Cytochalasin D Induces Increased Actin Synthesis in HEP-2 Cells

JANET TANNENBAUM* AND GABRIEL C. GODMAN

Department of Pathology, College of Physicians and Surgeons of Columbia University, New York, New York 10032

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In HEP-2 cells treated with 0.2 to 2.0 μ M cytochalasin D (CD) for 7.5 to 24 h there was a 20 to 50% relative increase in actin content (units of actin per microgram of total cell protein). This augmentation, which was concentration and time dependent, was prevented by treatment with cycloheximide during exposure to CD. A 15 to 20% increase in the relative rate of actin synthesis in CD-treated HEP-2 cells (0.2 to 2.0 μ M CD) was detectable after 1 h of treatment and increased to 30 to 50% by 24 h. This increased rate of actin synthesis was apparently responsible for the higher actin content of CD-treated HEP-2 cells. The concentration dependence of these effects of CD on actin metabolism correlated with the pattern seen for CD-triggered changes in cellular morphology and the underlying rearrangements of the actin-containing cytoskeletal structures, suggesting that the effects on metabolism and morphology were interrelated. Since the rapidly occurring cytoskeletal reorganization preceded the effects of CD on actin metabolism, it is proposed that actin synthesis is induced by the cytoskeletal rearrangement resulting from exposure to CD.

Cytochalasins inhibit cell motility and contractile system-dependent functions (review in reference 48). These drugs bind to actin, can reportedly inhibit *in vitro* polymerization (7, 8, 15, 29) and treadmilling (46) by associating with the rapidly growing end of the actin filament (30), cause scission of filaments (22, 31, 44), and dissolve gels of actin formed either by direct filament-filament interactions or associations mediated by cross-linking molecules (22, 30). Presumably, all of these effects also occur in the living cell. One of the effects of cytochalasins on living cells which is not observed with pure actin *in vitro* is a rearrangement of the actin-containing components of their cytoskeletons into aggregated masses (references 20, 21, 35, and 52, among others; review in reference 18). This rearrangement is dependent on metabolic energy (20, 21, 34, 43) and is accompanied by little or no depolymerization of cellular actin (3, 36). We have previously reported that treatment of HEP-2 cells with cytochalasin D (CD) induced an increase in the relative content of total cellular actin (36). We have now extended these observations and report here that this rise in actin content is accompanied by an elevation in the relative rate of actin synthesis. Both of these effects occur only at concentrations of CD capable of eliciting rearrangements of actin in these cells, suggesting that these effects may be interrelated.

MATERIALS AND METHODS

Cell culture. HEP-2 cells (ATCC CCL23) were grown in monolayers in Eagle minimum essential medium (Earle salts) supplemented with 10% newborn calf serum, penicillin (100 U/ml), and streptomycin (100 μ g/ml) (medium from Flow Laboratories, Inc.; all other components from GIBCO Laboratories). Monolayers were subcultured twice weekly by trypsinization. For all experiments except those shown in Table 1 and Fig. 3 and 4, cells were plated at a density of 4×10^5 to 5×10^5 cells per 60-mm plastic petri dish (Falcon Plastics), incubated in a humidified atmosphere of 5% carbon dioxide-95% air at 37°C, and used for experiments after 2 days (subconfluent). Cells for the experiments shown in Table 1 were plated at 3×10^5 (subconfluent) or 6×10^5 (confluent) per 60-mm dish and incubated for 2 days before use. The cells shown in Fig. 3 and 4 were plated on 22- by 22-mm glass cover slips in 35-mm plastic petri dishes at 4.5×10^4 and 1×10^5 cells per dish, respectively, and incubated for 2 days before drug treatment.

Drug treatments. Stock solutions of CD (Sigma Chemical Co.) were prepared at a concentration of 1 mg/ml in dimethyl sulfoxide (DMSO; Sigma) and diluted into growth medium immediately before use. For all dilutions of CD, the final concentration of DMSO was adjusted to 0.1%; control cultures were treated with 0.1% DMSO. Treatment of HEP-2 cells with CD was initiated by refedding them with growth medium containing CD at least 24 h after the cells were plated. For experiments in which CD treatment was for less than about 16 h, monolayers were refed with growth medium about 24 h before harvest and later refed with drug-containing medium at the appropriate time.

A stock solution of cycloheximide (Calbiochem), 10 mg/ml, was prepared in Earle balanced salt solution (EBSS) buffered with 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), sterile filtered through a 0.45- μ m Millex filter unit (Millipore Corp.), and stored at -20°C . The stock solution was diluted into growth medium immediately before use.

