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Isolation and Characterization of a *Clostridium botulinum* C2 Toxin-Resistant Cell Line: Evidence for Possible Involvement of the Cellular C2II Receptor in Growth Regulation

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***Clostridium botulinum* C2 toxin, which consists of the binding component C2II and the enzyme component C2I, acts on eukaryotic cells by selective ADP-ribosylation of G-actin. To obtain C2 toxin-resistant cells, we mutagenized CHO-K1 cells with *N*-nitroso-*N*-methylurea and selected for C2 resistance. Cells which survived the selection procedure with 50 ng of C2I and 100 ng of C2II per ml were obtained with a frequency of 30×10^{-6}. The colony-forming ability of CHO wild-type cells was reduced to 50% with 10 ng of C2I and 20 ng of C2II per ml. In contrast, the colony-forming ability of the isolated CHO mutant cells was not influenced by up to 200 ng of C2I and 400 ng of C2II per ml. Toxin-induced ADP-ribosylation of G-actin was not impaired in lysates of mutant cells. The C2 toxin-resistant phenotype remained sensitive to the cell-rounding activities of cytotoxins from *C. perfringens* (iota-toxin), *C. novyi*, *C. difficile*, and *C. botulinum* (C3) and to cytochalasin D. Binding of component C2II was impaired in resistant CHO cells, suggesting mutation of the toxin cell surface receptor. Serum factors protected wild-type cells against the cytotoxic effect of C2 toxin. Furthermore, the C2-resistant phenotype correlated with an increased serum dependency. The data suggest that the action of *C. botulinum* C2 toxin is mediated by its binding and uptake via a cell surface receptor which might be involved in growth regulation.**

Certain strains of *Clostridium botulinum* type C and type D produce C2 toxin, which is composed of two unlinked components, C2I and C2II (20). C2I is the enzyme component with ADP-ribosyltransferase activity, and C2II is the binding component (19, 26). The cytotoxic effect of C2 toxin is caused by ADP-ribosylation of monomeric G-actin by component C2I (molecular mass of ~45 kDa) (1, 6, 24). The toxin-catalyzed ADP-ribosylation of G-actin at arginine 177 inhibits actin polymerization (1) and actin ATPase activity (11). Moreover, the covalent modification turns ADP-ribosylated actin into a capping protein, which blocks the fast-growing ends of actin filaments (35). All of these toxin effects result in depolymerization of the actin microfilament network (3, 4). The binding component C2II has a molecular mass of ~100 kDa (20) and must be activated by trypsin treatment, thereby forming an about 80-kDa active fragment (18). The cytotoxic effects are apparently initiated by the binding of activated C2II to the cell surface of the target cells, followed by binding, internalization, and translocation of C2I into the cells (21, 27). Recent studies with doubly fluorescence-labeled toxin components indicate that C2I binds directly to activated C2II on the cell membrane and that the two components are internalized in the same endosomes (22). So far, less is known about the cellular receptor which enables the entry of C2 into cells. In a preliminary study, the involvement of glycoproteins in C2 uptake has been suggested (32).

A recently investigated example of a cellular receptor for bacterial ADP-ribosylating toxins is the diphtheria toxin receptor, which was cloned and identified as heparin-binding epidermal growth factor-like growth factor precursor (16, 17). Similar to C2 toxin, diphtheria toxin is internalized via receptor-mediated endocytosis (9, 10, 14, 29). One very important tool in analyzing and cloning the diphtheria toxin receptor was the use of toxin-sensitive and toxin-resistant cells. Because receptor-deficient eukaryotic cells also would be valuable for identification and analysis of the cellular C2 receptor, we attempted to generate toxin-resistant mutants. In this communication, we report on the isolation and characterization of C2 toxin-resistant, C2II receptor-deficient cells that were derived from toxin-sensitive wild-type CHO (CHO-WT) cells by chemical mutagenesis.

MATERIALS AND METHODS

Materials. *C. botulinum* C2 toxin, *C. perfringens* iota-toxin, and *C. botulinum* C3 exoenzyme were purified as described previously (2, 5, 20, 30). *C. difficile* toxin B was donated by C. Eichel-Streiber (Mainz, Germany); *C. novyi* alpha-toxin was donated by E. Habermann (Giessen, Germany). The alkylating agent *N*-methyl-*N*-nitrosurea (MNU) was purchased from Sigma (Deisenhofen, Germany). Cell culture media and ingredients were from Biochrom (Berlin, Germany). Iodination reagent (Iodo-Beads) was purchased from Pierce (Ba oud Beijerland, The Netherlands). $Na^{125}I$ was obtained from NEN-Du Pont (Dreieich, Germany).

Cell culture. CHO-K1 cells were cultured in Ham's F12 medium-Dulbecco's modified Eagle medium (1:1) containing 5% heat-inactivated (30 min, 56°C) fetal calf serum (FCS), 2 mM L-glutamate, 100 U of penicillin per ml, and 100 μg of streptomycin per ml. Cells were routinely trypsinized and reseeded twice a week.

