Microinjection of ADP-ribosylated actin inhibits actin synthesis in hepatocyte–hepatoma hybrid cells

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Treatment of hepatocyte–hepatoma hybrid cells with *Clostridium botulinum* C2 toxin led to a 167% increase in monomeric globular actin (G-actin) and to a 57% decrease in filamentous actin (F-actin) within 2 h. Simultaneously, the level of actin mRNA was specifically decreased to 49% and actin synthesis was significantly diminished. In contrast, treatment of hybrid cells with phalloidin led to a decrease in G-actin to 55% and to a reciprocal increase in actin mRNA to 244% and an increase in actin synthesis. These alterations of actin synthesis depending on the G-actin/F-actin ratio corresponded to the autoregulation of actin synthesis observed in primary cultures of rat hepatocytes. Microinjection of C2 toxin or of phalloidin into hepatocyte–hepatoma hybrid cells had the same effects on actin synthesis as incubation with

either toxin in the culture medium. Microinjection of non-polymerizable ADP-ribosylated G-actin into hepatocyte–hepatoma hybrid cells specifically decreased the incorporation of [³⁵S]methionine into newly synthesized actin within 1 h. This decrease continued for at least 19 h. Microinjection of ADP-ribosylated actin led to rounding of cells and obvious disaggregation of actin filaments, which might be due to capping of actin filaments by the ADP-ribosylated actin. Because stabilization of actin filaments by phalloidin before microinjection of ADP-ribosylated actin also resulted in decreased actin synthesis, the concentration of monomeric G-actin seems to be responsible for the regulation of actin synthesis in hepatocyte–hepatoma hybrid cells, which can be regarded as immortalized hepatocytes.

INTRODUCTION

Actin and tubulin represent the main components of the cytoskeletal framework in hepatocytes and in other non-muscle cells [1,2]. Moreover these proteins are involved in several processes of motility. Most of the functions of actin depend on the polymerization of monomeric globular actin (G-actin) and consecutive depolymerization of filamentous actin (F-actin) [3,4], a process characterized as ‘treadmilling’ [5].

There is evidence that the synthesis of actin is under autoregulatory control owing to the equilibrium between G-actin and F-actin, similar to other cytoskeletal proteins [2]. This dynamic equilibrium can be shifted under experimental conditions to the alternative form. Actin polymerization is impaired by toxin-dependent ADP-ribosylation of monomeric G-actin, most probably by structural hindrance. ADP-ribosylated G-actin acts as a capping protein that binds to the fast-growing end of actin filaments, thereby inhibiting polymerization of non-modified G-actin [6]. *Clostridium botulinum* C2 toxin, *Cl. perfringens* iota toxin, *Cl. spiroforme* toxin and an ADP-ribosyltransferase produced by *Cl. difficile* are examples of bacterial proteins that ADP-ribosylate G-actin. These toxins are therefore valuable tools for investigating the actin cytoskeleton [7,8].

In contrast, the mycotoxin phalloidin from *Amanita phalloides* stabilizes F-actin by decreasing the critical concentration for actin polymerization. Phalloidin shifts the G-actin/F-actin equilibrium specifically to the filamentous form and drastically decreases the cellular level of G-actin [9]. Thus loading phalloidin into mouse fibroblasts by mechanical alteration resulted in a decrease of monomeric actin, accompanied by enhancement of actin synthesis [10]. An inverse effect on the G-actin/F-actin ratio was observed in chicken embryo fibroblasts in the presence of *Cl. botulinum* C2 toxin, which shifted the G-actin/F-actin

equilibrium towards the monomeric form [11,12]. By using the antagonistic effects of C2 toxin and phalloidin, an autoregulatory control of actin synthesis was demonstrated in cultured rat hepatocytes [13]. An increase in the G-actin/F-actin ratio caused by C2 toxin was followed by a decrease in actin mRNA, whereas a decrease in G-actin/F-actin ratio caused by phalloidin was followed by an increase in actin mRNA [13]. Recently it has been demonstrated that the decrease in the G-actin/F-actin ratio in response to phalloidin treatment results in a transcriptional enhancement of actin synthesis, whereas the increase in the G-actin/F-actin ratio in response to C2 toxin treatment is accompanied by a decreased stability of actin mRNA [14]. The latter effect was confirmed in 3T3 cells and HeLa cells, which showed a post-transcriptional decrease in actin synthesis after treatment with C2 toxin and latrunculin A, a metabolite from the Red Sea sponge that binds G-actin and prevents its assembly [15]. The physiological relevance of this regulation was demonstrated in rat hepatoma cells and human leucocytes as well as in primary cultures of rat hepatocytes by other groups, which demonstrated the influence of hormones or osmotic stress on the cellular G-actin content and on the regulation of actin synthesis [16,17].

However, it remained unclear whether actin synthesis is regulated by the cellular level of G-actin or of F-actin or whether it is modulated by the G-actin/F-actin ratio. Moreover, direct effects of the toxins on actin synthesis have not yet been excluded. Therefore ADP-ribosylated G-actin was microinjected into hepatocyte–hepatoma hybrid cells to increase the cytosolic level of actin monomers independently of toxin treatment.

