# Alterations in protein synthesis induced by C2 toxin in 3T3 cells

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**Abstract** **We have studied the effect of actin skeleton depolymerisation induced by C2 toxin on protein synthesis in 3T3 cells. The toxin that was purified from culture medium of *Clostridium botulinum* type C was shown to specifically ADP-ribosylate actin in vitro and in vivo. Cells exposed to C2 toxin were rounded off, which was accompanied by disappearance of stress fibers. The rate of total protein synthesis decreased two-three times in the treated cells. This correlated with the reduction in amount of polyribosomes. The rates of specific protein synthesis were compared using 2D electrophoresis of pulse-labeled proteins. Dramatic changes were observed in the synthesis of a small group of cellular proteins. Our results indicate that actin filament depolymerization affects gene expression at the level of translation and/or through the control of mRNA concentrations.**

*Key words:* Actin; Tropomyosin; C2 toxin; ADP-ribosylation; Protein synthesis regulation

## 1. Introduction

Actin is one of the most prominent proteins in eukaryotic cells. Beside its participation in cytoplasm organization and cell locomotion actin filaments seem to play an important role in controlling gene expression. It is well documented that large population of mRNAs and polyribosomes are linked to the cytoskeleton [1]. At present it seems likely that untranslated mRNAs are associated with intermediate filaments [2,3], while most of the evidence suggests that cytoskeleton-bound polyribosomes are associated with the actin filaments [1,4–6]. Such an association is believed to have considerable physiological significance: it provides the basis for anisotropic spatial distribution of mRNAs and translational machinery. It has been shown that the extent of polyribosome interaction with microfilaments changes under conditions which induce alterations in protein synthesis [1,5].

Using phalloidin, latrunculin and C2 toxin, agents that change specifically the G to F actin ratio, we have recently shown that the rate of actin synthesis is linked to the state of actin polymerization in mammalian cells through the control of actin mRNA stability [7,8]. Here we further examined the effect of actin depolymerization on protein synthesis in 3T3 cells. To this end we used clostridial C2 toxin that is known to ADP-ribosylate non-muscle actin in vivo and in vitro, thus shifting actin to the monomer state [9,10]. Protein synthesis in the cells exposed to C2 toxin decreased two-three-folds when more than 80% of cellular actin was ADP-ribosylated. The effect of actin skeleton depolymerization on the pattern of synthesized proteins was also examined.

## 2. Materials and methods

### 2.1. Culture for toxin production

The *C. botulinum* type C strain was used for C2 toxin production. The medium for cell growth was based on a fortified chopped meat medium as described in [11,12]. The cells were incubated in tubes at 37°C. After 2-days growth the cultures were assessed for sporulation and purity by phase-contrast microscopy and for C2 toxin production by the ability to round off 3T3 cells. Tubes with the highest activity were selected for inoculating serum bottles. The cultures were cooled at 4°C overnight, heated for 15 min at 71°C and 0.5-ml portions were inoculated into serum bottles and incubated at 37°C for 2 days.

Contaminated cultures were purified by the roll tube method [13] in the same medium supplemented with 1.5% (w/v) agar but without cooked beef particles.

### 2.2. C2 toxin purification

The C2 toxin was purified from the culture medium of *Clostridium botulinum* as described in [14] with some modifications. Subunit II was activated by treatment for 30 min at 35°C in 20 mM phosphate buffer, pH 7.5, containing 0.2 mg/ml trypsin. Two assays were used to measure the C2 toxin activity. Subunit I was tested for ADP-ribosylation of actin as described in [9].

Subunit II was purified as a component helping subunit I to round off 3T3 cells. The fractions to be tested were treated with trypsin and 9 μl of each was mixed with 1 μl of pure subunit I (0.01 μg). 3T3 cells for the assay were plated onto 96-well plates at a density of 10,000 cells/cm$^2$. On the next day the medium was changed to a fresh one (100 μl) and the toxin was added. Changes in the cell shapes were observed after 1 h of incubation at 37°C in 5% $CO_2$.