Cytotoxicity assay. To determine the cytotoxic effects of bacterial toxins, we determined cell-rounding capacity and/or colony-forming ability. Percent cell rounding was calculated after counting the number of fully rounded cells. To determine colony-forming ability, cells were seeded in low density (500 to 1,000 per dish) and treated 8 to 12 h later with C2 toxin (the C2II concentration [nanograms per milliliter] used was always twice the C2I concentration). For these experiments, C2II was activated by trypsin as described previously (1). After the indicated times, toxin was removed and cells were grown for 7 to 10 days. The colony-forming ability of untreated control cells was set to 100%, and the cytotoxic effect of C2 was calculated from the number of colonies appearing after C2 pretreatment.

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Determination of serum dependence. CHO-WT or mutant cells (10^4 of each) were seeded in 24-well dishes and grown in the presence of 5 or 0.2% FCS. One to seven days later, cells were trypsinized and counted (Neubauer chamber). To determine the influence of serum factors on the plating efficiency, cells were seeded in low density (500 to 1,000 per dish) in the presence of 5 or 0.2% FCS and grown for 8 days. After this time, colonies were counted, and the number of colonies that grew in the presence of 5% FCS was compared with the number that grew in the presence of 0.2% FCS.

Isolation of C2-resistant CHO mutants. A total of 2×10^5 CHO-K1 cells were seeded per 10-cm-diameter dish; 24 h later, cells were treated for 4 h with 2 mM MNU (Sigma). After 3 days, cells were reseeded 1:5; 3 days later, 10^6 cells were reseeded for C2 treatment. C2 treatment was performed with 50 ng of C2I (100 ng of C2II) per ml for 8 h. Afterwards, medium was removed, and the cells were washed with phosphate-buffered saline (PBS) and grown for 10 days in fresh medium. Single colonies appearing after this time were picked, grown, and retested for C2 resistance (50 ng of C2I per ml, 8 h). To determine the mutation frequency on the *HGPRT* locus, MNU-mutagenized cells were selected for resistance to 6-thioguanine (2 mM; no medium change).

ADP-ribosylation. For ADP-ribosylation, cells were disrupted by sonication in ice-cold buffer containing 10 mM Tris-HCl (pH 7.4), 1 mM EDTA, 1 mM $MgCl_2$, and 0.1 mM phenylmethylsulfonyl fluoride. After centrifugation (10 min, 600 × *g*, 4°C), the supernatant was used for protein determination by the Bradford method (7). ADP-ribosylation of cell lysates was performed with either *C. botulinum* C3 exoenzyme (Rho-specific [^{32}P]ADP-ribosylation) or C2I toxin (G-actin-specific [^{32}P]ADP-ribosylation). Twenty micrograms of protein was incubated for 30 min at 37°C in 50 μl of a buffer containing 20 mM Tris-HCl (pH 7.4), 1 mM EDTA, 1 mM $MgCl_2$, 1 mM dithiothreitol, 10 mM thymidine, 0.2 μM (or 2 μM) NAD (0.5 μCi of [^{32}P]NAD), and 0.1 μg of C3 (or 0.5 μg of C2I). Reaction products were analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis as described by Laemmli (13), and the dried gels were exposed on Kodak X-Omat films.

Staining of actin with FITC-phalloidin. Logarithmically growing cells were fixed on dishes with 4% formaldehyde–0.2% Triton X-100 in 0.1 M phosphate buffer (pH 7.4) for 1 h at room temperature. After washing with PBS, cells were incubated with fluorescein isothiocyanate (FITC)-labeled phalloidin (0.5 μM in PBS) for 1 h at room temperature. Subsequently, cells were washed three times with PBS (for a total of 15 min), and actin filaments were detected by fluorescence microscopy.

Immunological detection of C2II. Logarithmically growing cells were treated with 200 ng of activated C2II (2) per ml and then incubated at 37°C for 30 min. Afterwards, cells were fixed with 4% formaldehyde (plus 0.2% Triton X-100) in 0.1 M phosphate buffer. Subsequently, fixed cells were incubated for 2 h at room temperature with a polyclonal anti-C2II (activated form) mouse antibody. After being washed with PBS, cells were incubated with an FITC-labeled anti-mouse immunoglobulin G antibody (2 h, room temperature). Afterwards, the uptake of C2II into the cells was monitored by immunofluorescence microscopy. Alternatively, the binding of C2II to cells was detected by Western blot (immunoblot) analysis. For this analysis binding was performed by incubation of the cells with 400 ng of C2II per ml in binding medium (Dulbecco's modified Eagle medium containing 50 μg of bovine serum albumin [BSA] per ml and 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [HEPES; pH 7.4]) for 4 h at 4°C. After being washed with PBS, cells were scraped off, resuspended in SDS-polyacrylamide gel electrophoresis buffer, and heated for 15 min at 95°C. After separation on 7% denaturing gels, proteins were blotted on nitrocellulose by semidry blotting. Bound C2II was detected after incubation with the polyclonal anti-C2II mouse antibody and subsequent incubation with peroxidase-coupled anti-mouse immunoglobulin G by chemiluminescence.