The present study demonstrates for the first time that microinjection of ADP-ribosylated G-actin decreases actin synthesis within 1 h. Stabilization of actin filaments by phalloidin before microinjection of ADP-ribosylated actin monomers did not

abolish this effect. The results indicate that monomeric G-actin is the regulatory protein of actin synthesis in hepatocyte–hepatoma hybrid cells, which can be regarded as a model of immortalized hepatocytes [18,19].

MATERIALS AND METHODS

Materials

DNA, DNase I, phalloidin, Protein G–Sepharose 4 fast flow, fluorescein isothiocyanate (FITC)-labelled phalloidin and rhodamine-labelled phalloidin were obtained from Sigma (München, Germany). Skeletal muscle α -actin (rabbit) was supplied by Sigma as standard for DNase inhibition assay, or it was prepared as described [20] for ADP-ribosylation and microinjection experiments. Medium 199 and hypoxanthine/aminopterin/thymidine (HAT) medium were supplied by Gibco (Karlsruhe, Germany), methionine-free medium 199 was purchased from Serva (Heidelberg, Germany), fetal calf serum was obtained from Seromed (Berlin, Germany), and collagenase was from Boehringer Mannheim (Germany). Monoclonal anti-actin antibodies (mouse IgG, kappa light chain) were from ICN Bio-medicals (Meckenheim, Germany), and Hybond C hybridization membrane, L-[35 S]methionine and [α - 32 P]dCTP were purchased from Amersham (Braunschweig, Germany). All other chemicals, obtained from commercial sources, were of analytical or molecular biological grade.

Cell culture

Hepatocytes were isolated from adult Wistar rats by collagenase perfusion and maintained on Falcon® culture dishes in medium 199, as described previously [21]. Before incubation with C2 toxin or phalloidin, hepatocytes were maintained in primary culture with serum-free medium for approx. 12 h.

Hepatocyte–hepatoma hybrid cells (clone 1E3), obtained by fusion of isolated hepatocytes and FAO-hepatoma cells [22], were donated by Professor E. Petzinger (Giessen, Germany). They were cultured in Falcon® culture flasks or on culture dishes in medium 199, supplemented with 10% (v/v) fetal calf serum, 0.5 nM insulin and 5 nM dexamethasone. After six to eight passages, spontaneously dedifferentiated cells were removed by recloning in a hypoxanthine/aminopterin/thymidine-containing medium as described previously [22]. For microinjection experiments, hepatocyte–hepatoma hybrid cells were cultured on 3 cm dishes until a semi-confluent layer was obtained. To facilitate localization of the cells, grids were engraved into the bottom of each dish with a marking comb, consisting of five thin steel needles approx. 100 μ m apart.

Before microinjection, most of the cells were scraped off by use of a small rubber 'policeman' under microscopic control, leaving a group of approx. 500 or 5000 cells in the middle of the dish. Cells that had been scraped off were removed by rinsing twice with culture medium.

Toxins

The two components, I and II, of *Cl. botulinum* C2 toxin were prepared and activated essentially as described [23]. For treatment of intact cells, the final concentration of component II (binding component) was twice that of the ADP-ribosylating component I. Microinjection was performed only with component I of C2 toxin. *Cl. perfringens* iota toxin was prepared as described [24]. Phalloidin was dissolved in water. The toxins were used in concentrations given in the Results section.

ADP-ribosylation of G-actin for microinjection

Globular skeletal muscle α -actin (rabbit) was prepared by the method of Spudich and Watt [20] and stored on ice. Before ADP-ribosylation, G-actin was dialysed against buffer G [0.2 mM CaCl_2 /0.25 mM ATP/5 mM Tris/HCl (pH 7.5)] for 12–24 h at 4 °C. ADP-ribosylation was started by incubating 2 ml of G-actin (2 mg/ml) on ice for 30 min with 10 μ l of NAD^+ (10 mM) and 5 μ l of iota toxin (200 μ g/ml). The reaction was continued on ice by adding 40 μ l of NAD^+ (10 mM) for 90 min. Thereafter, iota toxin was removed by incubating the reaction mixture twice for 30 min with 200 μ l of polyclonal anti-(iota toxin) antibodies (rabbit), coupled to CNBr-activated Sepharose 4b (Pharmacia, Freiburg, Germany). The supernatants, containing ADP-ribosylated G-actin, were separated by centrifugation and sterilized by ultrafiltration. Thereafter the solution was concentrated to 13 mg/ml by Centricon®-30 microconcentrators (Amicon, Beverly, MA, U.S.A.). The concentration of ADP-ribosylated actin was determined by measurement of the absorbance at 290 nm by using an extinction coefficient, ϵ_{290} , of 24900 $\text{M}^{-1}\cdot\text{cm}^{-1}$ [25]. A sample was tested for remaining iota toxin by proving the ADP-ribosylation activity, adding [32 P] NAD^+ and unmodified actin followed by incubation for 60 min at 37 °C. The proteins were separated by SDS/PAGE and evaluated by autoradiography. No ADP-ribosylation of actin was detectable, indicating that the samples did not contain iota toxin. The ADP-ribosylated actin was stored on ice. It migrated as a single 43 kDa band during SDS/PAGE, indicating that it was essentially free of contamination. Immediately before microinjection, small aliquots of the solution of ADP-ribosylated G-actin were clarified by centrifugation at 100000 g for 30 min.

For control, G-actin-free buffer G plus iota toxin was processed as described above, followed by injection into cells in parallel incubations to further exclude direct effects of undetected traces of remaining iota toxin.