### 2.3. Cell culture and cell labeling

Clone A31 of Balb/c 3T3 cells was grown in Dulbecco's modified Eagle medium (DMEM), supplemented with 10% fetal bovine serum, 1% non-essential amino acid mixture, 50 μg/ml of kanamycin and 1 μg/ml of fungizone (all from Flow Laboratories, UK) at 37°C in a humidified atmosphere containing 5% $CO_2$. The cells were split in 1:3 every 3 day and the medium was changed once between splits. The proteins were labeled by plating cells onto 24-well dishes at a density of 25,000 cells/cm$^2$ and after 24 h incubating them with [$^{35}$S]methionine (0.1 mCi/ml, 800 Ci/mM, Amersham, UK) for 60 min in methionine-free medium with dialyzed serum. The cultures were washed with PBS and lysed in the sample buffer for two-dimensional gel electrophoresis [15].

### 2.4. RNA isolation and in vitro translation

The total RNA from the control cells and those treated with C2 toxin was isolated by the guanidinium method [16]. Five μg of each RNA preparation was translated in a rabbit reticulocyte cell-free system in

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*Abbreviations:* 2D, two-dimensional; G-actin, globular actin; F-actin, filamentous actin.


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the presence of [$^{35}$S]methionine as described elsewhere [17]. Equal aliquots of the systems were subjected to 2D electrophoresis [15].

*2.5. Gel electrophoresis and immunoblotting*

Electrophoresis was performed in 10–20% polyacrylamide gel in the Laemmli discontinuous buffer system [18]. Two-dimensional gel electrophoresis was performed according to O'Farrell [15]. For immunoblotting analysis the proteins were transferred from 2D gels to a nitrocellulose membrane (Schleicher & Schuell) in Tris, 50 mM; glycine, 50 mM; SDS, 0.01%; and methanol, 20% (v/v). The filter was incubated with anti-actin polyclonal antibody or anti-tropomyosin polyclonal antibody (Sigma, USA) and then with secondary antibody linked to peroxidase.

*2.6. Visualization of Actin Filaments in 3T3 cells*

For actin filament staining the 3T3 cells grown on glass cover-slips were fixed in acetone for 5 min at −20°C, air-dried, immersed in PBS for 10 min and then incubated with FITC-phalloidin. The stained cells were photographed with a phase-contrast microscope equipped with a fluorescent microscopic device.

## 3. Results and discussion

*3.1. Purification and characterization of C2 toxin*

Clostridial C2 toxin is known to ADP-ribosylate cytoskeletal actin in vivo and in vitro [9]. ADPR-actin losses its ability to polymerize and can act like a capping protein to inhibit the polymerization of non-modified actin onto the barbed ends of actin filaments [10]. The toxin was first purified and characterized by Ohishi et al. from the culture medium of *Clostridium botulinum* [14]. It consists of two subunits that can be separated by ion-exchange or gel filtration chromatography. The 50 kDa subunit I catalyze ADP-ribosylation of cytoskeletal actin using NAD as a substrate. Subunit II, a 100 kDa polypeptide, helps the catalytic subunit to penetrate cellular membranes [9].

We have purified C2 toxin from the culture medium of *Clostridium botulinum* type C. This strain produces C2 toxin, but not that of C1 or C3 (Dr. Yu. Vertiyev, personal communication).