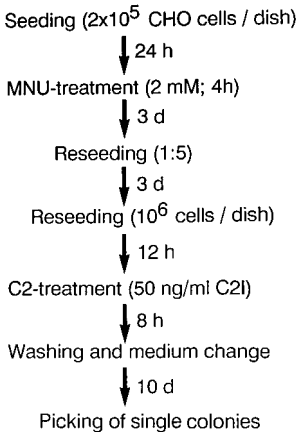


FIG. 1. Procedure for the isolation of C2 toxin-resistant CHO cells. d, days.

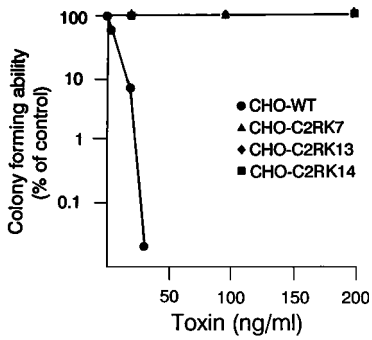


FIG. 2. Influence of C2 toxin on the colony-forming ability of CHO-WT and mutant (CHO-C2RK7, CHO-C2RK13, and CHO-C2RK14) cells. Twelve hours after seeding in low density (1,000 cells per dish), cells were treated with different concentrations of C2I toxin for 8 h (C2I/C2II concentration ratio of 1:2). Afterwards, the medium was changed and cells were grown for 1 week. After this period, the number of colonies appearing was counted. The colony-forming ability of untreated control cells was set to 100%. Data shown are the means from three independent experiments ($n = 6$).

Binding of ^{125}I-C2II. Iodination of the C2II component was performed with Iodo-Beads (Pierce) and Na^{125}I according to the manufacturer's protocol. The specific activity of iodinated C2II was 10^6 cpm/μg. After iodination, C2II was activated by trypsin cleavage as described previously (1). After incubation of cells with ^{125}I-C2II in binding medium for the indicated times at 4°C, cells were washed twice with PBS and harvested for SDS-polyacrylamide gel electrophoresis, and ^{125}I-C2II binding was detected by autoradiography.

RESULTS

To obtain cells resistant to *C. botulinum* C2 toxin, logarithmically growing CHO-K1 cells were treated for 4 h with 2 mM MNU. After a total expression period of 6 days, cells were selected for C2 resistance (50 ng of C2I per ml, 100 ng of C2II per ml, 8 h). Figure 1 schematically shows the procedure for selecting C2-resistant cell lines. The spontaneous mutation frequency for C2 resistance was $< 2 \times 10^{-6}$ (no C2-resistant clone out of 2×10^{-6} selected cells), while the MNU-induced mutation frequency was 30×10^{-6}. The mutation frequency on the *HGPRT* locus, which was determined by counting the number of cells surviving 6-thioguanine selection, was found to be

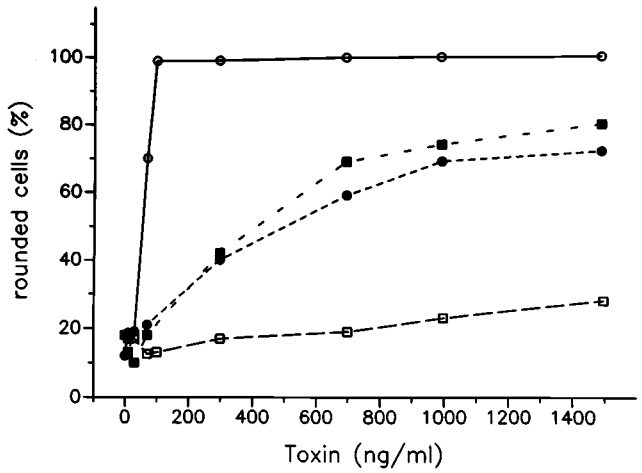
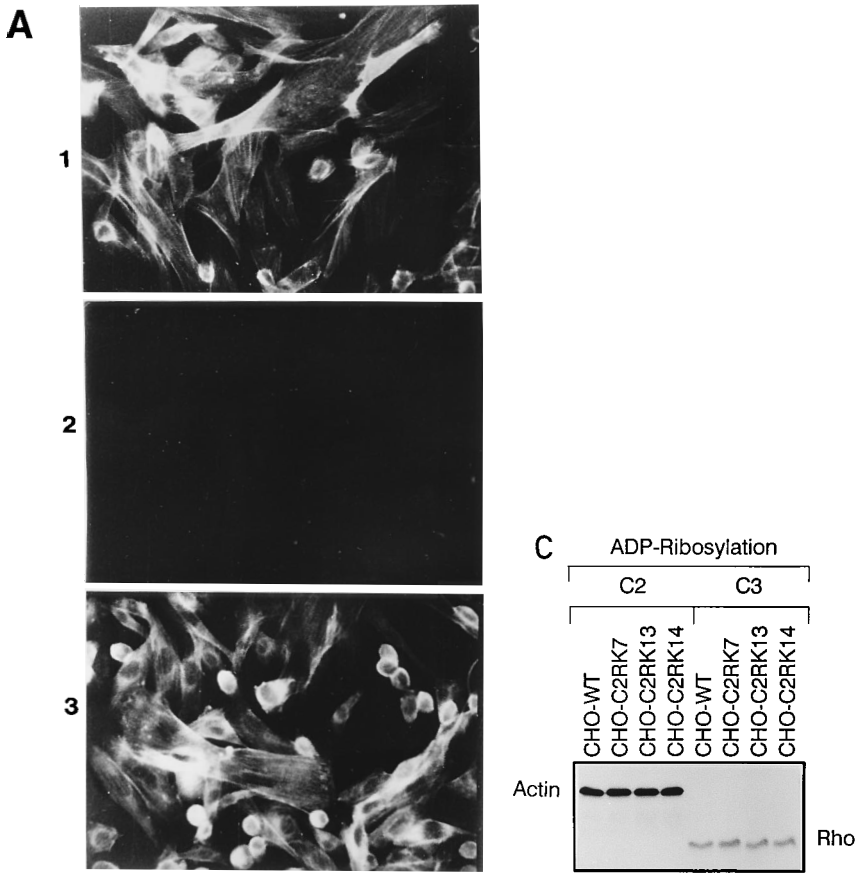


