On the Dynamics of the Microfilament System in HeLa Cells

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ABSTRACT We measured the pools of unpolymerized and filamentous actin in homogenates of HeLa cells made in several different lysis buffers, as well as after treatment of cells with a variety of chemicals or trypsin, and after adenovirus (type 2) infection. This was possible when a series of factors concerning the basic culture conditions were kept constant: e.g., serum type used, serum batch, cell density, time after subcultivation of cells, and buffering substance in the medium. Homogenates from untreated cells usually contain 35–45% of the total actin in an unpolymerized form. With some batches of cells this number can be as high as 50%. In sparse cultures (3×10^4 cells/cm^2), HeLa cells contain ~10 pg actin/cell, while the corresponding number is only 5 pg in dense cultures (3×10^5 cells/cm^2). Treatment of cells with cytochalasin B increases the pool of unpolymerized actin by ~30–40%, while colchicine decreases the fraction of unpolymerized actin by 20%. The oxidant diamide increases the filamentous actin pool 25–50%. Glucose, sodium azide, dinitrophenol, serum starvation, or thymidine treatment does not affect the distribution between unpolymerized and filamentous actin to any significant extent. Trypsin and EDTA induced rounding up of cells but did not change the actin distribution. The distribution of actin between G- and F-forms was unchanged after adenovirus infection. These results show that significant changes in the actin pools can be induced in nucleated cells. However, several treatments which alter the morphology and motility of cells are not accompanied by an alteration in the G-/F-actin ratio.

The cellular distribution of actin and the role that actin-containing structures has for a variety of cellular functions have been investigated for a number of years. It has been postulated that a shift in the equilibrium between actin in monomeric form and actin in polymeric form is responsible for many of these functions. In the last several years some direct biochemical evidence has been presented for the reorganization of actin between unpolymerized and filamentous forms in conjunction with changes in motility (5, 6, 14, 24, 26). These results have been obtained primarily by quantitative measurement of the different forms of actin by the DNAse inhibition assay (1). The specific aims of this investigation have been to answer questions such as the following: (*a*) How universal are changes in the G-/F-actin ratio in relation to motility and morphology changes? (*b*) How extensive might these changes be? And (*c*) can something be learned about activities regulating reorganizations of actin by applying inhibitors of various kinds? We therefore measured actin pools in HeLa cell homogenates to evaluate how they are influenced by the growth conditions of the cells, the lysis conditions, and treatment of cells with a variety of chemicals and biochemicals.

MATERIALS AND METHODS

Cell Culture

Stock cultures of HeLa cells (type S) were maintained at 37°C in closed plastic bottles (81 cm^2, Nunclon Labora, Gothenburg, Sweden) in Eagle's minimal essential medium (Eagle's MEM) supplemented with 10% calf serum (Gibco Europe Ltd., Glasgow, Scotland), 10 mM HEPES, pH 7.3, and 5 μg/ml gentamycin sulfate, if not otherwise stated. In some experiments, 10% fetal calf serum (FCS) or 10% newborn calf serum (NCS) was used. Replating of cells was done by applying 10 ml of Eagle's MEM containing 2.5 mg/ml of trypsin and 5 mM EDTA onto the cells, aspirating the trypsin/EDTA solution after 30 s, and incubating for a further 5 min, at 37°C. Then the cells were suspended by pipetting 10 ml of fresh Eagle's MEM containing serum into the bottles, pelleted at low speed (1,000 *g* for 45 s), and resuspended in Eagle's MEM (with serum). Cells used for an experiment were subcultured onto Nunclon plastic petri dishes (90 mm diameter [ϕ]) at ~1/10 the original concentration and incubated at 37°C in humidified 5% CO_2–95% air mixture until the cultures had reached confluence (usually 3–4 d). In later experiments cells were subcultured at a density of 10 or 5×10^6 cells/plate and incubated for 1 or 2 d, respectively, before the actin measurements were made. At this stage the cells were near confluence. For morphological observations of the lysis process, cells were subcultured onto round glass cover slips (24 mm ϕ) which previously had been soaked for 5 min in a poly-L-lysine solution (5 mg/ml; average mol wt 400,000).

Actin Measurements

Before actin pool measurements (1), each plate was thoroughly rinsed twice with phosphate-buffered saline (PBS) at room temperature unless otherwise stated. Cells from one plate were then directly scraped from the plate with a rubber policeman in 100 μl of lysis buffer (see below) and incubated at room temperature. Estimation of the total volume of the homogenates was performed with an isotope dilution technique. For measurements of total actin, an aliquot of the homogenates was routinely diluted five or ten times with lysis buffer before mixing with the guanidineHCl solution. To obtain reproducible results when

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measuring the total actin, aliquots must be treated with guanidineHCl before an extensive aggregation of cell components and nuclei occurs (see Results). Therefore, we usually treat an aliquot with the guanidineHCl solution immediately after lysis of the cells and before the first measurement of unpolymerized actin. The actual measurements of DNAse inhibitor activity were performed as described by Blikstad et al. (1), except that the Zeiss PMQ3 spectrophotometer was connected to a microcomputer (ABC 80, Luxor, Motala, Sweden) for which we have developed a program that takes care of the data recording, storage, and processing in a semiautomatic way. This allows one person to easily measure four samples every 3 or 4 min.

The DNAse (DN-100, Sigma Chemical Co., St. Louis, MO) was purified and calibrated against purified rabbit skeletal muscle actin as described earlier (1, 6). The specific inhibitor activity was estimated to 90,870 ± 890 (n = 14) inhibitor U/mg actin.