DNase 1 inhibition assay for actin. After drug treatment, cell monolayers were drained and quickly rinsed with warm HEPES-buffered EBSS. The cells were scraped by means of a rubber policeman into 0.3 to 0.4 ml of lysis buffer containing 0.5% Triton X-100 (4) and 1 mM phenylmethylsulfonyl fluoride as previously described (36). All other procedures for the DNase 1 assay were those of Blikstad et al. (4), with the modifications previously described (36). Briefly, lysates on ice were mixed with a guanidine hydrochloride buffer (4) to depolymerize all actin so that it could be assayed by inhibition of DNase 1 (36). For each determination, the amount of lysate used was adjusted to give inhibition of DNase 1 within the linear range of the assay. Values shown are the averages of four to five determinations per sample; one unit of actin is defined as the amount which gives 1% inhibition of DNase 1 in the standard assay. The DNase 1 was purchased from Sigma (DN-100); 0.2 μ g was used for each assay. Highly polymerized calf thymus DNA (Sigma) was used as the substrate. Other chemicals were reagent grade.

Since different lots of DNase 1 and DNA substrate were used for the experiments reported in Tables 1 and 2, the values for the units of actin per microgram protein measured are not directly comparable. For comparison among experiments, data are expressed as percentages of the value for the DMSO-treated control cells measured in each experiment.

Radioisotope labeling of cells. For labeling of cells with L-[^{35}S]methionine (1,340 Ci/mmol; Amersham Corp.), monolayers of CD- or DMSO-treated HEP-2 cells in 60-mm plastic Petri dishes were drained and refed with methionine-free growth medium (GIBCO) plus 10% newborn calf serum containing CD or DMSO and [^{35}S]methionine at a final concentration of 10 $\mu\text{Ci}/\text{ml}$. Cells were incubated at 37°C as described above for 60 min, drained, rinsed twice with warm HEPES-buffered EBSS, drained, and scraped into 0.3 ml of sodium dodecyl sulfate (SDS)-sample buffer (0.062 M Tris hydrochloride [pH 6.8], 0.1 M dithiothreitol, 10% glycerol, 2.3% SDS; with 1 mM phenylmethylsulfonyl fluoride and 1 mM L-1-tosylamide-2-phenylethyl chloromethyl ketone added immediately before use). Samples were sheared in a syringe with a 26-gauge needle to decrease viscosity and stored at -70°C . Stock solutions of 100 mM phenylmethylsulfonyl fluoride in isopropanol and 100 mM L-1-tosylamide-2-phenylethyl chloromethyl ketone in DMSO were stored at room temperature.

To determine total incorporation of [^{35}S]methionine into cellular proteins, the hot 5% trichloroacetic acid-precipitable material from 5- μ l samples of cell lysates in SDS-sample buffer was collected on glass fiber filters (Whatman no. 934-AH) and washed with 5% trichloroacetic acid. The filters were mixed with 10 ml of scintillation cocktail (4 g of Omnifluor per liter of toluene) plus 0.5 ml of Protosol tissue solubilizer (New England Nuclear Corp.) per vial and counted in a Packard Tricarb Scintillation counter. Omnifluor was

obtained from New England Nuclear Corp., and toluene (Scintianalyzed grade) was from Fisher Scientific Co.

To measure the inhibitory effect of cycloheximide on protein synthesis in HEP-2 cells, 35-mm plastic petri dishes planted at 2×10^5 cells per dish were incubated for 2 days and then refed with growth medium containing 0.5 to 20 μg of cycloheximide per ml plus 0.5 μCi of [^{14}C]amino acids (5 mCi/mg-atom of carbon; Amersham) per ml. After a 12-h labeling period, the monolayers were harvested as described above for [^{35}S]methionine-labeled cells, except that the cells were scraped into 0.4 ml of HEPES-buffered EBSS with 5 mM EDTA, and the entire sample was used for measurement of hot trichloroacetic acid-precipitable material. Treatment with 10 μg of cycloheximide per ml depressed protein synthesis by 95%; cell viability (by trypan blue exclusion) was not altered by up to 20 μg of cycloheximide per ml for 12 h.

Polyacrylamide gel electrophoresis. All SDS-polyacrylamide gel electrophoresis was by the procedures of Laemmli (28); separating gels were 9% polyacrylamide and stacking gels were 3% polyacrylamide. For the scans shown in Fig. 1, cylindrical gels (5.9 by 135 mm) were used; all other experiments employed slab gels (145 by 145 by 1.5 mm). Samples for cylindrical gels (portions of those used to obtain the data in Fig. 2) were precipitated from DNase 1 lysis buffer with cold acetone and dissolved in SDS-sample buffer; all other samples were harvested directly into SDS-sample buffer as described above. Cylindrical gels were run at 70 V for about 6 hours, fixed for 1 h in 50% methanol-10% acetic acid, stained overnight with 0.2% Coomassie blue R-250 in 50% methanol-10% acetic acid, and then destained for 4 days. Slab gels were similarly processed, except that they were run at 100 V for about 5 h, and destaining required only 1 day.