Microinjection procedure

Hepatocytes or hepatocyte–hepatoma hybrid cells were injected in Hepes-buffered culture medium at room temperature, essentially as described [26], with a Zeiss (Oberkochen, Germany) Axiovert 35 microscope, an Eppendorf (Hamburg, Germany) micromanipulator 5170, a microinjector 5242 and Eppendorf capillaries with an inner tip diameter of 0.5 μ m. The holding pressure was 35 hPa, the injection pressure was 80 hPa and the duration of injection was 400 ms. The injection volume of approx. 50 fl was about 1% of the volume of hepatocyte–hepatoma hybrid cells, which exhibit a volume similar to the volume of hepatocytes, described by Wiener et al. as being approx. 5000 μm^3 [27]. The relation of injected volume to cellular volume was in line with recommendations by other groups, which described the volume injectable into cultured cells without loss of viability to be up to 10% of the initial cell volume [28–30]. The concentration of microinjected ADP-ribosylated G-actin was 13 mg/ml. For microinjection of phalloidin or C2 toxin into hepatocyte–hepatoma hybrid cells, aqueous solutions of phalloidin (1 mg/ml) or of component I of C2 toxin (400 ng/ml) respectively were injected.

After microinjection, the cells were incubated in medium 199 at 37 °C as described above for the times indicated in the Results section. Thereafter the dishes were rinsed twice with methionine-free medium. For labelling of newly synthesized proteins, each 3 cm dish was incubated with 500 μ l of [35 S]methionine (more than 1000 Ci/mM, 2.5 mCi per ml of methionine-free medium) for 1 h at 37 °C. Thereafter the radioactive medium was removed,

the cells were rinsed twice with PBS and frozen at -25°C . A $10\ \mu\text{l}$ portion of Laemmli [31] gel sample buffer, containing 2% SDS and 100 mM dithiothreitol, was added to the frozen cells, and cellular material was removed with a pipette from the dishes after slow thawing. For removal of all cellular material, this procedure was repeated five times. Before SDS/PAGE, the pooled samples were boiled for 5 min and centrifuged for 3 min at $10000\ g$. Aliquots of $3\ \mu\text{l}$ were counted for ^{35}S in a liquid-scintillation counter and aliquots containing identical total amounts of radioactivity were subjected to SDS/PAGE.

For immunoprecipitation of actin, about 5000 cells were microinjected with ADP-ribosylated actin. After treatment as described above they were scraped off in $100\ \mu\text{l}$ of buffer containing 10 mM Tris/HCl, pH 7.4, 150 mM NaCl, 1% (w/v) deoxycholate, 1% (v/v) Triton X-100 and 0.1% (w/v) SDS. After homogenization, the lysates were centrifuged for 1 h at $100000\ g$ and actin in supernatants was immunoprecipitated as described below.

Immunoprecipitation of actin

After microinjection of hepatocyte–hepatoma hybrid cells with ADP-ribosylated actin or with control solutions, $100\ \mu\text{l}$ of $100000\ g$ supernatants were mixed with $1\ \mu\text{l}$ of solution containing anti-actin antibody and incubated for 1 h on ice. Thereafter $30\ \mu\text{l}$ of Protein G–Sepharose 4 fast flow beads were added, which had been washed twice before use in solution A [40 mM NaF/0.05% (v/v) Triton X-100/10 mM EDTA/100 mM potassium phosphate (pH 7.0)]. After another incubation for 1 h at 4°C , on a roller shaker, the beads were separated by centrifugation for 3 min at $10000\ g$ and washed sequentially with solution A, solution A containing 1% Triton X-100, solution A containing 0.5 M NaCl and finally again with solution A. Thereafter the beads were mixed with $50\ \mu\text{l}$ of Laemmli sample buffer containing 3% SDS, then vortex-mixed and boiled for 10 min. After centrifugation, supernatants were subjected to PAGE [11% (w/v) gel] and newly synthesized actin was detected by autoradiography of the dried gel on a BAS IIs detector (Fuji Photo, Japan), followed by scanning on a phosphorimager, using the TINA imaging software (Raytest, Straubenhardt, Germany).

Quantitative measurement of G-actin, F-actin and cellular protein

The amount of cellular G-actin was determined by DNase I inhibition assay [32] as described in detail previously [12]. G-actin of hepatocytes and of hepatocyte–hepatoma hybrid cells was referred to cytosolic protein in the $100000\ g$ supernatants, determined by the method of Bradford [33], with bovine gamma globulin as standard.

F-actin was quantified by binding of rhodamine–phalloidin to actin filaments [34] in permeabilized and formaldehyde-fixed cells with some modifications [35]. In brief, cells grown on 6 cm dishes were washed twice with stabilization buffer SB [75 mM KCl/3 mM MgSO_4 /1 mM EGTA/0.2 mM dithiothreitol/0.1 mM PMSF/10 mM imidazole/10 $\mu\text{g}/\text{ml}$ aprotinin (pH 7.2)] and permeabilized with 0.03% (w/v) saponin in SB for 10 min at room temperature. Cell monolayers were fixed in freshly prepared 3% (v/v) formaldehyde in SB for 20 min at room temperature, washed twice and stained in the dark with 0.175 $\mu\text{g}/\text{ml}$ rhodamine–phalloidin in SB for 30 min. After washing three times with SB, extraction of rhodamine phalloidin was initiated by addition of ice-cold methanol for 30 min at -20°C . Thereafter the cells were scraped off with a rubber ‘policeman’ and extraction was continued overnight at -20°C . The suspension was centrifuged for 10 min at $10000\ g$. Rhodamine in the supernatants was determined by means of a rhodamine phalloidin

standard curve with an Aminco-Bowman spectrophotofluorimeter (Columa, Lorch, Germany). Excitation and emission wavelengths were 542 and 563 nm respectively. Cellular protein was determined by the method of Bradford [33], with bovine gamma globulin as standard, in parallel cultures of hepatocytes lysed in the presence of 0.5% (v/v) Triton X-100.