Lysis Buffers

All lysis buffers were freshly made and stored at +4°C for not more than a week. The following buffers were used for lysis of cells:

MgISO buffer: 150 mM NaCl, 2 mM $MgCl_2$, 10 mM potassium phosphate, pH 7.6, 0.2 mM ATP, 0.2 mM dithioerythritol (DTE), 0.01 mM phenylmethylsulfonylfluoride (PMSF), and 0.5% Triton X-100.

PEG buffer: 140 mM NaCl, 5 mM $MgCl_2$, 10 mM Tris-HCl, pH 7.6, 4% polyethylenglycol (PEG) 6,000, 0.2 mM DTE, and 0.5% Triton X-100.

PolyAm buffer: 150 mM KCl, 5 mM $MgCl_2$, 5 mM ammonium acetate, 5 mM potassium phosphate, pH 7.0, 8 mM putresceine, 1 mM spermidine, 0.2 mM DTE, and 0.5% Triton X-100.

F buffer: 100 mM NaF, 50 mM KCl, 2 mM $MgCl_2$, 10 mM potassium phosphate, pH 7.6, 0.2 mM DTE, and 0.5% Triton X-100.

MT buffer: 100 mM PIPES, pH 6.75, 1 mM $MgCl_2$, 1 mM GTP, 1 mM EGTA, 0.2 mM DTE, 1 M sucrose, and 0.5% Triton X-100.

SF buffer: 100 mM NaF, 50 mM KCl, 2 mM $MgCl_2$, 1 mM EGTA, 10 mM potassium phosphate, pH 7.0, 0.2 mM DTE, 1 M sucrose, and 0.5% Triton X-100.

Chemicals and Other Reagents

Cytochalasin B was dissolved in dimethylsulfoxide. All other chemicals and biochemicals used to treat cells were dissolved in Eagle's MEM. Fine chemicals and drugs were obtained from Sigma Chemical Co. The adenovirus type 2 was a kind gift of Professor Lennart Philipson at the Biomedical Center, Uppsala.

RESULTS

Characterization of the MgISO Lysis Buffer

The lysis buffer for actin measurements was originally designed such that both filamentous and unpolymerized actin (possibly in the form of profilactin) should be kept stable (1). Therefore, the buffer contained both mono- and divalent cations, ATP, and a reducing agent, DTE (this buffer is called MgISO buffer).

A long series of measurements of actin pools performed on HeLa cell homogenates showed that a rather rapid increase in the pool of unpolymerized actin (depolymerization) occurred during the first 10–20 min after lysis. In some cases the first measured value of unpolymerized actin (1–3 min after lysis) was as low as 25–30% of the total actin, while in other experiments the initial value was 40–50%. Usually, a constant value which ranged between 50 and 60% unpolymerized actin was obtained after 20 min of incubation. One typical example is shown in Fig. 1. This apparent depolymerization of filamentous actin in HeLa cell homogenates is different from what has been observed in homogenates of pancreatic beta-cells where a polymerization has been observed (26). However, in some types of extracts of human platelets a depolymerization has been detected (6). The depolymerization observed in HeLa cell homogenates is, in contrast to platelet extracts, not affected by the free Ca^{2+} concentration, since inclusion of 5 mM EGTA in the lysis buffer did not have any significant effect. Several other modifications of the lysis buffer were tested but had no effect on the stability of the actin pools: increasing the Mg^{2+} concentration (up to 20 mM), adding a variety of protease inhibitors (PMSF, TPCK, and TLCK), incubating the homogenates at +4°C instead of at room temperature.

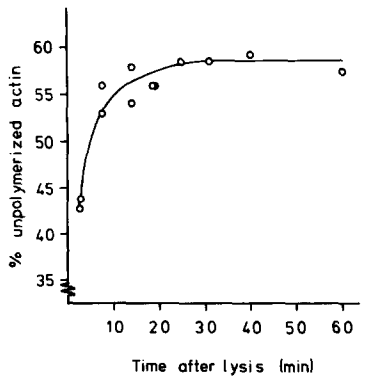

FIGURE 1 Determination of actin pools in homogenates of HeLa cells. Cells from one petri dish (90 mm ϕ) were lysed in 100 μl of MgISO buffer as described in Materials and Methods. DNAse inhibitor activity in the homogenate was determined at different times after lysis both before and after guanidineHCl treatment. The results are expressed as % unpolymerized actin of the total amount (+ guanidineHCl). The total actin concentration in the sample was ~0.7 mg/ml.

Characterization of the Growth Conditions

During these experiments it was obvious that various factors concerned with the culturing of the cells were important for the final result obtained in the actin measurements. We investigated this in some detail, while lysing the cells in the MgISO buffer as described above.

Long-term culture of cells (≥1 month) in different types of sera influences to a great extent the results obtained in the actin measurements. If HeLa cells were cultured in FCS instead of ordinary calf serum or NCS, both the lysis behavior and the actin pool measurements were affected. After application of the detergent-containing buffer to the cells, the remaining cellular debris and intact nuclei usually form large aggregates and eventually clump together into one piece. The time it takes for this aggregation to reach completion is much shorter when cells have been cultured in FCS (1/2–1 min), as compared with cells grown in ordinary calf serum (2–5 min). The fraction of actin in an unpolymerized form was only ~25–30% if the cells were grown in FCS, and this distribution was quite stable during 30–60 min of incubation of the homogenates (data not shown; compare Fig. 1). However, the fast and extensive aggregation of cellular debris represented a technical problem in taking a representative sample for measurement of the total amount of actin (+guanidineHCl).