Densitometry of cylindrical gels (Fig. 1) was performed by using a Beckman spectrophotometer equipped with a Gifford linear transport apparatus; scanning was at a wavelength of 590 nm. Sample loadings (50 μg of protein) were adjusted so that the staining intensity of the actin band would be within the range giving a linear response to the amount of actin present. For slab gels, 100,000 hot trichloroacetic acid-precipitable cpm of [^{35}S]methionine-labeled cell lysate was loaded in each well.

After destaining, slab gels were soaked in 10% acetic acid for 20 to 30 min and dried on Whatmann 3MM chromatographic paper under vacuum at about 70°C . To facilitate alignment of gel patterns with their radioautograms, dots of radioactive ink were painted onto the 3MM paper at the margins of the gel. The dried gels were exposed to Kodak X-ray film XR-5 for 3 to 4 days at room temperature, and the films were developed by standard techniques. For molecular weight markers, bovine serum albumin (66,200 daltons) and phosphorylase A (92,500 daltons) were obtained from Bio-Rad Laboratories; actin, myosin heavy chain, and filamin were obtained as a crude extract of chicken gizzard.

Two-dimensional polyacrylamide gel electrophoresis (isoelectric focusing followed by SDS-polyacrylamide slab gel electrophoresis) was performed as described by O'Farrell (38), but with longer (22-cm) isoelectric focusing gels, as previously described (49). Ampholytes (Bio-Lyte 5/7 and Bio-Lyte 3/10) were

purchased from Bio-Rad, urea (Ultra-Pure) was purchased from Schwarz-Mann, and Nonidet P-40 was purchased from Sigma. Solutions of Nonidet P-40 and acrylamide were deionized with AG 501-X8(D) mixed bed resin (Bio-Rad). HEP-2 cells were treated with 1 μ g of CD per ml or 0.1% DMSO for 26 h and then washed with EBSS and scraped into O'Farrell's lysis buffer A (38) plus 1 mM phenylmethylsulfonyl fluoride. Duplicate 20- μ g samples of each lysate were loaded onto prefocused 220- by 2-mm cylindrical gels and run for 16.5 h at 1,000 V and then 1 h at 1,500 V. A 120-mm segment of the gel, which included the focused actin bands, was cut from each and used for analysis in the second dimension (9% polyacrylamide gels, as described above).

Under these conditions, HEP-2 cell actin resolved into two spots corresponding to the β and γ isoelectric variants (see also reference 17). For equal protein loadings, the CD-treated cells gave darker Coomassie blue-staining actin spots than the DMSO-treated control cells, indicating that both species of actin underwent a relative increase in CD-treated HEP-2 cells. The difference in staining intensity between treated and control samples was readily apparent on visual inspection. In photographs, however, it was too small to be reproduced satisfactorily.

Measurement of [35 S]methionine-labeled actin in polyacrylamide slab gel patterns. For quantitation of the [35 S]methionine in actin bands, the bands were cut from the dried gel, rehydrated with 50 μ l of deionized water in scintillation vials, and suspended in 10 ml of Omnifluor-toluene scintillation cocktail with 0.3 ml of Protosol. This procedure is essentially the one described by Y. Kobayashi, (LSC Applications Notes no. 31-50, p. 9, New England Nuclear Liquid Scintillation Counting Applications Laboratory, Boston, Mass.), except that the samples in scintillation cocktail plus Protosol were incubated at room temperature for 3 days and then counted in a Packard Tri-Carb scintillation counter. Maximal elution was achieved by the second day; the counts per minute measured remained constant for at least 3 days thereafter. Background counts for each sample were measured by similarly processing for each lane a piece of gel from a location which lacked discernible bands on the radioautogram. All values reported were corrected for background counts.

Protein determinations. The method of Bradford (5) was used; Coomassie blue G-250 for preparation of the dye reagent was obtained from Eastman Kodak Co. Samples of cell lysates in Triton X-100 lysis buffer (DNase 1 assays), SDS-sample buffer (gel electrophoresis), and O'Farrell lysis buffer (two-dimensional gels) were diluted with distilled water (1:5, 1:20, and 1:20, respectively) before assay to decrease interference from the detergents present. Standard curves were prepared from bovine serum albumin similarly diluted with the appropriate buffers.

