Binding of *Clostridium botulinum* C2 Toxin to Asparagine-linked Complex and Hybrid Carbohydrates*

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*Clostridium botulinum* C2 toxin is a binary toxin composed of an enzymatic subunit (C2I) capable of ADP-ribosylating actin and a binding subunit (C2II) that is responsible for interaction with receptors on eukaryotic cells. Here we show that binding of C2 toxin depends on the presence of asparagine-linked carbohydrates. A recently identified Chinese hamster ovary cell mutant (Fritz, G., Schroeder, P., and Aktories, K. (1995) *Infect. Immun.* 63, 2334–2340) was found to be deficient in N-acetylglucosaminyltransferase I. C2 sensitivity of this mutant was restored by transfection of an N-acetylglucosaminyltransferase 1 cDNA. C2 toxin sensitivity was reduced after inhibition of α-mannosidase II. In contrast, Chinese hamster ovary cell mutants deficient in sialylated (Lec2) or galactosylated (Lec8) glycoconjugates showed an increase in toxin sensitivity compared with wild-type cells. Our results show that the GlcNAc residue linked β-1,2 to the α-1,3-mannose of the asparagine-linked core structure is essential for C2II binding to Chinese hamster ovary cells.

*Clostridium botulinum* C2 toxin is one of several binary toxins that recruit a binding component to deliver the enzymatic active component to the interior of eukaryotic cells (1). The 49-kDa catalytically active component (C2I) exhibits ADP-ribosyltransferase activity toward actin (2) and is translocated into cells through interaction of its N-terminal domain with the binding component C2II (3). Proteolytic cleavage by trypsin removes an N-terminal 20-kDa fragment, thereby activating the C2II binding component (4). The activated C-terminal 60-kDa fragment is then capable of binding both the enzymatic component and its receptor on eukaryotic cell membranes (5). After binding, the C2I-C2II complex is internalized by receptor-mediated endocytosis (6). Inside the cell, probably in an acidic endosomal compartment, C2I is translocated into the cytosol. In the cytosol, C2I ADP-ribosylates monomeric G-actin at arginine 177 (2, 7). ADP-ribosylated G-actin is not able to polymerize but binds to the growing end of actin filaments in a capping protein-like manner, resulting in depolymerization of microfilaments (8). At the morphological level, the breakdown of F-actin polymers is accompanied by the rounding up of toxin-treated cells. Toxins that act in a similar manner have been cloned from *Clostridium perfringens* (9), *Clostridium perfringens* ( iota toxin) (10), and *Clostridium difficile* CD196 (11). All are of binary structure, modifying G-actin, although they show some variations with respect to their substrate specificity. While C2I only ADP-ribosylates β- and γ-actin, iota toxin can modify all actin isoforms (12). The binding components of these toxins exhibit about 40% sequence homology to C2II (13), although the former three are more similar to each other than to C2II. The receptor structure for none of these toxins has been defined yet. Recently, a Chinese hamster ovary (CHO)1 cell mutant (RK14) resistant to C2 toxin has been isolated (14). The resistance of this mutant could be attributed to a lack of toxin binding rather than mutation of an intracellular factor that could inhibit the toxic action of C2 (14). In this study, we show that inability of RK14 cells to bind C2 toxin is based on deficiency in N-acetylglucosaminyltransferase I (GlcNAc-TI) activity, thus providing evidence that an N-linked complex (or hybrid) carbohydrate is essential for C2 toxin binding.

**EXPERIMENTAL PROCEDURES**

**Materials**—Lec2 and Lec8 cells (15) were obtained from the American Type Culture Collection (Manassas, VA). Lec13 (16), lIdLec1 (17), and 15B cells (18) were kindly provided by Dr. Pamela Stanley (Albert Einstein College of Medicine, Yeshiva University, New York) and Dr. Felix Wieland (Biochemie-Zentrum, Universität Heidelberg, Germany), respectively. Monoclonal antibody 735 directed against polysialic acid (19) was a kind gift of Dr. Rita Gerardy-Schahn (Medizinische Hochschule Hannover, Germany). An antisem against C2II was generated by immunization of rabbits with a peptide (ANARNRTDJDGIPDE) corresponding to the amino terminus of trypsin-activated C2II. Lectins, swainsonine, and tetramethylrhodamine isothiocyanate (TRITC)-phalloidin were obtained from Sigma. *Tuq* polymerase was from Roche Molecular Biochemicals. Recombinant *C. botulinum* toxin C2I and C2II were expressed in *Escherichia coli* as glutathione S-transferase fusion proteins, and the toxin components were liberated using thrombin as described recently (20). C2II was activated by incubation with 0.2 μg of trypsin/μg of C2II for 30 min at 37°C. The reaction was stopped by adding trypsin inhibitor (2 μg/μg of trypsin). Toxin components were stored at −20°C.