Not only differences between serum types have been observed. Different batches of calf serum, and even the same batch after prolonged storage (−20°C for >6 months), gave different results in the actin measurements. An increased percentage of unpolymerized actin and a more extensive depolymerization of filaments are observed when cells are cultured in the presence of older batches of serum or with aliquots that have been repeatedly freezed and thawed (data not shown).

Several other parameters are important to keep as constant as possible to get reproducible results in the actin determinations. Cell density, the protocol used for removal of cells from the substrate at subcultivation, time in culture after last sub-

cultivation, and choice of buffering substance in the culture medium (HEPES, PO_4^{2-} or H_2CO_3) must be controlled since all these parameters influence the growth of the cells and the result of the actin measurements.

Characterization of Lysis Buffers: Morphological Observations and Actin Measurements

Since the MgISO buffer was not sufficient to give a stable distribution of different forms of actin in HeLa cell homogenates, we started to test a series of other buffer compositions. Primarily, we used light microscopic observation of the lysis process as a rapid assay. We reasoned that if it were possible to find some conditions under which most of the cell morphology was kept intact, even after permeabilization of the cell membrane with detergent, these conditions might better stabilize the organization of actin as it was inside the cell.

Cells were cultured on poly-L-lysine-coated glass cover slips and, for an experiment, mounted either in a chamber where the medium can be replaced (total volume ~200 μl) or in a simple chamber constructed by directly inverting a glass cover slip on top of ~100 μl lysis buffer which has been placed on a microscope slide inside a thin teflon ring. Observing cells in a phase-contrast microscope continuously during the lysis process showed that complete disruption of cells is obtained within seconds after application of all the Triton X-100-containing buffers tested here. A more or less pronounced retraction of the cell edge and contraction of cell processes occurs during the first 10–20 s in most, but not all, of the solutions tested. Therefore, this is the most critical part of the lysis procedure, during which large changes in the organization of actin might be induced simply by breaking up the cells. Most of the cytoplasmic structures are rapidly solubilized in the MgISO buffer, and after 5–10 min many of the remaining nuclear residues lose their contact with the cover slip (Fig. 2 *b* and *c*). The cells are similarly rapidly solubilized and disrupted when lysed in a poly-amine-containing buffer (PolyAm; Fig. 2 *d* and *e*). If polyethyleneglycol (PEG) is included in the lysis buffer