Cell morphology. For Fig. 3, the cover slips of HEP-2 cells were rinsed in warm EBSS, fixed in 5% Formalin in phosphate-buffered saline (pH 7.1), and stained with hematoxylin-azure-crystal violet. For Fig. 4, cover slips were rinsed in warm EBSS, fixed in 2.5% methanol-free Formalin in phosphate-buffered saline, permeabilized successively with acetone-water (1:1), acetone, and acetone-water (1:1), and rinsed with phosphate-buffered saline. Sites of F-actin were

identified by staining with fluoresceinated phalloidin (a generous gift of T. Wieland) at 10 to 20 μ g/ml (54). The cover slips were mounted in a solution of Gelvatol (Monsanto Chemical Co.) and examined in the Leitz-Dialux 20 microscope with vertical illumination as previously described by Godman et al. (20).

RESULTS

Increased actin content in CD-treated HEP-2 cells. Treatment of HEP-2 cell monolayers with 2.0 μ M CD for 17 to 27 h produced a 20 to 30% increase in the content of actin relative to total cell protein, as measured by a DNase 1 inhibition assay (36). This observation was not the result of some possible artifactual effect of CD on the DNase 1 assay, because the actin content measured by DNase 1 assay in lysates of HEP-2 cells which had not been previously incubated with the drug while alive was not increased by the addition of 2.0 μ M CD to the lysates (data not shown). The possibility that *in vitro* interaction of CD with actin in the cell lysate might increase the inhibitory effect of this G-actin on DNase 1 was thus excluded. Densitometry of SDS-polyacrylamide gel patterns of cell lysates (Fig. 1) and patterns from two-dimensional gels (data not shown) confirmed that the relative content of actin (Fig. 1) increased in CD-treated HEP-2 cells. The identity of a second protein peak (ca. 77,000 daltons) (Fig. 1) which appeared to increase in CD-treated cells is not known.

Under the conditions used for these experiments, treatment with CD (0.2 or 2.0 μ M) for up to 24 h did not depress cell viability as measured by trypan blue exclusion. The total cell count for 0.2 μ M CD was the same as for the 0.1% DMSO-treated controls (about 3×10^6 cells per 60-mm

FIG. 1. Densitometric scan of Coomassie blue-stained SDS-polyacrylamide gel electrophoretic patterns of HEP-2 cell lysates. Cells treated for 19 h with 0.1% DMSO (—) or 2 μ M CD (---); 50 μ g of cell protein was separated on each cylindrical gel. The top of each gel is to the left. The portion of the scan below about 30,000 daltons was superimposed for both samples and has been deleted from the illustration. The position of the actin is indicated by an arrow; the position of a protein of ca. 77,000 daltons is indicated by the asterisk.

TABLE 1. Effect of cell density on content of actin in CD-treated HEp-2 cells

Treatment ^a	U of actin/ μ g of protein $\pm$ SD (% of control $\pm$ SD)	
	Subconfluent cells	Confluent cells
DMSO, 0.1%	4.74 $\pm$ 0.29 (100)	4.79 $\pm$ 0.30 (100)
CD, 0.2 μ M	6.37 $\pm$ 0.35 (134 $\pm$ 11)	6.44 $\pm$ 0.33 (134 $\pm$ 11)
CD, 1.0 μ M	6.37 $\pm$ 0.34 (134 $\pm$ 11)	6.76 $\pm$ 0.53 (141 $\pm$ 14)
CD, 2.0 μ M	7.21 $\pm$ 0.52 (152 $\pm$ 14)	6.85 $\pm$ 0.12 (143 $\pm$ 9)

^a Treatment was for 23 h.

dish), but for 2.0 μ M CD, the cell count was half the value of the controls. In the latter case, the protein content per cell had about doubled, consistent with the ability of 2.0 μ M CD to block cytokinesis and thus yield multinucleated cells (9). (Total protein content per dish of CD-treated cells was depressed about 10 to 15%, compared with DMSO-treated controls, with 24 h of treatment at either concentration of drug.)

The ability of CD to induce an increase in relative actin content was not altered by changes in cell density. The response to CD (0.2 to 2.0 μ M) was the same for subconfluent and confluent HEp-2 cell monolayers (approximately 2×10^6 cells versus 4×10^6 cells per 60-mm dish) (Table 1).

Dependence on CD concentrations. The effects of CD on actin content were observed at concentrations of CD within the range capable of altering cytoskeletal morphology (21, 34) and of inducing the various effects on actin assembly and association seen in vitro (see above). To explore relationships between effects of CD on cytoskeletal contractile systems and the CD-induced augmentation of actin levels, we examined the concentration dependence of each of these effects. The elevation of relative actin content after treatment with various concentrations of CD for 19 h is shown in Fig. 2. To quantitate the morphological effects of CD, we counted the number of cells exhibiting zeiosis (Fig. 2). The formation of zeiotic knobs, membrane-bound surface protrusions (Fig. 3c and d), on epithelioid cells is a sensitive indicator of morphological response to CD (19, 34). Since zeiosis occurs as a consequence of the rearrangement of underlying microfilamentous actin (see discussion in reference 19) it serves as a convenient assay for this reorganization of the cytoskeleton.