In addition, the ratio of F-actin to G-actin after toxin treatment was determined by separation of Triton-insoluble and Triton-soluble actin, as described previously [11,13]. Extraction with Triton was followed by SDS/PAGE. Immunoblotting of SDS gels was performed as described [13], with monoclonal mouse anti-actin antibodies (ICN), peroxidase-coupled rabbit anti-(mouse IgG) antibodies (Sigma) and 4-chloro-1-naphthol as peroxidase substrate. Quantification of Triton-insoluble cytoskeletal actin and Triton-soluble cytosolic actin was performed by densitometry with an Epson GT 6000 scanner and the Gel-Image program from Pharmacia (Freiburg, Germany).

Radioactive labelling of newly synthesized proteins

Newly synthesized proteins of cultured hepatocytes or hepatocyte–hepatoma hybrid cells were determined by incubating cells with [^{35}S]methionine (100 $\mu\text{Ci}/\text{ml}$, in methionine-free medium) for 1 h. Cellular proteins were then separated by SDS/PAGE (approx. 50 μg of protein per lane). Radioactively marked newly synthesized proteins were detected by autoradiography (Kodak X-Omat AR film). Quantification of radioactively marked proteins was performed by densitometry with an Epson GT 6000 scanner and the Gel-Image program from Pharmacia. The identity of the 43 kDa protein was demonstrated by immunological analysis.

Fluorescein-labelled phalloidin staining of actin microfilaments

FITC-phalloidin staining of actin filaments in cultured hepatocytes and in hepatocyte–hepatoma hybrid cells was performed essentially as described [36]. In brief, cells were washed with PBS [137 mM NaCl/2.7 mM KCl/8.0 mM Na_2HPO_4 /1.5 mM KH_2PO_4 (pH 7.4)] and fixed for 10 min with freshly prepared 3.7% (w/v) paraformaldehyde in PBS at room temperature. Thereafter the cells were washed with PBS, permeabilized for 1 min with 0.2% (v/v) Triton X-100 in PBS and washed twice again with PBS. Cytoskeletal actin was stained for 30 min with FITC-phalloidin (10 $\mu\text{g}/\text{ml}$ in PBS) in a moist chamber at room temperature. Unbound FITC-phalloidin was removed by washing twice for 10 min in PBS. Stained cells were mounted by covering with Mowiol and glass coverslips, viewed and photographed with a Zeiss fluorescence microscope (magnification $\times 630$) with Kodak Tri-X Pan films (33 DIN).

Detection and quantification of actin and albumin mRNA

RNA from rat hepatocytes and from hepatocyte–hepatoma hybrid cells was isolated by the method of Chomczynski and Sacchi [37]. RNA dot-blot hybridization was performed as described previously [13]. In brief, 10 μg of total RNA was dotted on nitrocellulose filters (Hybond-N, Amersham); hybridization was performed at 42°C in a mixture containing formamide (50%, v/v), with ^{32}P -labelled random primed cDNA. The probes were a 1.3 kb *Pst*I fragment of mouse β -actin cDNA and a 0.8 kb *Pst*I fragment of rat albumin cDNA (control), both cloned into standard Bluescript vector (Stratagene, La Jolla, CA, U.S.A.). The filters were washed and subjected to autoradiography for 12–24 h (Kodak X-Omat AR film). Hybridization signals were quantified by scanning densitometry or by counting of radioactively labelled dots in a liquid-scintillation counter.

Measurement of cellular enzyme activities

To determine the cellular integrity after toxin treatment, the enzymes alanine aminotransferase, aspartate aminotransferase and lactate dehydrogenase were measured in the culture medium in accordance with the recommendations of the German Society for Clinical Chemistry [38], using reagents from Boehringer Mannheim (Germany). Neither C2 toxin nor phalloidin led to a significant release of cellular enzymes.

RESULTS

Autoregulation of actin synthesis by the level of monomeric G-actin or polymeric F-actin has been demonstrated recently in primary cultures of rat hepatocytes [13,14]. To prove whether G-actin is responsible for this regulation, the cellular level of monomeric actin was increased by microinjection into isolated cells. Microinjection was performed with ADP-ribosylated monomeric actin, because native G-actin in concentrations sufficient to increase the cellular level of actin monomers showed polymerization even within the injection capillary. ADP-ribosylation of G-actin prevents polymerization of injected actin in the capillary as well as in the cells. However, injection into primarily cultured hepatocytes resulted in detachment of the cells from the bottom of the dish after 3–6 h and death within 24 h. This was apparently due to puncture of the cells rather than to injection of a volume increasing the cell volume by 50 fl, i.e. less than 2%. Conversely, microinjection of equal volumes into hepatocyte–hepatoma hybrid cells did not impair cell viability. Because hepatocyte–hepatoma hybrid cells are reported to show essential properties of hepatocytes [18,19], experiments were performed with this cell system.