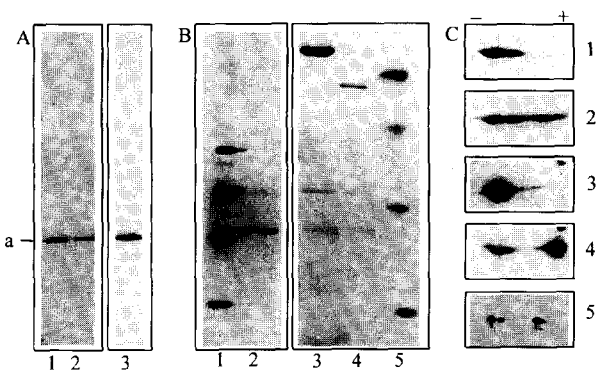


Fig. 1. Purification and properties of C2 toxin. (A) ADP-ribosylating activity of the toxin preparation. Cytosolic extract from 3T3 cells (1, 2) or gizzard actin (3) was incubated with the culture medium of *C. botulinum* (2, 3) or with the purified subunit I of the toxin (1) in the presence of [$^{14}$C]NAD. (a) The position of actin on the gel. (B) Electrophoretic mobility of C2 toxin subunits I (2), II (3), and subunit B that was pretreated with trypsin (4). 1, 5, molecular weight markers, from top to bottom: 93, 66, 45, and 30 kDa. (C) Two-dimensional electrophoretic analysis of actin ADP-ribosylated in vitro (1, 2) and in vivo (3, 4). Gizzard actin before (1) and after incubation with C2 toxin and NAD (2), and extract from 3T3 cells labeled with [$^{35}$S]methionine before (3) and after the treatment with the toxin for 3 h (4, 5) were subjected to 2D electrophoresis. 1, 2, Coomassie-stained gels; 3, 4, autoradiograph; 5, blotting with antibodies to actin.

Fig. 1A shows that the culture medium of *C. botulinum* contains toxin activity; it catalyzed the labeling of chicken gizzard actin and mouse fibroblast $\beta/\gamma$-actin in a reaction with [$^{14}$C]NAD. When the cytoplasm extracts from 3T3 cells were used as a substrate, only actin was ADP-ribosylated and no other ADP-ribosylating activities were observed in the culture medium (Fig. 1A, lane 2). Purified toxin consisted of two polypeptides: 100 kDa and 50 kDa (Fig. 1B). The 50 kDa subunit possessed the ADP-ribosylating activity in vitro (Fig. 1A, lane 1) but it was not toxic for cells (not shown). Only the mixture of both subunits was toxic for mice (not shown) and induced the rounding of 3T3 cells (see below). It is known that the in vivo activity of C2 toxin can be enhanced significantly by brief pre-treatment with trypsin [14]. This treatment did not affect the catalytic subunit (not shown) but transformed the 100 kDa-subunit to a 80 kDa polypeptide (Fig. 1B). Similar changes in $M_r$ of this subunit after trypsinization were described by Ohishi et al. [14].

In vitro ADP-ribosylated chicken gizzard actin can be seen on 2D gels as a tandem of more acidic spots in comparison with unmodified actin (Fig. 2C). A similar acidic form of actin appeared in the cells that were treated with the toxin, this form should be ADPR-actin since it can be labeled using [$^{14}$C]NAD (not shown). In the following experiments we used 2D electrophoretic analysis to monitor the extent of actin ADP-ribosylation in vivo.

*3.2. C2 toxin treatment leads to disappearance of actin filaments in 3T3 cells*

No morphological changes were observed in the 3T3 cells treated with either subunit I or II of C2 toxin. A mixture of both subunits (at a mass ratio of 1I to 5II) induced rapid alterations. A 1 h treatment rounded off 50% of the 3T3 cells; after 8 h nearly all the cells became spherical (Fig. 2C,D). These changes resulted from ADP-ribosylation of actin and the inability of modified actin to polymerize [10]. More than 80% of actin were ADP-ribosylated in 3T3 cells after 8 h of exposure to C2 toxin (see below). The rounded cells showed a drastic change in the actin skeleton (Fig. 2A,B). Instead of a well-developed system of parallel actin cables, typical for the control fibroblasts, C2-toxin-treated cells contained very few, if any, F-actin. The distribution of actin between the Triton X-100 soluble and insoluble fractions also changed significantly: 25% of actin in the control cells and over 80% in the toxin-treated cells was soluble. No variations were observed in the distribution of tubulin, another major cytoskeletal protein (calculated from 2D gels of Triton X-100 soluble and insoluble fractions, not shown).