The curves in Fig. 2 indicate a close correlation between the concentrations of CD which triggered cytoskeleton-dependent morphological changes and those which induced a rise in actin content. Comparison of Fig. 2 through 4 demonstrates that cells which had elevated levels of actin contained aggregated masses of actin (Fig. 4c and d) rather than the thin stress fibers seen in DMSO-treated cells (Fig. 4a) or in cells treated

with 0.02 μ M CD (Fig. 4b), a concentration which did not induce a rise in actin content (Fig. 2) or trigger zeiosis (Fig. 3b). Both 0.2 and 2.0 μ M CD induced zeiosis, aggregation of actin, and a rise in actin content, and the higher concentration of CD also triggered protrusion of nuclei from many of the cells (Fig. 3d), a process which on rare occasions results in spontaneous enucleation. It should be noted that the masses of actin in CD-treated cells, like the stress fibers from which most are derived, were visualized by staining with fluoresceinated phalloidin. Since phalloidin binds only to F-actin (54), these staining patterns give evidence that the rearranged actin has remained filamentous, as previously demonstrated by the ability of these masses to bind heavy meromyosin (35).

The cells in Fig. 4a through d were treated for 24 h. However, reorganization of the cytoskeleton is initiated rapidly upon treatment with CD (21). Smaller aggregates were seen within 1 h of treatment (Fig. 4e and f); these subsequently congregated to form the larger masses seen at

FIG. 2. Concentration dependence of response to CD in HEp-2 cells. Cells were treated with the indicated concentrations of CD for 19 h (---) or 20 h (—) and then harvested for determination of relative actin content by the DNase 1 inhibition assay (---) or fixed and stained for light microscopic examination of the percentage of cells displaying zeiotic knobs (Fig. 3) (—). Relative actin content was calculated as units of actin per microgram of protein in CD-treated cells compared with that in DMSO-treated control cells.

FIG. 3. CD-induced zeiosis in HEp-2 cells. Treatment with 0.1% DMSO (a), 0.02 μM CD (b), 0.2 μM CD (c), or 2.0 μM CD (d) was for 20 h. Cells were fixed and stained with hematoxylin-azure-crystal violet. Note clusters of zeiotic knobs (arrows) in c and d, and protruding nucleus (arrowhead) in d. Bars, 20 μm .

later times (Fig. 4c and d). This migration of actin masses entrains the simultaneous migration of the zeiotic knobs, which they underlie, into a cluster by a process resembling and presumably analogous to ligand-induced capping (see references 19 and 21 for discussion).

Time course of CD-induced rise in actin content. The time course for the rise in actin content during treatment of HEp-2 cells with 0.2 or 2.0 μM CD is illustrated in Fig. 5. Treatment for 5 min, sufficient time for CD to bind maximally to these cells (50), did not alter the actin content measured (data not shown); the slight apparent decline in actin content with 1.5 h of treatment was not statistically significant.

Dependence on protein synthesis. To determine whether this rise in actin content during treat-

ment with CD was dependent on new protein synthesis, HEp-2 cells were tested for their ability to accumulate actin while protein synthesis was inhibited with cycloheximide. Monolayers of HEp-2 cells were treated for 16.5 to 18.0 h with 2 μM CD or 0.1% DMSO (controls) in the presence or absence of cycloheximide (10 $\mu\text{g}/\text{ml}$). This concentration of cycloheximide inhibited protein synthesis by 95%, but did not depress viability (by trypan blue exclusion) or inhibit the characteristic morphological response to CD in these cells. Cycloheximide treatment prevented the CD-induced increase in relative actin content (Table 2), suggesting that new protein synthesis was required for the accumulation of "excess" actin in CD-treated cells.

Relative rate of actin synthesis in CD-treated

FIG. 4. Effect of CD treatment on distribution of filamentous actin in HEP-2 cells. Treatment was for 23 h with 0.1% DMSO (a), 0.02 μM CD (b), 0.2 μM CD (c), or 2.0 μM CD (d) or for 1 h with 0.2 μM CD (e) or 2.0 μM CD (f). Cells were stained with fluoresceinated phalloidin to visualize filamentous actin. Note that stress fibers are present in cells treated with 0.1% DMSO (a) or 0.02 μM CD (b), but are replaced by aggregated masses of actin-containing material in cells treated with 0.2 μM CD (c and e) or 2.0 μM CD (d and f). The small masses present after 1 h of treatment (e and f) migrate together to form the larger masses seen after longer treatment (c and d). Bars, 20 μm .