To investigate whether the mechanism of regulation of actin synthesis in the hybrid cells is similar to that in primary cultures of hepatocytes, the dependence of actin synthesis on the G-actin/F-actin equilibrium was compared in both types of cell. Treatment of primary hepatocytes with C2 toxin-containing culture medium for 2 h increased the level of G-actin to 157% and lowered the F-actin content to 59% (Table 1). Similar effects were observed during incubation of hepatocyte–hepatoma hybrid cells with the toxin (Table 1). In both cases, actin mRNA decreased to less than 50% (Table 1).

In contrast, treatment of hepatocytes as well as of hepatocyte–hepatoma hybrid cells with phalloidin-containing medium led to a significant decrease in G-actin and a simultaneous increase in actin mRNA to approx. 250% (Table 1). The effects of either toxin on actin synthesis were specific, as demonstrated by

Table 1 Influence of C2 toxin or phalloidin on the levels of G-actin, F-actin and actin mRNA in primarily cultured hepatocytes and hepatocyte–hepatoma hybrid cells

Cells were treated with or without C2 toxin (C2T; 200 ng/ml component I and 400 ng/ml component II) or with phalloidin (PHAL; 2.5 µg/ml) for 2 h (determination of G-actin or F-actin) or for 12 h (determination of actin mRNA). Thereafter G-actin, F-actin or actin mRNA was determined as described in the Materials and methods section. Results are shown as percentages of controls and represent means ± S.D. for three to six separate determinations of a typical experiment that was repeated twice.

	G-actin (%)		F-actin (%)		Actin mRNA (%)	
	C2T	PHAL	C2T		C2T	PHAL
Hepatocytes	157 ± 12	32 ± 4	59 ± 8		25 ± 12	250 ± 13
Hybrid cells	167 ± 4	55 ± 9	57 ± 6		49 ± 10	244 ± 17

Figure 1 Influence of C2 toxin or phalloidin on the synthesis of cellular protein

Primary cultures of rat hepatocytes (A) or hepatocyte–hepatoma hybrid cells (B) were treated with or without C2 toxin (C2T) or phalloidin (PHAL; concentrations as indicated in Table 1) for 12 h. Thereafter the cells were incubated with [³⁵S]methionine for 1 h and lysed, and equal amounts of lysates were subjected to SDS/PAGE. Actin was detected by its relative molecular mass (43 kDa). Each experiment was repeated at least twice. Typical autoradiograms are shown.

Figure 2 Influence of microinjected C2 toxin or phalloidin on protein synthesis in hepatocyte–hepatoma hybrid cells

Approx. 500 cells were microinjected with component I of C2 toxin (C2-I, 400 ng/ml) or with phalloidin (PHAL, 1 mg/ml). After injection, the cells were incubated for 10 h, followed by labelling with [³⁵S]methionine for 1 h. Lysates were subjected to SDS/PAGE as described in the Materials and methods section. Newly synthesized actin was detected by its relative molecular mass. The experiments were repeated twice. Typical autoradiograms are shown.

incorporation of [³⁵S]methionine into newly synthesized proteins (Figure 1). Synthesis of actin was increased or decreased in a similar manner in both cell systems, whereas the synthesis of other proteins was essentially not influenced by C2 toxin or

Figure 3 Influence of microinjected G-actin on protein synthesis in hepatocyte-hepatoma hybrid cells

Cells maintained as controls (lanes a) or injected with ADP-ribosylated G-actin (13 mg/ml; lanes b) or with ADP-ribosylation buffer without G-actin (lane c) were incubated for the indicated times. After incubation, newly synthesized proteins were labelled with [35 S]methionine for 1 h, and actin was detected as described in the legend to Figure 2. Results represent a typical experiment that was repeated twice.

Figure 4 Immunoprecipitation of radioactively marked actin after microinjection of hepatocyte-hepatoma hybrid cells with ADP-ribosylated G-actin

Hepatocyte-hepatoma hybrid cells were microinjected with ADP-ribosylated G-actin (13 mg/ml) or with ADP-ribosylation buffer without actin for control, followed by incubation for 3 h. Newly synthesized proteins were then labelled with [35 S]methionine for 1 h. Actin was precipitated and detected as described in the Materials and methods section. Phosphorimaging data of the SDS/PAGE (one of two experiments) are shown.

phalloidin, either in primary cultures of hepatocytes (Figure 1A) or in hepatocyte-hepatoma hybrid cells (Figure 1B).

Microinjection of the G-actin ADP-ribosylating component I of C2 toxin into hepatocyte-hepatoma hybrid cells resulted in a decrease in actin synthesis (Figure 2), similar to the decrease observed when both components of C2 toxin were present in the culture medium (Figure 1B). A reciprocal effect was observed after injection of phalloidin (Figure 2), similar to the effect of phalloidin in the culture medium (Figure 1B).

