucine into acid precipitable materials. Growth-arrested GC-7 cells were stimulated with serum at 0 time (solid lines) or left unstimulated (broken lines) and treated with cytochalasin D (0.6 μg/ml) from 0 h (●), 5 h (▲), 10 h (■), or 15 h (×), or left untreated (○). The cells were labeled with [3H]leucine for 1 h. Each value represents a mean of two determinations.

Fig. 2. Incorporation of [3H]leucine into acid precipitable materials. Growth-arrested GC-7 cells were unstimulated (●) or serum-stimulated (○) and cultured for 25 h in the presence of (a) cytochalasin D or (b) cycloheximide. The cells were labeled with [3H]leucine for the last 1 h. Each value indicates a mean of two determinations.
Cytochalasin D at 1.0 µg/ml induced an extensive rounding in cell morphology, but inhibited protein synthesis by only about 50%. We have reported in the previous paper (7) that the incorporation of [3H]leucine into serum-stimulated cells markedly decreased after the addition of cytochalasin D, but the data were found to have been misleading. Incorporated radioactivities were measured by immersing cells on coverslips directly in scintillation cocktail after they were fixed, washed, and dried. This procedure was simple and quick, but radioactivities in cytochalasin D-treated cells were underestimated due to the increased self-absorption of β-rays due to rounding of these cells.

Inhibition of the progression in prereplicative phase. Cytochalasin D at 0.6 µg/ml inhibited protein synthesis by about 25%, where about 70% of the serum-stimulated cells were prevented from entering S phase by 30 h. Cycloheximide at 60 ng/ml inhibited protein synthesis by 50%, where only about 30% of the cells did not enter S phase. Thus cytochalasin D inhibits induction of DNA synthesis more specifically than cycloheximide.

When arrested GC-7 cells were stimulated with serum in the presence of different concentrations of cytochalasin D, the rate of entrance into S phase (slope of the curve in Fig. 3a) was reduced dose dependently without changing the length of the prereplicative period. On the other hand, cycloheximide, at the concentrations used, prolonged the prereplicative phase but did not change the slope of the curves (Fig. 3b), in agreement with the previous report that the entrance into S phase is delayed by low concentrations of cycloheximide (1, 2, 9). The presence of cytochalasin D during 0–8 h after serum stimulation did not affect the entrance of cells into S phase.
at all (7). On the other hand, the presence of cycloheximide at 30–90 ng/ml during the same period retarded initiation of S phase by about 2–5 h (data not shown), confirming the previous results that the progression of cells is sensitive to cycloheximide almost throughout the prereplicative phase (10). These kinetic results clearly show that the inhibitory effects of the two drugs on the progression of prereplicative phase in serum-stimulated cells are different.

Two-dimensional polyacrylamide gel analysis of cellular proteins. Even if cytochalasin D blocked total protein synthesis only partially, the possibility cannot be ruled out that the drug inhibits specifically the synthesis of labile proteins that are assumed to be required for the progression of the prereplicative sequence (1, 2, 9, 10). Since the stage sensitive to cytochalasin D was around 8–10 h after serum stimulation (7), proteins in serum-stimulated GC-7 cells were labeled with [35S]methionine for 1 h from 8 to 9 h poststimulation in the presence or absence of cytochalasin D, and analyzed by two-dimensional polyacrylamide gel electrophoresis. If the accumulation of labile proteins occurred at this stage, the protein spots from serum-stimulated cells would have higher intensity than those of unstimulated cells and be affected by cytochalasin D. Increase (solid arrows in Fig. 4) and decrease (open arrows in Fig. 4) in the intensity of several protein spots were observed after serum stimulation, but these changes were not modified by cytochalasin D treatment (Fig. 4). In the area of the gel between pH 7.0–10.0, all protein spots were almost identical among these samples (data not shown).

Protein degradation. If cytochalasin D accelerates degradation of cellular proteins, any accumulation of labile proteins required for the progression of G1 phase would be abolished. Serum-stimulated GC-7 cells were pulse-labeled for 30 min with
During 7.5-8 h after the stimulation, and then the decrease in the radioactivities in acid precipitable materials was monitored in the presence or absence of cytochalasin D. This decrease was not accelerated by cytochalasin D (Fig. 5). Similar experiments by labeling cells with [3H]leucine from -16 to 0, from 0 to 8, or from 7 to 9 h of serum stimulation confirmed no acceleration of total protein degradation by cytochalasin D treatment (data not shown).

**DISCUSSION**

Our knowledge of the sequence of biochemical events operating in mid/late prereplicative phase is still limited. Cytochalasin D, which blocks the progression of cells from growth-arrest to S phase at mid prereplicative stage, 8-10 h after growth-stimulation (7), is a unique tool for understanding the process of growth induction. Taking previous observations together, as mentioned in the introduction, it seems likely that the block of entrance into S phase of serum-stimulated cells by low concentrations of cytochalasin D is due to the partial inhibition of protein synthesis. The following kinetic results, however, suggest that the mechanism of the blockage by cytochalasin D is different from that by cycloheximide: 1) cytochalasin D reduced the rate of entry of cells into S phase without affecting the length of the prereplicative phase, whereas cycloheximide extended the prereplicative phase without affecting the rate of entry into S phase; and 2) progression of the prereplicative phase is sensitive to cytochalasin D only at around 8-10 h after serum stimulation (7), whereas it is sensitive to cycloheximide for almost the whole prereplicative period (unpublished data and (10)). In addition, the results from the two-dimensional gel electrophoresis of the proteins synthesized from 8 to 9 h after stimulation and from the experiments on protein degradation are consistent with, although they do not prove, that cyto-
Cytochalasin D does not inhibit the accumulation of labile proteins which is assumed to take part in the growth regulation.

Actin-containing structures are probably involved in the transduction of growth signal from the cell surface into the nucleus, but the expression of such cell cycle-regulated genes as c-fos, c-myc, β-actin, or ornithine decarboxylase occurred in serum-stimulated GC-7 cells regardless of the presence of cytochalasin D (11). Other early events such as an increase in uridine transport also occurred after serum stimulation in the presence of the drug (7). Among the various effects of cytochalasin D on GC-7 cells, the following are the most prominent: cytoplasmic actin cables were disorganized within 30 min after the addition of 0.6 µg/ml of cytochalasin D (7), and cell locomotion activated after the serum stimulation was completely abolished by the drug (unpublished data). These two occurred, however, independently of the cell cycle. At present, we can not distinguish between the following two possible mechanisms for the inhibition by cytochalasin D of cell cycle progression: direct effects on cell cycle-specific events and indirect effects on these events through action on cell cycle-independent events; for example, disruption of actin cables.

REFERENCES

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