FIG. 5. Time course of actin accumulation in CD-treated HEP-2 cells. After treatment with 0.2 μ M CD (---), 2.0 μ M CD (—), or 0.1% DMSO (controls) for the indicated time, cells were harvested, and their actin content was determined by DNase 1 inhibition assay. The percentage of control indicates units of actin per microgram of protein in CD-treated cells compared with that in DMSO-treated (control) cells.

HEP-2 cells. The possibility that this required protein synthesis represented a preferential increase in the rate of actin synthesis relative to other proteins was tested by pulse-labeling CD-treated cells with [35 S]methionine for 1 h before harvest. Figure 6 is a radioautogram of the pattern of proteins from lysates of HEP-2 cells separated on a 9% polyacrylamide slab gel. An equal amount (100,000 cpm) of hot trichloroacetic acid-precipitable labeled material was loaded into each well to allow visual comparison. The lysate depicted in lane 2 was from cells treated with 0.2 μ M CD for 23.5 h (1-h labeling period in the continued presence of CD commenced at 23.5 h); cells for lane 1 were treated with 0.1% DMSO, and cells for lane 3 were treated with 2 μ M CD. Under these conditions, the relative rate of actin synthesis (arrow indicates actin band) appeared to increase in CD-treated cells. The rate of synthesis of two high-molecular-weight proteins, which comigrated with smooth muscle myosin heavy chain and filamin, also appeared to be increased (Fig. 6).

The apparent increase in the relative rate of actin synthesis with CD treatment seen in this

gel pattern was confirmed by cutting the actin band from each lane, eluting the labeled material into scintillation cocktail (see above), and counting the [35 S]methionine-labeled actin. Table 3 is a compilation of results from this gel pattern and several others. There was a concentration-dependent increase in the relative rate of actin synthesis when cells were treated with 0.2 or 2.0 μ M CD for 23.5 to 24.0 h. At 0.02 μ M, however, CD did not produce an increase in the relative rate of actin synthesis, correlating well with the failure of this low concentration of CD to induce an increase in actin content (Fig. 2). An elevation of the relative rate of actin synthesis in HEP-2 cells treated with 0.2 or 2.0 μ M CD could be detected as early as 1 h after the beginning of CD treatment (i.e., a 1-h labeling period in the presence of CD began after a 1-h prior CD treatment) (Table 3). In contrast to the response at about 24 h, this early response of actin synthesis to CD treatment did not appear to be concentration dependent for 0.2 or 2.0 μ M CD.

DISCUSSION

Treatment of HEP-2 cells with CD at concentrations which elicit changes in cellular and cytoskeletal morphology produced an elevation both in the relative content of actin (units of actin per microgram of protein) and in the relative rate of actin synthesis (proportion of total [35 S]methionine incorporation into actin). Like the effects of CD on morphology, these effects on actin metabolism were reversible when the drug was washed from the cells and they were allowed to recover (unpublished observation). The data on actin content were primarily obtained by the DNase 1 inhibition assay (Tables 1 and 2, Fig. 2 and 5), because this technique allowed precise quantitation of multiple samples. The relative increase in actin content was also confirmed by densitometry of one-dimensional polyacrylamide gel electrophoretic patterns (Fig. 1) and inspection of two-dimensional patterns (not shown), which indicated that both the β and γ species of actin were affected. This response to CD is not unique to HEP-2 cells; a rat fibroblast line, Hmf, also exhibited a CD-

TABLE 2. Effect of cycloheximide on accumulation of actin in CD-treated HEP-2 cells

Treatment	U of actin/ μ g of protein $\pm$ SD (% of control $\pm$ SD)	
	Without cycloheximide	With cycloheximide
DMSO, 0.1%	3.57 $\pm$ 0.12 (100)	4.07 $\pm$ 0.14 ^a (100)
CD, 2 μ M	4.34 $\pm$ 0.08 (122 $\pm$ 5)	3.51 $\pm$ 0.20 ^a (86 $\pm$ 6)
DMSO, 0.1%	3.54 $\pm$ 0.23 (100)	3.25 $\pm$ 0.06 ^b (100)
CD, 2 μ M	4.45 $\pm$ 0.26 (126 $\pm$ 11)	2.98 $\pm$ 0.16 ^b (92 $\pm$ 5)

^a Cycloheximide (10 μ g/ml) treatment for 18 h.

^b Cycloheximide treatment for 16.5 h.

FIG. 6. Relative rate of actin synthesis in CD-treated HEP-2 cells. Radioautogram shows pattern of polypeptides from cell lysates separated on an SDS-polyacrylamide slab gel. Cells were treated with 0.1% DMSO (lane 1), 0.2 μ M CD (lane 2), or 2.0 μ M CD (lane 3) for 23.5 h and then labeled with [35 S]methionine for 1 h in the continued presence of the drug. To facilitate comparison, an equal number of counts per minute of each sample was loaded on the gel. The arrow indicates the location of the actin marker. Other markers indicated by bars are as follows (top to bottom): chicken smooth muscle filamin, myosin heavy chain, phosphorylase A (92,500 daltons), and bovine serum albumin (66,200 daltons).

induced increase in actin content (J. Brett and G. C. Godman, manuscript in preparation).