Microinjection of ADP-ribosylated rabbit muscle G-actin into hepatocyte-hepatoma hybrid cells led to a drastic decrease in newly synthesized actin without remarkable changes of other cellular proteins. This decrease in actin synthesis occurred within 1 h of incubation of microinjected cells and persisted for at least 19 h (Figure 3). In contrast, microinjection of ADP-ribosylation buffer without G-actin did not alter actin synthesis, indicating that the effects of microinjection were due to ADP-ribosylated

Figure 5 Influence of microinjection of ADP-ribosylated G-actin into hepatocyte-hepatoma hybrid cells on the cell shape and on the actin cytoskeleton

Hepatocyte-hepatoma hybrid cells were maintained as controls (a, c) or injected with ADP-ribosylated G-actin (b, d), followed by incubation for 5 h. Cells were then photographed by phase-contrast microscopy (a, b) or by fluorescence microscopy after staining the actin cytoskeleton with FITC-phalloidin (c, d) as described in the Materials and methods section. Scale bar, 16 μ m.

actin and not to a contamination with iota toxin used for preparation of ADP-ribosylated actin (Figure 3).

To demonstrate that the 43 kDa protein that was down-regulated after microinjection of ADP-ribosylated actin was actually actin, immunoprecipitation of actin from microinjected hybrid cells was performed after labelling with [35 S]methionine. Cells microinjected with ADP-ribosylated actin showed down-regulation of actin synthesis by approx. 65 % in comparison with cells microinjected with ADP-ribosylation buffer, which showed no decrease in actin synthesis (Figure 4).

Microinjection of ADP-ribosylated actin into hepatocyte-hepatoma hybrid cells caused rounding of cells (Figure 5b). The actin cytoskeleton was obviously rearranged, showing diffuse labelling of actin stained by fluorescein-labelled phalloidin (Figure 5d). This might have been due to a shortening of actin filaments caused by capping of F-actin by ADP-ribosylated actin. Thus the autoregulation of actin synthesis could be due to a depolymerization of actin filaments rather than to an increase in free actin monomers within the cytosol. To exclude this possibility, cells were preincubated for 18 h with phalloidin (10 μ g/ml) to prevent depolymerization of F-actin by stabilization of actin fibres [9]. Thereafter ADP-ribosylated actin was microinjected as described above. Even under this condition, actin synthesis was decreased to about 50 % as demonstrated by densitometry (Figure 6).

To demonstrate that F-actin in hepatocyte-hepatoma hybrid cells was actually stabilized by phalloidin and protected against depolymerization, cells were treated with phalloidin (10 μ g/ml) for 18 h, followed by incubation with C2 toxin (200 ng/ml component I and 400 ng/ml component II) for 2 h. Thereafter F-actin and G-actin were identified as described in the Materials and methods section. The effect of C2 toxin, which caused a decrease in F-actin to 57 % and an increase in G-actin to 167 %

Figure 6 Influence of microinjected G-actin on protein synthesis after pretreatment of hepatocyte-hepatoma hybrid cells with phalloidin

Hepatocyte-hepatoma hybrid cells were preincubated with phalloidin (10 μ g/ml) for 18 h to stabilize the actin filaments. The cells were then microinjected with ADP-ribosylated G-actin (13 mg/ml) or with ADP-ribosylation buffer without actin as a control, followed by further incubation for 5 h. Thereafter newly synthesized proteins were labelled with [35 S]methionine for 1 h, and actin was detected as described in the legend to Figure 2. Quantification of radioactively marked actin was performed by densitometry. It revealed a density of 0.35 units (100%) after injection of buffer compared with 0.17 units (48%) after injection of ADP-ribosylated G-actin. A typical experiment is shown that was repeated twice.

within 2 h (Table 1), was completely abolished by pretreatment of cells with phalloidin, which led to a decrease in G-actin to 46% and an increase in F-actin to 142%, as demonstrated by scanning densitometry of Triton-soluble and Triton-insoluble actins. No quantitative differences in the distribution of actin could be observed whether the cells were treated only with phalloidin or with phalloidin followed by incubation with C2 toxin, indicating that the F-actin-stabilizing effect of phalloidin was not reversible. This is in line with previous studies, which demonstrated that treatment of G-actin with phalloidin impaired the C2 toxin-induced ADP-ribosylation of actin [7] and that incubation of endothelial cells with phalloidin prevented the effects of C2 toxin on endothelial permeability [35].

DISCUSSION

It was recently demonstrated that autoregulation of actin synthesis in cultured hepatocytes is due to transcriptional as well as post-transcriptional mechanisms depending on the increase or decrease in the G-actin/F-actin equilibrium effected by C2 toxin and phalloidin [14]. However, it could not be excluded that direct effects of the toxins might influence actin synthesis. Moreover it was not clear whether the actual cellular concentrations of G-actin or of F-actin, or even the G-actin/F-actin ratio, is responsible for the regulation of actin synthesis in hepatocytes [14].

In the present study, we attempted to inject G-actin into primarily cultured hepatocytes to increase the pool of free actin monomers selectively. In a similar experiment the elevation of tubulin levels by microinjection suppressed the synthesis of tubulin in an autoregulatory manner [30]. Moreover, microinjection of low concentrations of rhodamine-labelled actin or of actin-binding proteins was reported to be a useful experimental tool to investigate processes involved in the formation of the actin cytoskeleton [39–44]. However, microinjection of high concentrations of G-actin, sufficient to enhance the cellular G-

actin level significantly, was hampered in the present study by the fact that even siliconized injection needles were obstructed by the rapidly polymerizing actin as soon as the needles dipped into the culture medium. Therefore ADP-ribosylated G-actin was applied instead of G-actin to avoid this polymerization [8]. Actin monomers that were altered in this way passed through the injection needles in concentrations of 13 mg/ml.