FIG. 3. Rounding up of wild-type and mutant CHO cells by C2 toxin and *C. perfringens* iota toxin. CHO-WT cells (●, ○) and C2-resistant cells (CHO-C2RK14; ■, □) were treated with increasing concentrations of C2 toxin (○, □) and iota-toxin (●, ■) for 8 h. Thereafter, the numbers of rounded cells were determined.



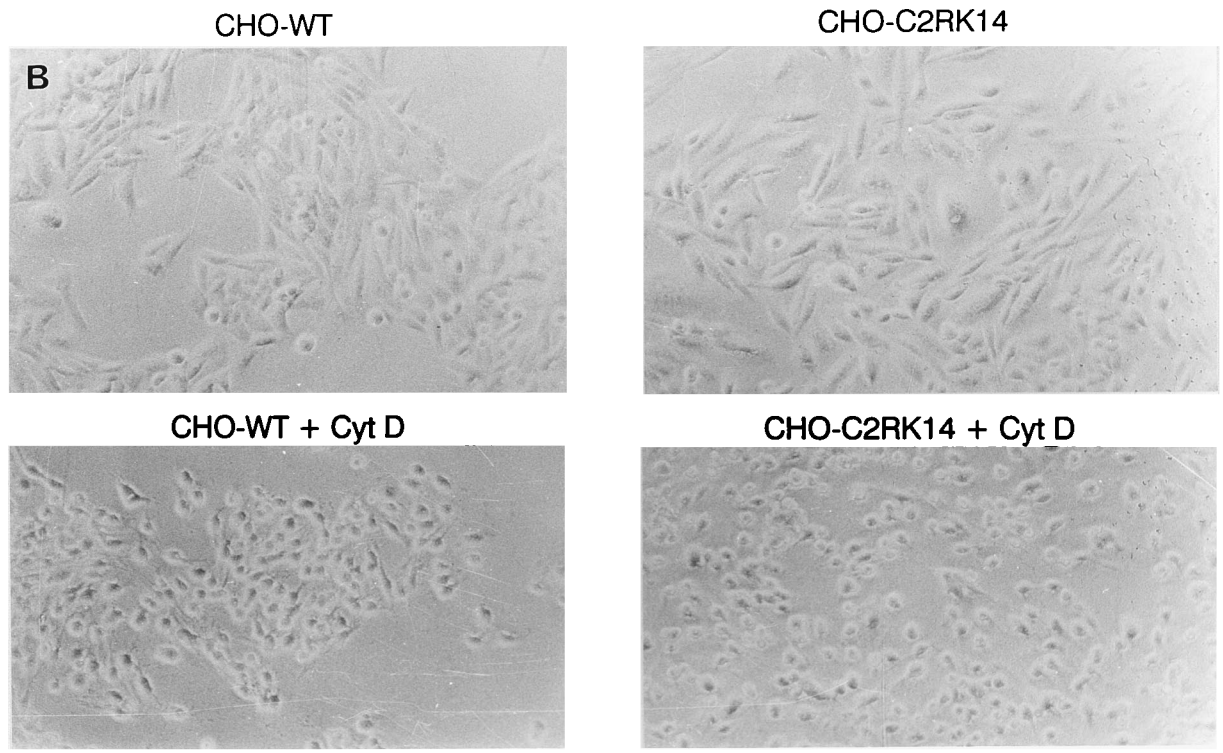


FIG. 4. CHO-WT and C2-resistant cells do not differ in the actin cytoskeleton. (A) CHO-WT and C2-resistant (CHO-C2RK14) cells were treated with 50 ng of C2I (plus 100 ng of C2II) per ml; 4 h later, cells were fixed and the actin cytoskeleton was stained with FITC-phalloidin. 1, untreated CHO-WT cells; 2, C2-treated CHO-WT cells; 3, C2-treated CHO-C2RK14 cells. (B) CHO-WT and C2-resistant mutant (CHO-C2RK14) cells were treated with cytochalasin D (Cyt D; 5 μg/ml, 1 h, 37°C) and photographed. (C) Total cell extract was prepared from logarithmically growing CHO-WT cells and C2-resistant clones (CHO-C2RK7, CHO-C2RK13, and CHO-C2RK14); 20 μg of protein from total cell extracts was used for ADP-ribosylation as described in Materials and Methods. Reaction products were separated by SDS-polyacrylamide gel electrophoresis, and [^{32}P]ADP-ribosylated proteins were detected by autoradiography.