The elevation in relative actin content in CD-treated HEP-2 cells appears to result from an increased relative rate of actin synthesis. Increased synthesis of actin was observed at concentrations of CD (2.0 and 0.2 μ M) which induced a rise in actin content and not at a concentration (0.02 μ M) which did not elevate actin content (Fig. 2, Table 6). In addition, the increased rate of actin synthesis preceded the accumulation of extra actin in the cell (Fig. 5, Table 6), and this accumulation of actin did not occur in the absence of protein synthesis (Table 2). We might also consider the possibility, though, that the formation of actin-containing aggregates in CD-treated cells might render the actin in them less accessible to the usual mecha-

nisms of protein degradation. Then increased relative actin content would be caused by a selective decrease in its rate of turnover. Such a mechanism was invoked to explain the increased actin content in the liver of phalloidin-treated rats (R. B. Low and G. Gabbiani, *J. Cell. Biol.* **87**:223a, 1980). However, the ability of cycloheximide to block the CD-induced increase in actin content argues against this mechanism in the case of CD (Table 2). In addition, phalloidin reportedly decreased the susceptibility of F-actin to *in vitro* proteolysis, whereas cytochalasin B, at least, increased this susceptibility (12). If these *in vitro* results on pure F-actin apply to relative rates of degradation of actin *in vivo*, phalloidin would be expected to decrease the rate of turnover for actin, but cytochalasins would be expected to increase it. Further work is planned to determine whether CD alters the rate of actin turnover.

Changes in actin content and synthesis have been observed in a variety of nonmuscle systems undergoing development and differentiation (10, 11, 24, 26, 32, 37, 39, 45) and in some cases of transformation (14), but not in others (42). Small, transient changes in the relative rate of actin synthesis (33; but see reference 6) and in its content (53) as a function of position in the cell cycle have been reported for synchronized HeLa cells. Stimulation of noncycling Swiss 3T3 cells to enter S-phase triggered a preferential increase in actin synthesis (40) which was not seen during the G1 to S transition in cycling cells (41). These latter observations suggest that small, temporary adjustments in the level of actin and its rate of synthesis may be a normal accompaniment of cell growth. It has been proposed that cytoskeletal organization contributes to such regulation of actin synthesis. When suspension cultures of 3T6 fibroblasts, which are anchorage dependent for growth, were allowed to attach to a solid substrate, they synthesized actin at an elevated rate during the initial hours of spreading (2); this enhanced synthesis appeared to depend on new mRNA synthesis (13).

We do not yet know whether the CD-induced response in HEP-2 cells is unique to actin. The densitometric scans gave evidence that the content of a second protein of unknown identity (ca. 77,000 daltons) might be selectively increased in CD-treated HEP-2, but pulse-labeled samples (Fig. 6) did not show an obvious increased rate of synthesis for protein bands in this region of the gel. A comparison of the radioautograms of lysates from pulse-labeled, CD-treated cells and from control cells (Fig. 6) suggested that some high-molecular-weight proteins, which comigrated with chicken gizzard myosin heavy chain and filamin, also might exhibit an increased

TABLE 3. Effect of CD on relative rate of actin synthesis in Hep-2 cells

Treatment	Time of treatment (h)	Relative rate of actin synthesis ^a	% of control
0.1% DMSO	23.5	5.04	100
0.2 μ M CD	23.5	5.66	112
2.0 μ M CD	23.5	7.21	143
0.1% DMSO	1.0	5.26 $\pm$ 0.11 ^b	100
0.2 μ M CD	1.0	5.98 $\pm$ 0	114 $\pm$ 2 ^b
2.0 μ M CD	1.0	6.22 $\pm$ 0.22	118 $\pm$ 4
0.1% DMSO	23.5	4.53 $\pm$ 0.25	100
0.2 μ M CD	23.5	5.87 $\pm$ 0.47	130 $\pm$ 13
2.0 μ M CD	23.5	6.96 $\pm$ 0.54	154 $\pm$ 15
0.1% DMSO	24.0	5.31	100
0.02 μ M CD	24.0	5.61	106
2.0 μ M CD	24.0	7.85	148

^a Percentage of total trichloroacetic acid-precipitable counts per minute incorporated into actin band in polyacrylamide slab gels (1-h pulse-label with [³⁵S]methionine).

^b Data are presented $\pm$ standard deviations for duplicate loadings of sample on separate polyacrylamide slab gels.

relative rate of synthesis in CD-treated Hep-2 cells. Since the densitometric scans shown in Fig. 1 did not completely resolve these bands from others in the same region at the top of the cylindrical gels, we have not determined whether the relative content of these proteins increased with CD treatment. Further work to identify the proteins involved is planned.