Injection of 50 fl of buffer with or without ADP-ribosylated G-actin into primarily cultured hepatocytes resulted in detachment of the cells from the culture dishes and, finally, in cell death. In contrast, hepatocyte-hepatoma hybrid cells did not detach after microinjection. These proliferating cells, generated by fusion of isolated rat hepatocytes with FAO-hepatoma cells, exhibit the essential properties of hepatocytes [18,19] as well as the autoregulation of actin synthesis in the same manner as hepatocytes (Table 1 and Figure 1). Therefore they were used as a model of immortalized hepatocytes in the present study.

The cellular concentration of total actin is reported to be approx. 5–7 pg per cell [45–47] consisting of 25% G-actin in cultured hepatocytes [17]. Corresponding concentrations can be assumed to exist in hepatocyte-hepatoma hybrid cells. The injection volume of ADP-ribosylated actin (13 mg/ml) was approx. 50 fl, which is about 1% of the total cellular volume. Thus the level of actin could be enhanced via microinjection by 10% and that of G-actin could be elevated by 40%. This is in line with the results of Cleveland et al. [30], who demonstrated that the cellular level of tubulin could be enhanced by 25–50% by microinjection of this cytoskeletal protein.

Injection of ADP-ribosylated G-actin into hepatocyte-hepatoma hybrid cells specifically caused a significant decrease in actin synthesis, comparable to suppression of tubulin synthesis after injection of tubulin into cells [30]. Because ADP-ribosylated G-actin acts as a capping protein at the growing end of actin filaments [6], it shifts the G-actin/F-actin equilibrium towards unpolymerized G-actin. Thus the observed autoregulatory decrease in actin synthesis could be due to the injection of ADP-ribosylated G-actin as well as to the enhancement of G-actin and decrease in F-actin by net depolymerization. To prove these possibilities, hepatocyte-hepatoma hybrid cells were preincubated with phalloidin, which was shown to stabilize actin filaments. Subsequent microinjection of ADP-ribosylated G-actin also suppressed actin synthesis by approx. 50%, which was less pronounced than the decrease of actin synthesis without preincubation of cells with phalloidin. The more pronounced decrease in the absence of phalloidin seems to be due to an additional increase in G-actin by net depolymerization of F-actin or to a decrease in F-actin itself. Regardless of this possibility, the selective increase in G-actin seems to be sufficient to mediate down-regulation of actin synthesis.

In the present study, ADP-ribosylated α -skeletal muscle G-actin was injected into hepatocyte-hepatoma hybrid cells, which physiologically contain β - and γ -non-muscle actin. In contrast with other observations [48,49], autoregulation of actin synthesis in hepatocyte-hepatoma hybrid cells apparently did not depend on specific isoforms of actin, e.g. β - or γ -actin.

In contrast with a recent study using 3T3 cells [50] as well with our own investigations with chicken embryo fibroblasts, we did not observe a significant depression of the overall protein synthesis after treatment with C2 toxin in hepatocytes and in hepatocyte-hepatoma hybrid cells. This might be due to differences between fibroblasts and hepatic cells.