~10-fold higher (20×10^{-5}). The cells which survived C2 selection were picked, grown, and rechecked for C2 resistance under the same conditions. In total, 16 clones were isolated. Whereas 50 ng of C2I (100 ng of C2II) per ml caused a complete rounding of wild-type cells within 2 h, all 16 isolated mutant cells showed no rounding up to 8 h after C2 treatment (last time point tested). Even 16 h after treatment with 100 ng of C2I (200 ng of C2II) per ml, no rounding activity was observed on C2-resistant mutants (CHO-C2RK7, CHO-C2RK13, and CHO-C2RK14). In contrast, CHO-WT cells treated with 2 ng of C2I (4 ng of C2II) per ml exhibited rounding activity. To determine the cytotoxic effects of C2 more quantitatively, colony-forming ability after C2 treatment was measured (Fig. 2). Whereas 10 ng of C2I (20 ng of C2II) per ml was sufficient to cause a 50% reduction in the colony-forming ability of CHO-WT cells, more than 98% of the mutant cells from all three single clones tested (CHO-C2RK7, CHO-C2RK13, and CHO-C2RK14) survived treatment with 200 ng of C2I (400 ng of C2II) per ml. Interestingly, no intermediate resistance behavior of the C2-selected CHO clones was observed. The C2-resistant phenotype was stable over 3 months and was not affected by storage of the cells at −80°C.

The resistance to C2 was found to be specific. The rounding activities of several other bacterial toxins (*C. novyi* alpha-toxin [1 μg/ml, 4 h], *C. difficile* toxin B [10 ng/ml, 4 h], and *C. botulinum* C3 transferase [30 μg/ml, 24 h]) which are known to destroy the cellular cytoskeleton were not influenced. Interestingly, the C2-resistant CHO cells were still sensitive to the cytopathic activity of *C. perfringens* iota-toxin. As shown in Fig. 3, whereas C2 toxin was effective in wild-type cells but not in mutants, iota-toxin induced rounding up in both cell types. The concentration effect curve of iota-toxin was almost identical for wild-type and mutant cells. As expected, each separated toxin component did not cause rounding up in either cell type (not shown). After C2 treatment, CHO-WT cells rounded up and their actin cytoskeleton disappeared completely, whereas the actin cytoskeleton of C2-resistant CHO mutants was not influenced at all (Fig. 4A). However, C2 resistance did not protect cells from the rounding activity of cytochalasin D (Fig. 4B). Furthermore, the MNU-induced mutation leading to C2 resistance did not interfere with the ability of C2I to ADP-ribosylate G-actin (Fig. 4C). Also, C3-mediated ADP-ribosylation of Rho proteins, which are assumed to be involved in the regulation of the microfilamental network (8, 23, 36), was not different in lysates from mutant cells from that in lysates from wild-type cells (Fig. 4C). Altogether, these data strongly indicate that a mutation on actin is not responsible for the C2-resistant phenotype.

The simplest explanation for the observation that all isolated mutants show a largely enhanced C2 resistance is a functional loss of the cellular C2II receptor. Cells lacking the receptor are not able to take up C2I efficiently and survive at high concentrations of the toxin. To test this hypothesis, we investigated the binding of C2II to wild-type and mutant cells. As shown in Fig. 5, C2 resistance apparently correlates with a lack of C2II binding. By immunofluorescence, Western blotting (not shown), and use of ^{125}I-labeled C2II, no binding of C2II to mutant cells was detectable (Fig. 5). These data indicate that the C2-resistant phenotype is most likely based on a mutation in the cellular receptor for C2II, thereby preventing uptake of C2I toxin into cells. To study the physiological function of the involved cellular receptor, we investigated its interference with cellular growth. Therefore, we analyzed the serum dependency and plating efficiency of wild-type and mutant cells. The growth rate of the C2-resistant mutants CHO-C2RK7 and CHO-C2RK14 was lower than that of CHO-WT cells in the presence