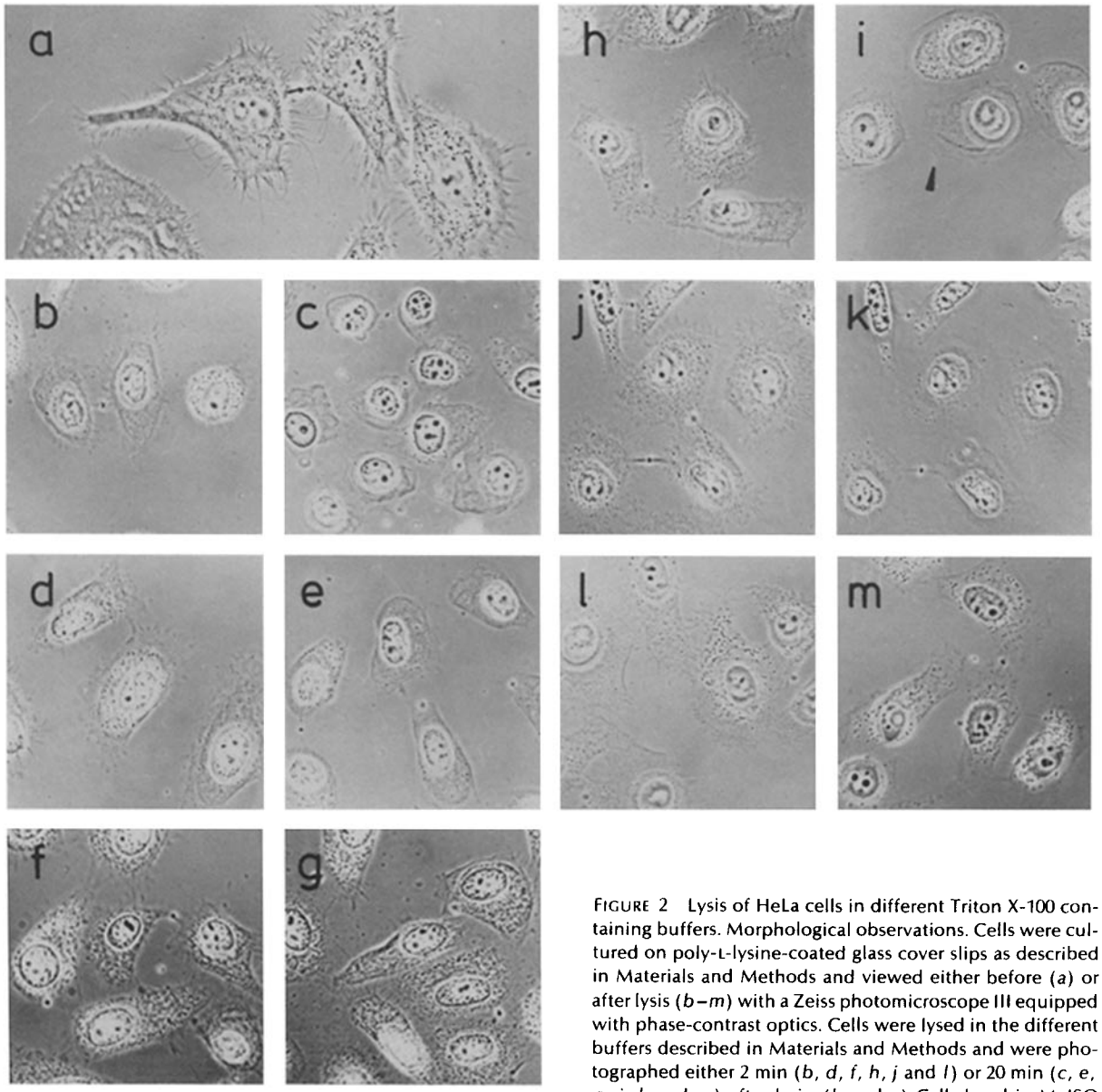

FIGURE 2 Lysis of HeLa cells in different Triton X-100 containing buffers. Morphological observations. Cells were cultured on poly-L-lysine-coated glass cover slips as described in Materials and Methods and viewed either before (*a*) or after lysis (*b–m*) with a Zeiss photomicroscope III equipped with phase-contrast optics. Cells were lysed in the different buffers described in Materials and Methods and were photographed either 2 min (*b*, *d*, *f*, *h*, *j* and *l*) or 20 min (*c*, *e*, *g*, *i*, *k* and *m*) after lysis. (*b* and *c*) Cells lysed in MgISO buffer. (*d* and *e*) Cells lysed in PolyAm buffer. (*f* and *g*) Cells lysed in PEG buffer. (*h* and *i*) Cells lysed in F buffer. (*j* and *k*) Cell lysed in MT buffer. (*l* and *m*) Cell lysed in SF buffer. *a*, × 750. *b–m*, × 450.

(as in Figure 2*f* and *g*), much of the original phase density of the cells is preserved, or even increased after lysis. However, during the first 2 min after application of the PEG buffer to the cells, a large number of spherical surface protrusions (or blebs) are formed which later seem to disrupt and disappear. Because of this unexpected vacuolization and extensive contraction of the cell borders in PEG buffer, this solution was not further used for actin measurements.

The next solution tested for lysis of cells contained a rather high concentration of sodium fluoride (100 mM) instead of sodium chloride (the F buffer). As seen in Fig. 2*h* and *i*, more of the original cell morphology is preserved early after lysis when compared with the above described buffers. After prolonged incubation (10–20 min), material is solubilized and the cell residues lose phase density. However, under these conditions two parts of the cytoplasm can be distinguished with different solubility properties: one thin and easily extractable peripheral part (at the arrow in Fig. 2*i*), and a second more centrally located and thicker part perhaps corresponding to the keratin and vimentin fiber distribution (10, 15).

In a microtubule (MT)-stabilizing buffer (containing EGTA and a high concentration of sucrose), most of the original cellular morphology and the detailed outlines of the cell periphery are retained for at least 30 min of incubation (Fig. 2*j* and *k*). However, the images rapidly lose phase density to a great extent. This might be caused by changes in the refractive index as a result of the high sucrose concentration in the solution. We finally tested a buffer containing both fluoride and sucrose (called SF buffer; see Fig. 2*l* and *m*). In principle, with this buffer, the results was similar to that with the MT buffer. However, one major difference was observed when cells were scraped from the plates in SF or MT buffers. As described earlier for the MgISO buffer, the cell debris and nuclei aggregate after some time of incubation of the homogenates. In the presence of sucrose (as in the MT buffer), this process is slowed down and takes usually about 10 min to reach completion. In the SF buffer this aggregation is completely inhibited, even after 24 h of incubation of the homogenates.

Fig. 3 shows representative results of actin pool determinations from cells lysed in the different buffers described above. It is obvious that lysing cells in the MT or SF buffer results in a more stable distribution of unpolymerized and filamentous actin in the homogenates as compared with the MgISO buffer or any of the other buffers tested. This result could be expected from the morphological observations. The mean value of the percentage of unpolymerized actin is 43.7 ± 1.7% (n = 10) and 40.4 ± 1.9% (n = 4) in the MT and SF buffers, respectively.

One other factor of importance for the stability of actin pools in HeLa cell homogenates became clear during these experiments. The initial rate of depolymerization in MgISO buffer always showed large variations between different experiments. Therefore, we tested whether the washing procedure (two times with PBS) affected the results of the actin measurements. Excluding the wash, or washing with fresh Eagle's MEM (without serum), seemed to induce a rapid and even further depolymerization of actin. The measured value of unpolymerized actin was ~60–70% at the first measurement (see Fig. 3*a*). Furthermore, if cells were washed with PBS or MgISO buffer (without Triton X-100) and then lysed with Eagle's MEM (−serum) containing Triton X-100, the high level of unpolymerized actin was also obtained (data not shown). These experiments show that one part of the apparent depolymerizing factor, or factor accelerating a depolymerizing activity, can be washed away (or inhibited) by PBS but added back with