**Cell Culture**—CHO mutants Lec2, Lec8, Lec13, 15B, and IdLd.Lec1 were maintained in α-modified Eagle’s medium supplemented with 10% fetal calf serum, 2 mM 1-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. All cells were incubated at 37°C in 5% CO2. CHO-K1 wild-type and RK14 cells were maintained in Dulbecco’s modified Eagle’s medium/Ham’s F-12 (1:1) supplemented with 5% fetal calf serum and antibiotics described above. In experiments where cytotoxic effects of C2 toxin were compared among CHO-K1, RK14, and the other CHO mutants, all cells were maintained in α-modified Eagle’s medium to exclude differences in C2 cytotoxicity due to different medium components or pH values.

**Cytotoxicity Assays**—Semiconfluent monolayer cultures were treated with increasing concentrations of equimolar amounts of C2I and trypsin-activated C2II. After appropriate incubation at 37°C, cells were

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1 The abbreviations used are: CHO, Chinese hamster ovary; GlcNAc-TI, UDP-GlcNAc:α-1,2-mannoside-α-1,2-N-acetylglucosaminyl transferase I (EC 2.4.1.101); LCA, lens culinaris agglutinin; PCR, polymerase chain reaction; TRITC, tetramethylrhodamine isothiocyanate; PBS, phosphate-buffered saline.
fixed in 4% paraformaldehyde/PBS, and the percentage of rounded cells was determined. In a second approach, cells were seeded at low density into 96-well cell culture plates (2 × 10^4 cells/well) in the presence of increasing amounts of C2 toxin. When the untreated control wells became confluent, the C2 concentration that led to more than 90% cell death was determined. Lectin sensitivity of CHO mutants was determined in the same way, using increasing concentrations of the ricin B chain, lens culinaris agglutinin (LCA), and concanavalin A. To determine the protective effect of α-mannosidase II inhibition, cells were first incubated with 10 μM swainsonine for 48 h, before treatment with C2 toxin. The influence of toxin treatment on the colony forming ability was determined by incubating CHO cells at a density of 500 cells/6-cm cell culture dish with increasing amounts of C2 toxin for 4 h. Thereafter, cells were washed three times with PBS, medium was added, and the appearing colonies were counted 12–14 days later.

Actin Staining with TRITC-Phalloidin—Toxin-treated or untreated cells grown on glass coverslips were fixed in 4% paraformaldehyde, 0.1% Triton X-100 in PBS for 20 min at room temperature. After washing three times with PBS, cells were incubated with 0.75 μg/ml TRITC-phalloidin for 30 min at room temperature. Thereafter, cells were washed four times for 10 min each with PBS followed by a short rinse in water. Specimens were embedded in Kaiser’s gelatin.


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cell fusion was induced using 50% polyethylene glycol 4000 in 75 mM Hepes (pH 8.0). Another 24 h later, cells were harvested and analyzed by SDS-polyacrylamide gel electrophoresis and Western blotting. After transfer onto nitrocellulose membranes, nonspecific binding sites were blocked in 5% nonfat dry milk in PBS. Polysialic acid was detected using monoclonal antibody 735 and anti-mouse peroxidase conjugate. Bound secondary antibodies were revealed using ECL chemiluminescence detection.