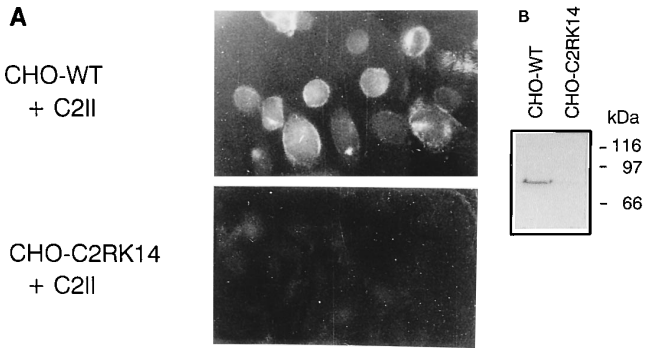


FIG. 5. C2-resistant (CHO-C2RK14) cells lack C2II binding. (A) Logarithmically growing cells were incubated with 200 ng of C2II per ml for 30 min at 37°C. Subsequently, cells were washed twice with PBS and fixed with formaldehyde as described in Materials and Methods. Bound C2II was detected by immunofluorescence after incubation of the cells with a polyclonal anti-C2II antibody followed by incubation with FITC-conjugated anti-mouse immunoglobulin G. (B) C2-sensitive and -resistant CHO cells were incubated for 4 h at 4°C with 200 ng of ^{125}I-C2II per ml. Afterwards, cells were washed twice with PBS, harvested for SDS-polyacrylamide gel electrophoresis as described for panel A, and subjected to autoradiography.

of 5% FCS (Fig. 6A). In the presence of 0.2% FCS, the growth of CHO-C2RK7 and CHO-C2RK14 cells ceased and the growth of CHO-C2RK13 cells was inhibited (Fig. 6A). Furthermore, plating efficiency studied in the presence of 0.2% FCS was consistently decreased with the attainment of C2 resistance. With the number of colonies growing in the presence of 5% FCS set to 100%, 8% of the wild-type cells but less than 1% of the mutant cells were able to form colonies at a reduced serum concentration (0.2% FCS) (Fig. 6B). The clonability in soft agar (with 5% FCS) was 70 to 80% for CHO-WT and CHO-C2RK13 cells, whereas the clonability of the mutant cell lines CHO-C2RK7 and CHO-C2RK14 was reduced to 40 to 60%. Altogether, these data show a correlation between C2 resistance and enhanced serum dependency, suggesting involvement of the cellular C2II receptor in growth.

The extent of the cytotoxic effect of C2 toxin changed with the concentration of serum factors. Increasing serum concentrations reduced the cytotoxicity of C2 toxin (Fig. 7). Furthermore, preincubation of wild-type cells in the presence of FCS (at 4°C) reduced the subsequent binding of C2II (not shown). To analyze the serum component which protects cells against C2 toxicity, effects of FCS pretreatment and of various serum components were investigated. Table 1 summarizes these results. Apparently, the protective serum factor needed heat activation, was heat resistant, and was resistant to trypsin but sensitive to proteases like proteinase K or pronase. From these data, we suggest that the protective serum component is a heat-resistant peptide. This view is in line with the observation that charcoal-extracted FCS kept its protective function, whereas protein-free, chloroform-methanol extracts from FCS were inactive. Concanavalin A, lysophosphatidic acid, or *N*-acetylneuraminic acid showed no protective effect against the cytotoxicity of C2. Also, negatively charged heparin and poly-L-glutamate did not reduce the toxicity of the positively charged C2II. This finding indicates that the protective function of serum is not simply due to a charge-dependent, nonspecific binding of C2II to serum components. Pretreatment of heat-inactivated FCS with hyaluronidase or glucuronidase reduced the ability of FCS to protect against C2 toxin. Furthermore, the protective serum factor bound to heparin-Sepharose (eluting at 0.2 to 0.4 M NaCl). Preliminary studies performed on spin columns with matrices of different exclusion M_rs (G25