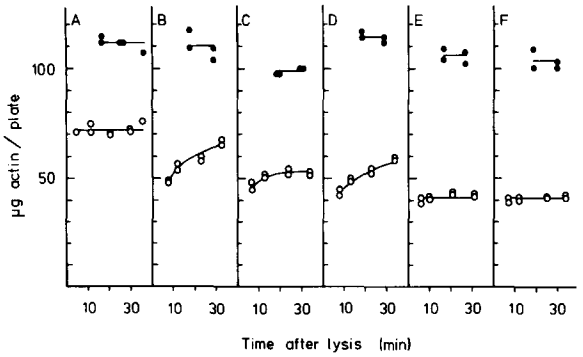

FIGURE 3 Lysis of HeLa cells in different Triton X-100 containing buffers. Actin pool determinations. For each experiment, one petri dish (90 mm ϕ) with HeLa cells was taken to actin determinations as described in Materials and Methods. Cells were lysed either without (*A*) or with a prior wash with PBS (*B–F*) in the MgISO buffer (*A* and *B*), in the PolyAm buffer (*C*), in the F buffer (*D*), in the MT buffer (*E*), or in the SF buffer (*F*). DNAse inhibitor activity (unpolymerized actin) in the homogenates was determined at different times after lysis (○). Directly after lysis (2 min), 20 µl of the homogenates was diluted five times with lysis buffer and treated with guanidineHCl solution (Blikstad et al. 1978), before the total amount of actin was determined (●). The results are expressed as µg actin/plate, calculated from the estimated specific inhibitor activity of 90,870 (see Materials and Methods).

Eagle's MEM at the moment of lysis. This factor is not Ca^{2+} ions, since inclusion of 2 or 10 mM EGTA in the Eagle's MEM used for lysis did not influence the depolymerization observed. We have not yet identified what component(s) in the Eagle's MEM is (are) responsible for this effect on the actin pools. The same result has been observed when cells are lysed in MT buffer in the presence of growth medium (data not shown). A rapid depolymerization was observed, reaching up to 70–80% unpolymerized actin after 10 min as compared with a stable level ~35% (compare Fig. 3*e*) in homogenates of cells washed with PBS before lysis.

As has been described above, there are many factors that influence actin measurements on cell homogenates. For the following experiments we chose to wash cells twice with PBS before lysis in either MT or SF buffer. With each batch of cells used (each experimental day) untreated controls were measured in parallel with cells treated in different ways. Such pairs of plates were always used in the calculations of possible effects of the different treatments (see below and Tables I–IV).

Effect of Chemicals, Proteolytic Enzymes and Viral Infection on the Organization of Actin

After the growth and lysis conditions had been established as described above, we performed some physiological experiments to test the hypothesis that the distribution of actin between unpolymerized and filamentous forms might be changed after various treatments affecting the morphology or motility of cells. The mushroom poison cytochalasin B is known to disrupt the distribution of actin-containing stress-fibers and impair motility of various kinds (for review, see reference 27). When actin pools were measured after treating cells with relatively low concentrations of cytochalasin B, an increase was seen in the unpolymerized pool of actin (Table I). If the amount of unpolymerized actin of treated cells is expressed as percent of the unpolymerized pool of untreated plates (measured the same day), then a significant increase of 30%–40% of the unpolymerized pool is observed, depending on

TABLE I

Effect of Cytochalasin B on Actin Pools

Buffer	Treatment	% Unpolymerized ± SEM (*n*)	Δ%	μg actin/plate (*n*)	No. of cells/plate	pg actin/cell
MT	UT	41.5 ± 0.2 (7)	—	96.4 ± 0.8 (4)	ND	—
MT	20 μM, 60 min	55.6 ± 0.7 (6)	+14.1	98.5 ± 1.3 (4)	ND	—
MT	UT	37.9 ± 0.4 (12)	—	76.2 ± 1.1 (5)	10.5×10^6	7.25
MT	20 μM, 60 min	48.0 ± 0.5 (12)	+10.1	66.8 ± 0.3 (4)	10.5×10^6	6.36
MT	20 μM, 60 min	48.0 ± 0.6 (12)	+10.1	83.5 ± 0.7 (5)	10.5×10^6	7.96
SF	UT	39.9 ± 0.4 (8)	—	103.3 ± 2.0 (4)	ND	—
SF	20 μM, 60 min	49.9 ± 1.0 (8)	+10.0	105.7 ± 1.4 (4)	ND	—
SF	UT	35.2 ± 0.4 (8)	—	99.7 ± 0.6 (4)	ND	—
SF	40 μM, 60 min	51.0 ± 1.0 (6)	+15.8	95.3 ± 0.9 (4)	ND	—
SF	40 μM, 60 min	47.4 ± 0.7 (6)	+12.2	97.5 ± 1.0 (4)	ND	—

Actin pool measurements were performed as described in Fig. 3 and Materials and Methods. The results represent mean values ±SEM, using for the calculation all time-points from curves such as in Fig. 3 *E* or *F*. Untreated (*UT*) and treated plates measured the same day was paired (see further Table IV). The number of cells per plate was determined on duplicate petri dishes cultured under identical conditions as the plates used for actin determinations. *ND*, not determined.

TABLE II

Effect of Colchicine on Actin Pools

Buffer	Treatment	% Unpolymerized ± SEM (*n*)	Δ%	μg actin/plate (*n*)	No. of cells/plate	pg actin/cell
SF	UT	43.3 ± 0.5 (8)	—	99.4 ± 1.7 (4)	ND	—
SF	5×10^{-4} M, 3 h	33.1 ± 0.3 (8)	−10.2	101.9 ± 0.6 (4)	ND	—
MT	UT	39.2 ± 0.5 (8)	—	105.9 ± 1.7 (4)	ND	—
MT	5×10^{-4} M, 3 h	33.0 ± 0.5 (8)	−6.2	108.3 ± 2.6 (4)	ND	—
MT	UT	38.1 ± 0.7 (9)	—	68.7 ± 1.8 (5)	9.2×10^6	7.46
MT	10^{-6} M, 3 h	29.1 ± 0.4 (11)	−9.0	67.0 ± 0.9 (6)	9.2×10^6	7.29
MT	10^{-7} M, 3 h	30.3 ± 0.5 (11)	−7.8	72.1 ± 0.9 (6)	9.2×10^6	7.84
MT	UT	49.8 ± 0.6 (11)	—	88.7 ± 1.5 (6)	18.0×10^6	4.93
MT	10^{-7} M, 3 h	36.6 ± 0.6 (9)	−13.2	100.6 ± 1.3 (6)	18.0×10^6	5.59
MT	10^{-8} M, 3 h	39.8 ± 1.0 (11)	−10.0	102.2 ± 0.6 (5)	18.0×10^6	5.68

See legend of Table I for details.