*3.3. Changes in protein synthesis induced by C2 toxin*

There is a great body of data on the involvement of the actin skeleton in the spatial and temporal organization of protein synthesis in a living cell [1,5]. Most of the results obtained, however, were either correlative or achieved by using drugs (cytochalasins) that did not change the G- to F-actin ratio [4]. To explore the dependence of protein synthesis on the actin assembly status, we first compared the kinetics of protein synthesis in the control cells and the cells exposed to C2 toxin. Toxin treatment of the cells for 8 h resulted in nearly complete depolimerization of actin filaments and was accompanied by a 2–3-fold inhibition of the total protein synthesis (Fig. 3, insert). The polyribosomes were reduced in amount but exhibited a normal sedimentation distribution (Fig. 3). The decrease of the

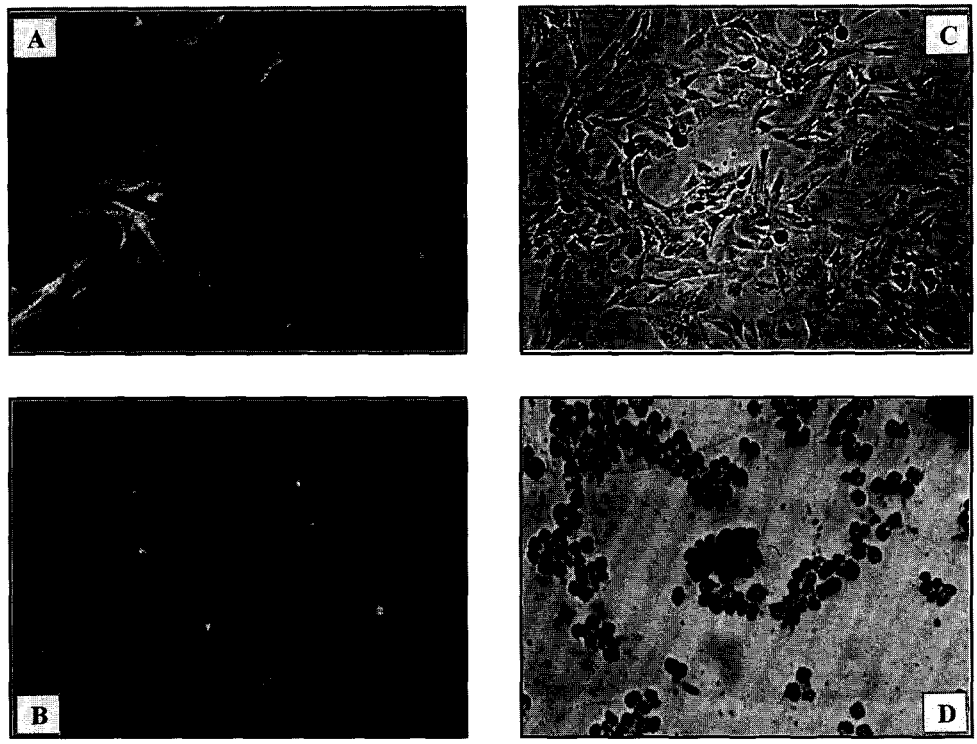


Fig. 2. Changes in cell morphology induced by C2 toxin. Control cells (A, C) and cells exposed to the toxin for 8 h (B, D) were fixed and stained with FITC-phalloidin (A, B). C, D, phase contrast.

amount of the polyribosomes correlated well with the reduction in the protein synthesis. This means that portion of mRNA molecules was removed from the translation in the treated cells, while the remaining polyribosomes were functioning at normal rates. The cessation of mRNA function might be the result of its release from the microfilament network due to actin depolimerization. These data are consistent with the previous study of the functional significance of mRNA association with the cytoskeleton [1,2,4].