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REFERENCES

- 1 Feldmann, G. (1989) *J. Hepatol.* **8**, 380–386
- 2 Bershadsky, A. D. and Vasiliev, J. M. (1988) *Cytoskeleton*, Plenum Press, New York
- 3 Pollard, T. D. and Cooper, J. A. (1986) *Annu. Rev. Biochem.* **55**, 987–1035
- 4 Cooper, J. A. (1991) *Annu. Rev. Physiol.* **53**, 585–605
- 5 Neuhaus, J. M., Wanger, M., Keiser, T. and Wegner, A. (1983) *J. Muscle Res. Cell Mot.* **4**, 507–527
- 6 Wegner, A. and Aktories, K. (1988) *J. Biol. Chem.* **263**, 13739–13742
- 7 Aktories, K., Bärmann, M., Ohishi, I., Tsuyama, S., Jakobs, K. H. and Habermann, E. (1986) *Nature (London)* **322**, 390–392
- 8 Aktories, K. and Wegner, A. (1989) *J. Cell Biol.* **109**, 1385–1387
- 9 Cooper, J. A. (1987) *J. Cell Biol.* **105**, 1473–1478
- 10 Serpinskaya, A. S., Denisenko, O. N., Gelfand, V. I. and Bershadsky, A. D. (1990) *FEBS Lett.* **277**, 11–14
- 11 Reuner, K. H., Presek, P., Boschek, C. B. and Aktories, K. (1987) *Eur. J. Cell Biol.* **43**, 134–140
- 12 Aktories, K., Reuner, K. H., Presek, P. and Bärmann, M. (1989) *Toxicon* **27**, 989–993
- 13 Reuner, K. H., Schlegel, K., Just, I., Aktories, K. and Katz, N. (1991) *FEBS Lett.* **286**, 100–104
- 14 Reuner, K. H., Wiederhold, M., Dunker, P., Just, I., Bohle, R. M., Kröger, M. and Katz, N. (1995) *Eur. J. Biochem.* **230**, 32–37
- 15 Bershadsky, A. D., Glück, U., Denisenko, O. N., Sklyarova, T. V., Spector, I. and Ben-Ze'ev, A. (1995) *J. Cell Sci.* **108**, 1183–1193
- 16 Rao, K. M., Betschart, J. M. and Virji, M. A. (1985) *Biochem. J.* **230**, 709–714
- 17 Theodoropoulos, P. A., Stournaras, C., Stoll, B., Markogiannakis, E., Lang, F., Gravanis, A. and Häussinger, D. (1992) *FEBS Lett.* **311**, 241–245
- 18 Katz, N., Immenschuh, S., Gerbracht, U., Eigenbrodt, E., Föllmann, W. and Petzinger, E. (1992) *Eur. J. Cell Biol.* **57**, 117–123
- 19 Petzinger, E., Föllmann, W., Blumrich, M., Walther, P., Hentschel, J., Bette, P., Maurice, M. and Feldmann, G. (1994) *Eur. J. Cell Biol.* **64**, 328–338
- 20 Spudich, J. A. and Watt, S. (1971) *J. Biol. Chem.* **245**, 4866–4871
- 21 Katz, N. R. and Giffhorn, S. (1983) *Biochem. J.* **212**, 65–71
- 22 Polokoff, M. A. and Everson, G. T. (1986) *J. Biol. Chem.* **261**, 4085–4089
- 23 Ohishi, I., Iwasaki, M. and Sakaguchi, G. (1980) *Infect. Immun.* **30**, 668–673
- 24 Stiles, B. G. and Wilkens, T. D. (1986) *Infect. Immun.* **54**, 683–688
- 25 Wegner, A. (1976) *J. Mol. Biol.* **108**, 139–150
- 26 Müller, H., v. Eichel-Streiber, C. and Habermann, E. (1992) *Infect. Immun.* **60**, 3007–3010
- 27 Wiener, J., Loud, A. V., Kimberg, D. and Spiro, D. (1968) *J. Cell Biol.* **37**, 47–62
- 28 Stacey, D. W. and Allfrey, V. G. (1976) *Cell* **9**, 725–732
- 29 Graessmann, A., Graessmann, M. and Mueller, C. (1980) *Methods Enzymol.* **65**, 816–825
- 30 Cleveland, D. W., Pittenger, M. F. and Feramisco, J. R. (1983) *Nature (London)* **305**, 738–740
- 31 Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- 32 Blikstad, I., Markey, F., Carlsson, L., Persson, T. and Lindberg, U. (1978) *Cell* **15**, 935–943
- 33 Bradford, M. (1976) *Anal. Biochem.* **72**, 248–254
- 34 Wysolmerski, R. B. and Lagunoff, D. (1988) *Am. J. Pathol.* **132**, 28–37
- 35 Suttorp, N., Polley, M., Seybold, J., Schnittler, H., Seeger, W., Grimminger, F. and Aktories, K. (1991) *J. Clin. Invest.* **87**, 1575–1584
- 36 Hooser, S. B., Beasley, V. R., Waite, L. L., Kuhlenschmidt, M. S., Carmichael, W. W. and Haschek, W. M. (1991) *Vet. Pathol.* **28**, 259–266
- 37 Chomczynski, P. and Sacchi, N. (1987) *Anal. Biochem.* **162**, 156–159
- 38 German Society for Clinical Chemistry (1972) *Z. Klin. Chem. Klin. Biochem.* **10**, 182–192
- 39 Kreis, T. E., Geiger, B. and Schlessinger, J. (1982) *Cell* **29**, 835–845
- 40 Füchtbauer, A., Jockusch, B., Maruta, H., Kilimann, M. W. and Isenberg, G. (1983) *Nature (London)* **304**, 361–364
- 41 Cooper, J. A., Bryan, J., Schwab, B., Frieden, C., Loftus, D. J. and Elson, E. L. (1987) *J. Cell Biol.* **104**, 491–501
- 42 Symons, M. H. and Mitchison, T. J. (1991) *J. Cell Biol.* **114**, 503–513
- 43 Cao, L. G., Babcock, G. G., Rubenstein, P. A. and Wang, Y. L. (1992) *J. Cell Biol.* **117**, 1023–1029
- 44 Cao, L. G., Fishkind, D. J. and Wang, Y. L. (1993) *J. Cell Biol.* **123**, 173–181
- 45 Snabes, M. C., Boyd, A. E., Pardue, R. L. and Bryan, J. (1981) *J. Biol. Chem.* **256**, 6291–6295
- 46 Blikstad, I. and Carlsson, L. (1982) *J. Cell Biol.* **93**, 122–128
- 47 Heacock, C. S. and Bamberg, J. R. (1983) *Anal. Biochem.* **135**, 22–36
- 48 Leavitt, J., Ng, S. Y., Aebi, U., Varma, M., Latter, G., Burbeck, S., Kedes, L. and Gunning, P. (1987) *Mol. Cell Biol.* **7**, 2457–2466
- 49 Lloyd, C., Schevzov, G. and Gunning, P. (1992) *J. Cell Biol.* **117**, 787–797
- 50 Sklyarova, T., Kostyukovski, V., Sharov, V., Prisyazhnoy, V. and Denisenko, O. (1995) *FEBS Lett.* **363**, 273–276