TABLE III

Effect of Diamide on Actin Pools

Buffer	Treatment	% Unpolymerized ± SEM (*n*)	Δ%	μg actin/plate (*n*)	No. of cells/plate	pg actin/cell
MT	UT	48.9 ± 0.7 (8)	—	109.3 ± 1.2 (4)	ND	—
MT	5×10^{-4} M, 10 min	39.7 ± 0.8 (8)	−9.2	108.2 ± 1.0 (4)	ND	—
MT	UT	49.8 ± 0.6 (11)	—	88.7 ± 1.5 (6)	18.0×10^6	4.93
MT	5×10^{-4} M, 10 min	32.7 ± 0.4* (9)	−17.1	93.9 ± 0.8 (6)	18.0×10^6	5.22
MT	5×10^{-4} M, 60 min	26.2 ± 0.5 (10)	−23.6	100.8 ± 1.2 (6)	19.7×10^6	5.12

See legend of Table I for details.
* Slight polymerization.

the concentration of cytochalasin B used (Table IV).

As a control we also tested colchicine, which is known to disturb microtubule organization. Colchicine also had a completely unexpected effect on the organization of actin (Tables II and IV). Treatment of cells with colchicine leads to an increase of ~20% of the filamentous pool of actin, apparently irrespective of the concentration used in these experiments.

Both actin itself and unpolymerized actin in the form of the profilin:actin complex are sensitive to oxidation (4, 7, 8). We therefore tested the oxidant diamide for possible effects on the actin distribution in cells. Brief treatment with diamide results in an extensive increase of the filamentous pool of actin as measured in the homogenates (Tables III and IV). In one case, nearly 50% of the unpolymerized actin pool was converted to filaments after a 60-min incubation.

However, different manipulations with the energy metabolism of the cells, by glucose treatment (Table IV) or inhibition of oxidative phosphorylation by azide or the uncoupler dinitrophenol (data not shown), did not affect the G-/F-actin ratio to any significant extent. Neither trypsin added to the culture medium nor trypsin/EDTA-induced rounding up of cells had any effect on the actin distribution (Table IV). Other treatments that have no significant effect on the G-/F-actin ratio include blocking cells in S-phase by 10 mM thymidine (Table IV) and serum starvation (0.5% calf serum for 24 h) (data not shown). Furthermore, we have been unable to detect any significant change in the G-/F-actin ratio during infection of HeLa cells with adenovirus type 2 (data not shown).

One further observation that can be made from the collective number of experiments is that the amount of actin per cell varies considerably depending on the cell density (Fig. 4). Cells from sparse cultures (3.0×10^4 cells/cm^2) contain 10.7 ± 0.3 pg actin/cell, while cells from dense cultures (28.5×10^4 cells/cm^2) contains 5.4 ± 0.1 pg actin/cell.

TABLE IV

Effect of Different Treatments on Actin Pools

Treatment	% Unpolymerized actin of control	(n)
Cytochalasin		
20 μM, 1 h	128 ± 2*	(4)
40 μM, 1 h	139 ± 6	(2)
	132 ± 3‡	(6)
Colchicine		
5×10^{-4} M–10^{-8} M, 3 h	78 ± 2‡	(6)
Diamide		
5×10^{-4} M, 10 min	73 ± 8	(2)
5×10^{-4} M, 1 h	53	(1)
Glucose		
20 mM, 1 h	99 ± 0.2§	(2)
Trypsin		
0.1 mg/ml, 4 h	109 ± 8§	(2)
Trypsin/EDTA		
2.5 mg/ml, 6 min		
5 mM, 6 min	93 ± 2§	(5)
Thymidine		
10 mM, 20 h	97 ± 0.5§	(2)

Actin pool measurements were performed as described previously. All time points from curves such as in Fig. 3 *E* and *F* were used for the calculation of the initial percentage of unpolymerized actin. For calculations of changes in the unpolymerized pool of actin, each experimental plate was paired with an untreated plate measured the same day. The amount of unpolymerized actin of treated HeLa cells is expressed as % ± SEM of the unpolymerized pool of actin in the paired untreated control plates. Statistical significancies were calculated by Student's *t* test.

* $P < 0.01$.

‡ $P < 0.001$.

§ $P > 0.2$.

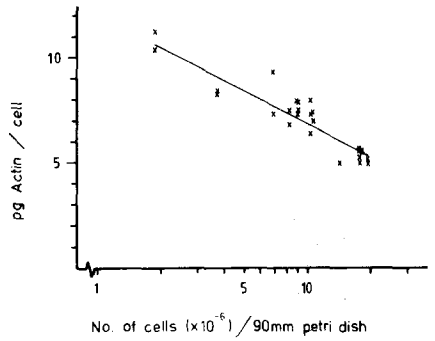

FIGURE 4 Determination of the total amount of actin per cell at various cell densities. The total amount of actin (+ guanidineHCl) per cell (as determined in experiments such as in Fig. 3 and Table I–IV) was plotted against the logarithm of the number of cells per 90 mm ϕ petri dish. The straight line drawn was fitted by linear regression analysis. The coefficient of correlation was estimated to be 0.92.