Cloning and Expression of Hamster GlcNAcTI—Hamster GlcNAc transferase I was cloned from genomic DNA from CHO-K1 wild-type cells and mutants RK14 and 15B by PCR using Tag polymerase and primers 5'-GCCATTACCATGCTAGAAGAGAAGCTGAGTC-3' and 5'-GGGCTGATCCTAGCTAGATCATAGGC-3' (the KpnI and EcoRI restriction sites introduced to facilitate subcloning are underlined). The PCR product was subcloned into the KpnI and EcoRI sites of the vector pcDNA3 (Invitrogen), resulting in the plasmid pcGnT1. CHO-RK14 cells were transfected with pcGnT1 using LipofectAMINE, following the instructions of the manufacturer. G418-resistant colonies were isolated and examined for C2 toxin sensitivity.


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ADP-ribosylation of Actin—Cells were lysed in 0.5% Triton X-100, 50 mM Hepes (pH 7.4). Lysates were diluted 10-fold with 50 mM Hepes (pH 7.4) and subjected to an ADP-ribosylation assay as described (20). Briefly, samples (50 μg of protein) were incubated with 300 ng of C2I in 35 mM Hepes (pH 7.4), 0.1 mM dithiothreitol, and 0.5 μM [α-32P]NAD for 30 min at 37°C. The reaction was stopped by methanol-chloroform precipitation (21). Precipitated proteins were dissolved in Laemmli buffer and subjected to SDS-polyacrylamide gel electrophoresis (22) followed by PhosphorImager analysis.

Binding of C2II to CHO Cells—Confluent monolayers were prechilled at 4°C and then incubated with different amounts of C2II in Dulbecco’s modified Eagle’s medium for 2 h at 4°C. Thereafter, cells were washed six times with PBS and lysed in 20 mM Tris-HCl (pH 8.0), 150 mM NaCl, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1% Triton X-100. Protein concentrations were determined according to Bradford (23). Equal amounts were subjected to SDS-polyacrylamide gel electrophoresis and Western blotting as described above. C2II was detected by sequential incubation with anti-C2II antiserum and anti-rabbit peroxidase-conjugate followed by ECL detection, as described (3).

RESULTS

C2 Toxin Binding Depends on N-Linked Complex or Hybrid Carbohydrates—Binding of several bacterial toxins to target cells has been shown to be carbohydrate-dependent (24), and it has been suggested that cell binding of C2 toxin might be carbohydrate-dependent (25). To characterize the receptor for

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**TABLE I**

| Mutant | Gene affected | Phenotype |
|--------|---------------|-----------|
| 15B    | N-Acetylglucosaminyltransferase I | No complex and hybrid N-glycans |
| RK14   | N-Acetylglucosaminyltransferase I | No complex and hybrid N-glycans |
| Lec2   | CMP-sialic acid transporter | No sialylated glycans |
| Lec8   | UDP-galactose transporter | No galactosylated glycans |
| Lec13  | GDP-D-mannose-4,6-dehydratase | No fucosylated glycans |
the C2II binding component of C2 toxin, we used several mutant CHO cell lines with well defined glycosylation defects (see Table I). Lec2 and Lec8 mutants lack sialylated and galactosylated glycoproteins and glycolipids, due to genetic defects in the CMP-sialic acid and UDP-galactose transporter, respectively (26, 27). Lec13 cells cannot produce GDP-fucose and therefore lack fucosylated glycoconjugates (16). 15B cells are deficient in GlcNAc-TI (18). As a result, they produce only high-mannose and hybrid N-glycans, whereas O-linked glycans and glycolipids are normal. The cytotoxic effect of these mutants was compared with that of CHO-K1 wild-type cells and the C2 toxin-resistant CHO mutant RK14, described previously (14).

**TABLE II**

**C2 toxin sensitivity of CHO mutants**

Cells were seeded into 96-well plates at a density of 10³ per well in the presence of increasing concentrations of C2 toxin. Cells were then cultivated until the untreated control wells reached confluency, and the lowest C2 concentration that causes more than 90% cell death was determined. Pretreatment with 10 μM swainsonine was for 48 h. -Fold resistance (R) and sensitivity (S) are indicated with respect to CHO wild-type cells (CHO-wt). The highest toxin concentration tested did not affect growth of RK14 and 15B cells (R > 16). Data are the mean from three experiments. n.d., not done.

|                  | CHO-wt | Lec8 | Lec2 | RK14 | 15B | Lec13 |
|------------------|--------|------|------|------|-----|-------|
| Untreated        | R/S = 1| S = 5| S = 4| R > 16 | R > 16 | R = 4 |
| Swainsonine-treated | R = 4  | R = 4 | ND   | ND   | R = 4  |

* ND, not done.