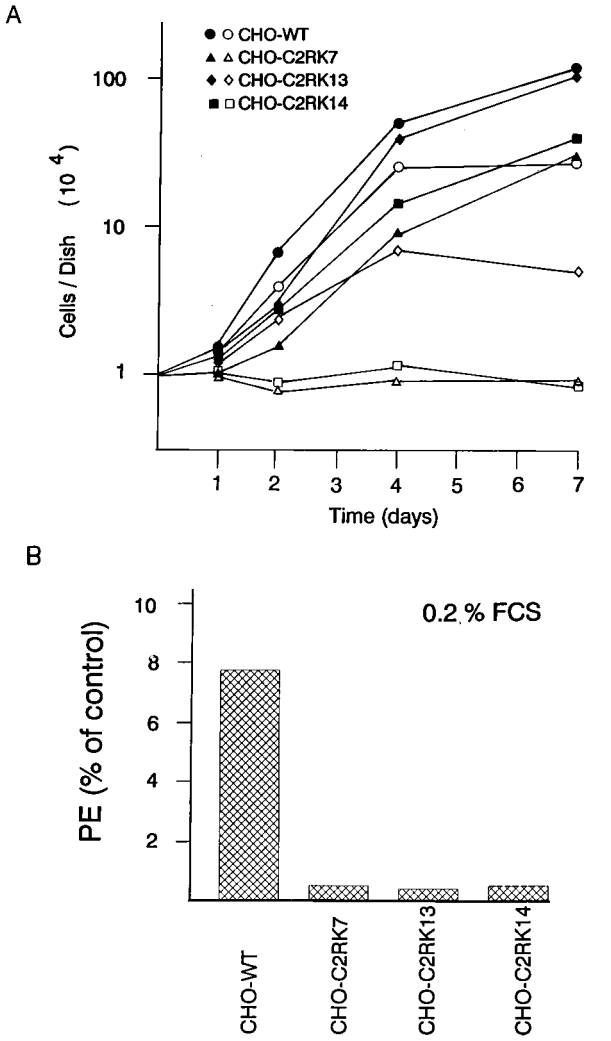


FIG. 6. The C2-resistant phenotype is correlated with an increased serum dependency. (A) Cells (10^4) were seeded and grown in the presence of 5% (closed symbols) or 0.2% (open symbols) FCS; 1 to 7 days after seeding, cell number was counted with a Neubauer chamber. CHO-C2RK7, CHO-C2RK13, and CHO-C2RK14, C2-resistant mutants. Data are derived from at least two independent experiments. (B) Cells were seeded in low density (1,000 per dish) and grown in the presence of 5 and 0.2% FCS. Colonies were counted after 1 week. The relative plating efficiencies (PE) with 0.2% FCS were calculated with respect to those with 5% FCS, which were set to 100%. Data shown are the means of three independent experiments.

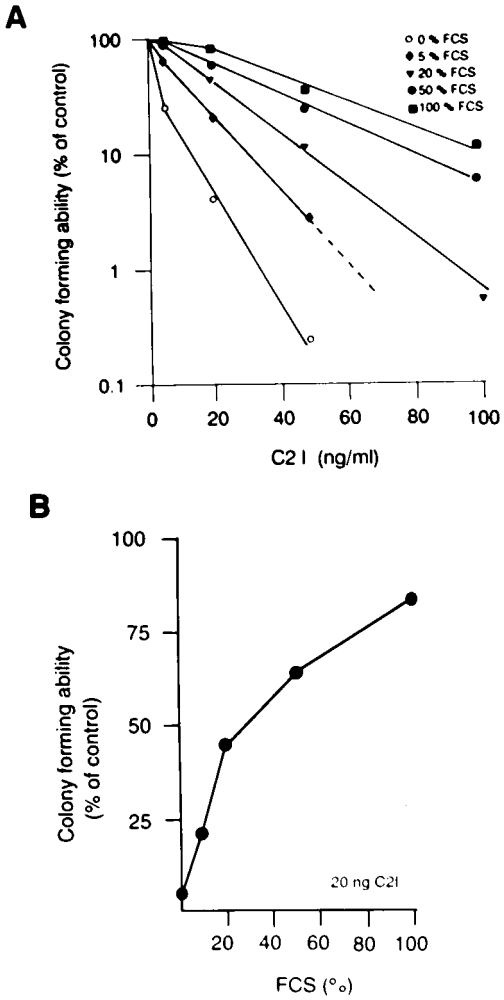


FIG. 7. Serum factors protect CHO-WT cells against the cytotoxic effects of C2. (A) Low-density (1,000 per dish)-seeded CHO-WT cells were preincubated for 5 min at room temperature with different concentrations of FCS. Subsequently, C2 toxin was added at increasing concentrations, and cells were further incubated for 2 h at 37°C. Afterwards, medium was replaced by fresh medium containing 5% FCS, and cells were allowed to grow for 1 week. Thereafter, colonies were counted, and the colony-forming ability of toxin-treated cells was compared with that of untreated control cells, which was set to 100%. The data shown were derived from at least two independent experiments. (B) Colony-forming ability of C2-treated CHO-WT cells as function of serum concentration. The data shown are transformed from those in panel A.

to G150) suggest a molecular mass of the protective serum component of 50 to 100 kDa (not shown).

DISCUSSION

Toxin-resistant cells have been shown to be extremely valuable for elucidating the mechanism of toxin action on target cells. For example, the cellular receptor of diphtheria toxin has been identified by analysis of toxin-sensitive and toxin-resistant cell lines (16, 17). Similar to diphtheria toxin or other intracellularly acting bacterial protein toxins, C2 toxin is constructed according to the A-B model and consists of two components, C2I and C2II. C2II, the binding component (B) of C2 toxin, enables the receptor-mediated uptake of the biologically active enzyme component C2I (A) into cells (22, 27). However, in contrast to other toxins (diphtheria toxin, tetanus toxin, and cholera toxin), both components of C2 toxin are separated proteins which are neither covalently nor noncovalently linked. By selection of MNU-mutagenized CHO cells with high concentrations of C2 toxin, we have isolated C2-resistant cell clones. With respect to cell rounding and colony-forming ability, all clones obtained tolerated >100-fold-higher concentrations of C2I than wild-type cells. Various observations indicate that the C2-resistant phenotype is not due to changes in the organization of the actin cytoskeleton or to mutations inhibiting the ADP-ribosylation of G-actin by C2I. First, the typical morphology was not changed after MNU-induced mutation, and F-actin stained by FITC-phalloidin exhibited no changes compared with controls. Second, C2I-induced [^{32}P]ADP-ribosylation of G-actin in cell lysates of wild-type and mutant cells was independent of C2 resistance. Third, C2-resistant cells were sensitive to other bacterial toxins, including *C. perfringens* iota-toxin (25), *C. difficile* toxin B (12, 15), and *C. botulinum* C3