DISCUSSION

Several complicating facts have been observed when one is trying to measure actin pools in cell homogenates. Both depolymerization and polymerization reactions (1, 6, 26) have been detected, which makes it difficult to decide how large the original pool sizes might have been. Here, we have searched for conditions which both morphologically and biochemically stabilize filamentous structures in the cell. Two buffers, the MT and SF buffer, have been found to give a reliably constant distribution of unpolymerized and filamentous actin. Under these lysis conditions significant shifts in the G-/F-actin ratio have been revealed after specific treatments of the cells.

Much work has been directed toward an understanding of how cytochalasins might act to inhibit motility. Recently, several laboratories have reported on the detailed biochemical interaction between cytochalasin and the fast polymerizing end of actin filaments (2, 3, 16–18). Some years ago it was also proposed that the interaction between several actin binding proteins and filamentous actin is highly sensitive to cytochalasins (13, 25, 29). From our experiments and others (19), it is clear that the rapid and dramatic change in morphology is not accompanied by an extensive depolymerization of actin. However, contrary to Morris and Tannenbaum (19), we detect a statistically significant increase of the unpolymerized actin pool. This difference can be explained by the different lysis conditions used. The increase in the unpolymerized pool we detected might be explained simply by a disaggregation of filament bundles, thus making them more sensitive to depolymerization after lysis.

The increase in the fraction of filamentous actin obtained after colchicine treatment of cells was quite unexpected. Colchicine has been widely used as a specific probe for microtubule function. Cytoplasmic microtubules disappear (11, 22, 28) and the pool of free tubulin increases rapidly after colchicine treatment of cells (23). However, apparently normal microfilament bundles are still present in spread-out cells (11) and will form if cells are allowed to spread in the presence of colchicine (12). No direct interaction between colchicine and actin has so far been presented. Our results indicate that the organization of microtubules and microfilaments is coordinatively regulated. In an earlier communication we proposed (5) that a function or activity regulating the depolymerization of actin might be linked to intact microtubules (maybe at the peripheral ends). The relatively small increase seen in the pool of filamentous actin might be explained if the depolymerizing activity is not inhibited by the breakdown of microtubules but only compartmentalized to a less efficient position in the cell. There could also, *a priori*, be several types of depolymerization activities in the cell, and only one of these linked to microtubules. We do not yet know anything about the possible nature of this activity.

It is known that one form of unpolymerized actin, the profilin:actin complex, is sensitive to oxidants (7, 8) and can be induced to form filaments if treated with stoichiometric amounts of, for example, diamide (4). Therefore it was interesting to test whether changes in the redox potential in the cells affected the pools of actin. The increase in filamentous actin seen after treatment of cells with diamide could indicate that oxidation reactions are of prime importance in the formation of actin filaments. Several investigators have also proposed that oxidation-reduction reactions might be involved in the regulation of microtubule organization (20, 21).

One could visualize that reorganization of fibrous structures in the cell would be energy-requiring processes. Ultrastructural and biochemical observations have shown that lymphocytes respond to azide by accumulating large numbers of actin filaments (9, 14). However, the fact that no alteration in actin pools was observed after changes in the energy metabolism of HeLa cells does not necessarily exclude a tight coupling between an energy requirement and reorganization of actin. The use of other metabolic inhibitors may give a different result.

It is clear that the effects of cytochalasin, colchicine, and diamide represent significant changes in the actin pools. On the other hand, it is also clear that several treatments of cells which lead to extensive alterations in cell morphology and motility (virus infection, blocking cells in S-phase, or trypsin/EDTA induced rounding up of cells) are not coupled to an

alteration in the G-/F-actin ratio. These observations can be interpreted in several ways: (*a*) there is no need for alterations in the microfilament system; (*b*) most rearrangements involve shuffling of filaments or pieces of filaments; (*c*) only a small part of the actin participates in the necessary reorganizations; or (*d*) the turnover rate of actin monomers is so fast that the overall equilibrium is not extensively altered.

At present, it is difficult to judge what relative importance the shifts in the G-/F-actin ratio have in relation to changes in motility and morphology in general. There are clearly situations where changes do not occur. In these situations, alterations in actin filament organization (shuffling of filaments) or changes in the other fiber systems and membrane components could be of prime importance. Taken together, the effect of colchicine on the organization of actin and the effect of oxidants on both the stability of microtubules and formation of microfilaments strengthen the idea that the assembly/disassembly of microtubules and microfilaments are coordinatively regulated. Obviously, much more has to be learnt about how the fiber systems interact with each other. By applying other drugs and metabolic inhibitors, it might be possible to delineate regulatory enzymatic activities and to describe in more detail the effects of colchicine and diamide on the actin organization.