To compare the patterns of synthesized proteins, control and C2 toxin-treated cells were pulse-labeled with [$^{35}$S]methionine and proteins were separated using O'Farrell's method. Autoradiographs of the gels are displayed in Fig. 4A,B. The patterns of the synthesized proteins were quite similar in both cell cultures with the obvious exception that synthesis of more than a dozen proteins was markedly altered in the toxin-treated sample. The synthesis of actin, tropomyosin (spot No. 6 was identified by antibody to tropomyosin on 2D blots, not shown), p27/5.4 ($M_r$/pI), and p43/4.45 was depressed whilst the synthesis of a small set of proteins, p28/5.9, p32/4.5, p34/4.45, p35/6.3–6.8 (most probably, annexin-I), p38/4.5, p55/5.8 and p60/5.4, was induced in the toxin-treated sample. These dramatic changes might be a consequence of actin depolimerization and/or the changes in the assembly state of actin-associated proteins, since the disruption of microfilament framework by cytochalasin D (not shown) or by detachment of cells from the substrate has only a slight effect on the synthesis of these proteins [8]. However, we can not exclude the possibility that the changes in protein synthesis observed in C2 toxin-treated cells might be the result of other yet unknown activities of the toxin subunits.

We have previously shown that the synthesis of actin and vinculin in the cell is autoregulated by the actin polymerization state through the control of the mRNA amounts [7,8]. Since actin filament depolymerization induced by toxin is accompanied by alterations in the synthesis of some other proteins, we used in vitro translation to estimate changes in the levels of their mRNAs. Two-dimensional maps of the in vivo labeled proteins were compared with the patterns of in vitro translational products of mRNA preparations from both cell cultures. In vitro translation was done at a sub-saturating mRNA concentrations to be sure that quantities of the synthesized proteins were

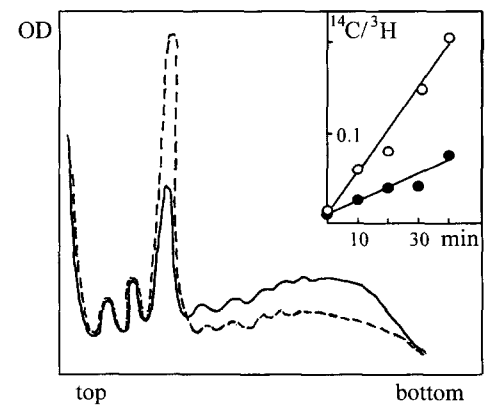


Fig. 3. Polyribosome centrifugation in sucrose gradients. Cytosolic extracts (4 OU $A_{260}$) from control cells (line) or from cells treated with C2 toxin for 8 h (dashed line) were centrifuged in 15–50% sucrose gradients for 2 h at 4°C in a Beckman SW-41 rotor. The gradients were scanned at 260 nm. OD, optical density at 260 nm. Insert, kinetics of [$^{14}$C]leucine incorporation into control cells (open circles) or cells exposed to the toxin for 8 h (closed circles). Before [$^{14}$C]leucine addition the cells were prelabeled with [$^{3}$H]leucine for 4 h. The $^{14}$C/$^{3}$H ratio was calculated in each sample.