TABLE 1. Effects of different serum pretreatments and serum factors on the cytotoxicity of C2 toxin[a]

Treatment	Cytotoxic effect of C2[b]
FCS (untreated)	++
FCS (30 min, 56°C)	0
FCS (15 min, 95°C)	0
*Trypsin (50 μg/ml)	++
*Proteinase K (1 mg/ml)	0
*Pronase (50 mg/ml)	0
*Hyaluronidase (5 mg/ml)	+
*Glucuronidase (3 U/ml)	+
*Phospholipase A or C (20 U/ml)	++
FCS (chloroform-methanol supernatant)	++
FCS (charcoal extracted)	0
Lysophosphatidic acid (0.1–10 μg/ml)	++
Heparin (0.01–5 mg/ml)	++
Poly-L-glutamate (0.5 mg/ml)	++
Concanavalin A (0.01–0.5 mg/ml)	++
Lectin (0.5 mg/ml)	++
N-Acetylneuraminic acid (1–10 mg/ml)	++
Glutathione (2 mM)	++
Dithiothreitol (5 mM)	++
BSA (5 mg/ml)	++
Transferrin (0.01–100 μg/ml)	++

[a] Low-density-seeded CHO-WT cells were pretreated with the indicated substances or with pretreated, heat-denaturated FCS for 5 min at room temperature. Enzymatic pretreatment (*) of heat-inactivated FCS for 2 h at 37°C was followed by a denaturating step (15 min, 95°C). Subsequently, 20 ng of C2I (40 ng of C2II) per ml was added, cells were further incubated for 4 h at 37°C, and the medium was changed; colonies were counted 1 week later. The influence of each pretreatment was calculated by comparing the colony-forming ability after pretreatment with the colony-forming ability in the presence of 0 and 20% heat-inactivated FCS.

[b] 0, no cytotoxic effect; +, weak cytotoxic effect; ++, strong cytotoxic effect.

exoenzyme (8, 23), which are known to act on the actin cytoskeleton. Finally, the actin cytoskeleton of C2-resistant CHO cells was still sensitive to cytochalasin D. Thus, it appears that the mutation does not affect the actin cytoskeleton itself but rather affects the transport of the toxin into the cells. It is generally accepted that the transfer of bacterial toxins into cells depends on at least three steps: toxin binding, endocytosis, and membrane translocation. Our finding that C2-resistant mutants lost the ability to bind activated C2II indicates that the receptor for toxin binding was affected by the MNU treatment. *C. perfringens* iota-toxin and *C. difficile* toxin B enter eukaryotic cells also by endocytosis and membrane translocation. The observation that these toxins are still active suggests that the internalization machinery is still functioning in C2-resistant CHO cells and therefore corroborates the hypothesis that the C2II receptor is affected in the mutated cells. The effectiveness of *C. perfringens* iota-toxin is noteworthy. Also, iota-toxin is a binary toxin consisting of the enzyme component Ia and the binding component Ib (28, 31). Although iota-toxin ADP-ribosylates G-actin at the same amino acid (Arg-177) as C2 toxin (33, 34), the binding components of the toxins are different and are apparently not interchangeable. Our findings that C2-resistant CHO cells are sensitive to iota-toxin indicate that the two toxins act via different membrane receptors on eukaryotic cells.

To analyze a possible function of the C2 toxin receptor in growth, we studied the influence of serum and serum factors on wild-type and mutant CHO cells. We observed an increased serum dependency of C2-resistant mutant cells. In the presence of 0.2% FCS, both growth and colony-forming ability of the mutant cells were largely reduced in comparison with wild-type cells. These data suggest that the nonmutated C2II receptor may be involved in growth control, indicating that its physiological ligand is a growth-related factor. In line with this hypothesis are the findings that (i) serum factors protected wild-type cells from the cytotoxic effects of C2 and (ii) preincubation of wild-type cells with high serum concentrations reduced subsequent C2II binding. By analyzing the protective capacities of differently pretreated sera, we preliminarily characterized the putative serum ligand for the cellular C2II receptor as a heat-resistant glycoprotein of 50 to 100 kDa, which, as often observed for growth factors, binds to heparin-Sepharose. Interestingly, this serum factor needed heat activation for its C2 protective function. A possible explanation for this finding is that heat treatment activates the factor from serum components.

In summary, we characterized CHO cell mutants which were resistant to *C. botulinum* C2 toxin. The loss of binding of C2II toxin and the ability of other cytotoxins to affect the actin cytoskeleton in mutant cells indicate a defect of the C2II cell surface receptor. Serum protection of CHO-WT cells to C2 toxin and the enhanced serum dependency of C2II receptor-deficient CHO mutants suggest that the C2II receptor is involved in growth regulation. For further studies to identify the nature of the C2 toxin receptor, the mutant C2-resistant CHO cells described herein will be of high value.

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