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REFERENCES

1. Blikstad, I., F. Markey, L. Carlsson, T. Persson, and U. Lindberg. 1978. Selective assay of monomeric and filamentous actin in cell extracts, using inhibition of deoxyribonuclease I. *Cell.* 15:935–943.
2. Brenner, S. L., and E. D. Korn. 1979. Substoichiometric concentrations of cytochalasin D inhibit actin polymerization. *J. Biol. Chem.* 254:9982–9985.
3. Brown, S. S., and J. A. Spudich. 1979. Cytochalasin inhibits the rate of elongation of actin filament fragments. *J. Cell Biol.* 83:657–662.
4. Carlsson, L. 1979. Cell motility. The possible role of unpolymerized actin. *Acta Univ. Ups.* 537:1–65.
5. Carlsson, L., and I. Blikstad. 1981. Colchicine treatment of HeLa cells alters the G/F actin ratio. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 124:282–284.
6. Carlsson, L., F. Markey, I. Blikstad, T. Persson, and U. Lindberg. 1979. Reorganization of actin in platelets stimulated by thrombin as measured by the DNase inhibition assay. *Proc. Natl. Acad. Sci. U. S. A.* 76:6376–6380
7. Carlsson, L., L.-E. Nyström, I. Sundkvist, F. Markey, and U. Lindberg. 1976. Profilin, a low-molecular-weight protein controlling actin polymerizability. *In Contractile Systems in Non-Muscle Tissues.* S. V. Perry, A. Marzella, and R. S. Adelstein, editors. 39–49. *Abstr. Upps. Diss. Sci.*
8. Carlsson, L., L.-E. Nyström, I. Sundkvist, F. Markey, and U. Lindberg. 1977. Actin polymerizability is influenced by profilin, a low molecular weight protein in non-muscle cells. *J. Mol. Biol.* 115:465–483.
9. de Petris, S. 1977. Distribution and motility of plasma membrane components on lymphocytes. *In Dynamic Aspects of Cell Surface organization.* G. Poste and G. L. Nicolson, editors. North-Holland, Amsterdam. 643–728.
10. Franke, W. W., E. Schmid, K. Weber, and M. Osborn. 1979. HeLa cells contain intermediate-sized filaments of the prekeratin type. *Exp. Cell Res.* 118:95–109.
11. Goldman, R. D. 1971. The role of three cytoplasmic fibers in BHK-21 cell motility. I. Microtubules and the effects of colchicine. *J. Cell Biol.* 51:752–762.
12. Goldman, R. D., and D. M. Knipe. 1973. Functions of cytoplasmic fibers in nonmuscle cell motility. *Cold Spring Harbor Symp. Quant. Biol.* 37:523–534.
13. Hartwig, J. H., and T. P. Stossel. Interactions of actin, myosin, and an actin-binding protein of rabbit pulmonary macrophages. III. Effects of cytochalasin B. *J. Cell Biol.* 71: 295–302.
14. Laub, F., M. Kaplan, and C. Gitler. 1981. Actin polymerization accompanies Thy-1-capping on mouse thymocytes. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 124:35–38.
15. Lazarides, E. 1980. Intermediate filaments as mechanical integrators of cellular space. *Nature (Lond.).* 283:249–256.
16. Lin, D. C., and S. Lin. 1979. Actin polymerization induced by a motility-related high affinity cytochalasin binding complex from human erythrocyte membrane. *Proc. Natl. Acad. Sci. U. S. A.* 76:2345–2349.
17. Lin, D. C., K. D. Tobin, M. Grumet, and S. Lin. 1980. Cytochalasins inhibit nuclei-induced actin polymerization by blocking filament elongation. *J. Cell Biol.* 84:455–460.
18. MacLean-Fletcher, S., and T. D. Pollard. 1980. Mechanism of action of cytochalasin B on actin. *Cell.* 20:329–341.
19. Morris, A., and J. Tannenbaum. 1980. Cytochalasin D does not produce net depolymerization of actin filaments in HEp-2 cells *Nature (Lond.)* 287:637–639.
20. Nath, J., and L. I. Rebhun. 1976. Effects of caffeine and other methylxantines on the development and metabolism of sea urchin eggs. Involvement of $NADP^+$ and glutathione. *J. Cell Biol.* 68:440–450.
21. Oliver, J. M., D. F. Albertini, and R. D. Berlin. 1976. Effects of glutathione-oxidizing agents on microtubule assembly and microtubule-dependent surface properties of human neutrophils. *J. Cell Biol.* 71:921–932.
22. Osborn, M., and K. Weber. 1976. Cytoplasmic microtubules in tissue culture cells appear to grow from an organizing structure towards the plasma membrane. *Proc. Natl. Acad. Sci. U. S. A.* 73:867–871.
23. Ostlund, R. E., J. T. Leung, and S. V. Hajek. 1980. Regulation of microtubule assembly in cultured fibroblasts. *J. Cell Biol.* 85:386–391.
24. Otto, J. J., R. E. Kane, and J. Bryan. 1980. Redistribution of actin and fascin in sea urchin eggs after fertilization. *Cell Motility.* 1:31–40.
25. Pollard, T. D. 1976. Cytoskeletal functions of cytoplasmic contractile proteins. *J. Supramol. Struct.* 5:317–334.
26. Swanston-Flatt, S. K., L. Carlsson, and E. Gylfe. 1980. Actin filament formation in pancreatic beta-cells during glucose stimulation of insulin secretion. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 117:299–302.
27. Tanenbaum, S. F. 1978. Cytochalasins: Biochemical and Biological Aspects. North-Holland, Amsterdam.
28. Tilney, L. G. 1968. Studies on the microtubules in Heliozoa. IV. The effect of colchicine on the formation and maintenance of the axopodia of *Actinosphaerium nucleofilum. J. Cell Sci.* 3:549–562.
29. Weihing, R. R. 1976. Cytochalasin B inhibits actin-related gelation of HeLa cell extracts. *J. Cell Biol.* 71:303–307.