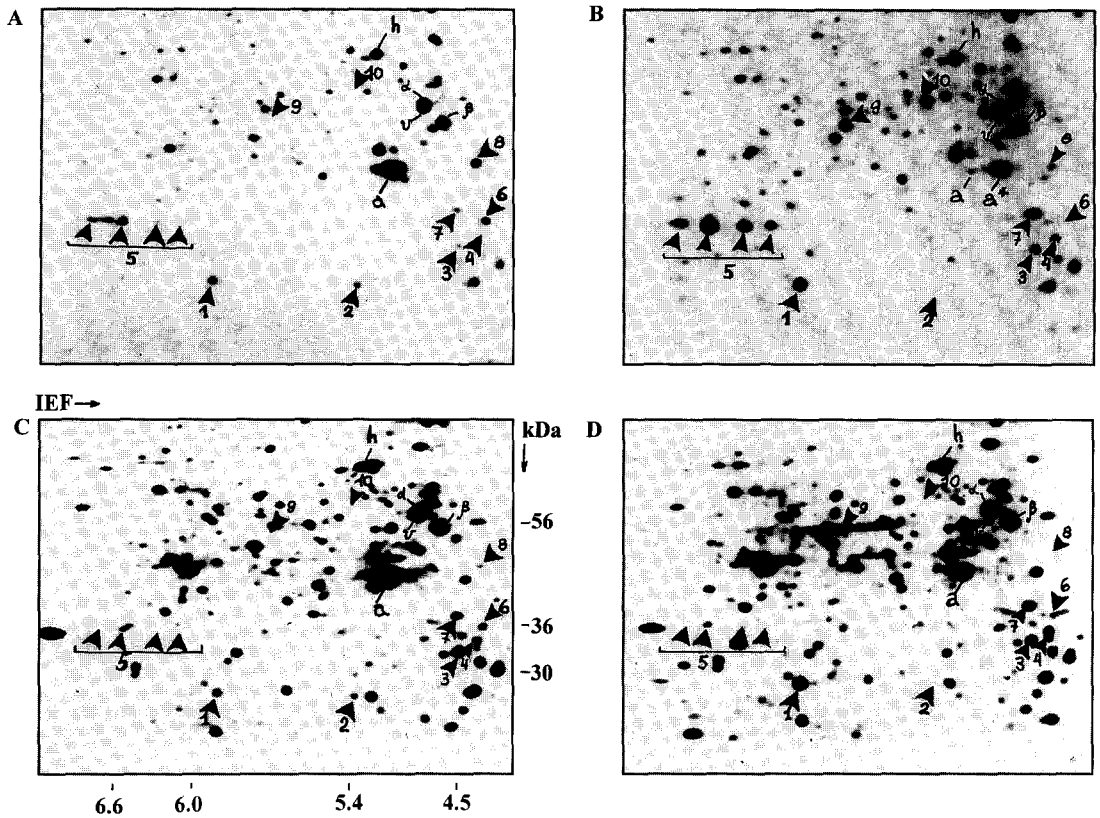


Fig. 4. (A,B) The effect of C2 toxin treatment on the pattern of proteins synthesized in 3T3 cells. Control cells (A) and cells exposed to C2 toxin for 8 h (B) were pulse-labeled with [$^{35}$S]methionine and analyzed by two-dimensional gel electrophoresis. Equal amounts of radioactivity were loaded on each gel. (C, D) In vitro translation of total RNA preparations from control cells (C) and cells exposed to C2 toxin for 8 h (D). 5 $\mu$g of each RNA was translated in the rabbit reticulocyte system in the presence of [$^{35}$S]methionine. Equal amounts of the system were loaded on each gel. a, actin; a*, ADPR-actin; $\alpha$, $\beta$, $\alpha$- and $\beta$-tubulin; h, hsc72; v, vimentin; 1, p28/5.9; 2, p27/5.4; 3, p32/4.5; 4, p34/4.45; 5, p35/6.3–6.8; 6, p36/4.5 (tropomyosin); 7, p38/4.5; 8, p43/4.45; 9, p55/5.8; 10, p60/5.4.

proportional to the amounts of their mRNA. As it can be seen in Fig. 4C and D, the changes in synthesis of p27/5.4, p28/5.9, p43/4.45 and p55/5.8 in vivo correlated with the alterations in the mRNA quantities. Little or no variations were observed in the levels of mRNAs for tropomyosin, p32/4.5, p34/4.45, p35/6.3–6.8 and p38/4.5. This suggests that synthesis of the first group of proteins was regulated at the level of mRNA quantity, while of the second – at the translational level. Thus, our data indicate that the rate of actin and some other proteins' synthesis is tightly linked to the actin skeleton polymerization state. This regulation is exerted through the changes in the translational control and/or through the control of mRNA concentrations.

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