The mechanism for the CD-induced effects on actin metabolism remains to be determined, but our present results allow us to exclude some possible explanations. Because 2 μ M CD caused nuclear protrusion (which infrequently resulted in spontaneous enucleation), the question is raised of whether the relative increase in actin synthesis might be dependent on impairment of nuclear function. If production of new mRNA were decreased as a result of incapacitation of the protruding nucleus or outright enucleation, there could be a selective relative increase in translation of actin, assuming that its mRNAs were more stable than some other remaining in the cytoplasm. However, this explanation is unlikely, because augmentation of relative actin content and synthesis was also observed at 0.2 μ M CD, a concentration which did not trigger nuclear protrusion or enucleation (Fig. 3) (34).

Various agents are known to be able to induce synthesis of the characteristic pattern of "heat-shock proteins" triggered by exposure to elevated temperatures (1). Since actin synthesis is reportedly not increased during heat shock (23, 27), the effect of CD on actin synthesis is evidently not related to this phenomenon. The possibility that there is a relationship between the ca. 77,000-dalton protein affected by CD

(Fig. 1) and the 72,000- to 74,000-dalton (47) or 68,000- to 70,000-dalton (23, 51) heat shock protein of HeLa cells, which may be associated with the cytoskeleton (25, 51), remains to be explored.

Hep-2 cells treated with CD initially exhibited extreme retraction, becoming less flat and well spread than DMSO-treated control cells (data not shown). These changes were most evident within the first few hours of treatment, before a secondary, "pseudospreading" occurred (21, 34) to give the morphology illustrated in Fig. 3. Cells did not detach, but they were less closely associated with their neighbors. As a tumor-derived cell line, Hep-2 cells may not be absolutely dependent on substrate attachment for growth and would not be expected to exhibit density-dependent regulation of growth. But it could be proposed that actin content and synthesis would be influenced to some extent by cell-cell or cell-substrate associations, because the organization of microfilaments was shown to be influenced by cell density in the closely related HeLa tumor cell line (16).

Creation of areas of cell-free substrate between cells upon CD-induced cell retraction might thus have been responsible for triggering a difference between the actin content of CD-treated cells compared with the more crowded (i.e., not retracted) DMSO-treated control cells. However, the data in Table 1 demonstrated that CD increased actin content to an equal degree in subconfluent and confluent cultures. This lack of difference in response to CD was despite the fact that the subconfluent cultures already had areas of cell-free substrate around many cells,

whereas such areas were only exposed in the confluent cultures after CD treatment. It therefore seems unlikely that decrease in the area of cell-cell contact because of CD-induced cell retraction was responsible for the observed changes in actin metabolism. In addition, we have found that inhibition of normal spreading may not in itself be sufficient to induce changes in actin metabolism. When HEP-2 cells were cultured in bacteriological-grade plastic dishes, on which they spread poorly, the relative actin content did not increase; preliminary results indicated that the relative rate of actin synthesis also was not increased (unpublished observation). Thus, inhibition of normal adherence and spreading by growth on an altered substrate did not mimic the effects of CD on actin metabolism.

The observation that the same range of CD concentrations elicited rearrangement of cytoskeletal actin into aggregated masses (Fig. 4), increases in actin content (Fig. 2), and elevation of the rate of actin synthesis (Table 3) suggests that these effects on the morphology and metabolism of cellular actin may be interrelated. One possible relationship, that the aggregates of actin form solely as a consequence of the presence of extra actin in the cells, is clearly ruled out by the time course of the two responses. Whereas the rearrangement of actin occurred rapidly, within 1 h of treatment (Fig. 4e and f), the rise in actin content was gradual (Fig. 5). Conversely, though, the rapid cytoskeletal reorganization brought about by CD might trigger the changes in metabolism of actin. Since the morphological response to CD begins within minutes of exposure to the drug (21, 35), the changes in cytoskeletal arrangement are a reasonable candidate to be the inducer of actin synthesis in CD-treated HEP-2 cells.

As mentioned previously, anchorage-dependent 3T6 fibroblasts which had been in suspension cultures were reported to undergo an induction of actin synthesis during the process of respreading on solid substrate (2, 13). It is tempting to speculate that our observations on the effects of CD may also be an example of this induction of actin synthesis, apparently in response to a signal generated by rearrangement of the cytoskeleton. Our present observations do not allow us to choose which of the observed effects of cytochalasins on actin and cytoskeletons (see above) might be the direct trigger of this change in the metabolism of actin. In any case, though, CD-treated cells offer a useful model system for examining the factors which regulate cellular actin content and synthesis.

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