**TABLE III**

**Lectin sensitivity of RK14, CHO-15B, and Lec8 cells**

Cells were seeded into 96-well plates at a density of 10³ per well in the presence of increasing concentrations of the lectins indicated. Cells were then cultivated until the untreated control wells became confluent, and the lowest lectin concentration that causes more than 90% cell death was determined. -Fold resistance (R) and sensitivity (S) to the lectins are indicated with respect to CHO wild-type cells.

| Lectin                     | Binds to                                 | RK14 | CHO-15B | Lec8 |
|----------------------------|------------------------------------------|------|---------|------|
| Ricin B chain              | Gal β1–4GlcNAcβ1-R                       | R = 32| R = 64  | R = 2 |
| LCA                       | Bi- and triantennary complex N-glycans containing a core fucose | R > 8 | R > 16 | S = 4 |
| Concanavalin A            | High mannose/hybrid N-glycans            | S = 3 | S = 4   | Not done |
measure the cytotoxic effect of C2 toxin, the cell rounding activity of increasing C2 toxin concentrations was determined for all cell lines (Fig. 1). CHO wild-type cells as well as the mutants Lec8 and Lec13 were sensitive against C2 toxin. However, significant differences in the sensitivity were observed. Lec8 cells were approximately 4-fold more sensitive than wild-type cells (Fig. 1j), and the same was true for Lec2 cells (data not shown). In contrast, Lec13 cells were found to be slightly (2-fold) more resistant than wild-type cells. As reported previously (14), RK14 cells were strongly resistant to C2 toxin. Moreover, the CHO mutant 15B, which lacks complex and hybrid N-linked carbohydrates, was C2-resistant. This was also true for an independent mutant belonging to the same complementation group (ldID.Lec1; data not shown). The above results could be confirmed by determination of the colony forming ability of toxin-treated cells (Fig. 2). Again, Lec13 cells were found to be more resistant, whereas Lec2 and Lec8 cells exhibited increased sensitivity to C2 toxin compared with wild-type cells. In a different approach, cells were seeded at low density in 96-well plates and treated with increasing concentrations of C2 toxin. When untreated control wells were confluent, the lowest C2 concentration that causes more than 90% cell death was determined. The results obtained further confirmed the above results (Table II).

To show the specificity of the cytotoxic effects and to exclude the possibility that differences in the organization of the cytoskeleton are responsible for the differences observed, mutants were treated with other clostridial toxins known to affect actin fibers by glucosylating Rho GTPases (28, 29). All mutants tested (Lec13, Lec2, Lec8, 15B, and RK14) were equally sensitive to the action of *C. difficile* toxins A and B (data not shown).

Binding of C2II to CHO cells was examined by Western blotting using a specific antiserum directed against the carboxyl terminus of the toxin (Fig. 3a). Wild-type, Lec13, and Lec8 cells bound C2II in a concentration-dependent manner. However, significant differences in C2II binding were not evident. Only a faint, nonspecific binding to RK14 cells was observed. In order to correlate the rounding activity of C2 toxin with the ADP-ribosylation of actin, we determined the amount of modified actin in toxin-treated cells. Cell lysates from toxin-treated cells were incubated with C2I to [32P]ADP-ribosylate the unmodified actin fraction. As expected, actin from untreated cells was effectively ADP-ribosylated (Fig. 3b, -C2II).

**FIG. 4.** Complementation analysis of RK14 cells. RK14, 15B, Lec8, wild-type cells, and fusion products of the mutants induced by polyethylene glycol (marked by ×) were analyzed by Western blotting using the polysialic acid-specific antibody 735. 100 μg of protein were applied per lane except for wild-type cells, where only 30 μg were used.

**FIG. 5.** RK14 cells stably transfected with GlcNAc-TI are C2 toxin-sensitive. RK14 cells transfected with pGnT1, encoding hamster GlcNAc-TI, parental RK14, and wild-type cells were treated with 200 ng/ml C2I and 400 ng/ml C2II, and the number of rounded cells was determined at the indicated time points (mean ± S.D., n = 3).

**FIG. 6.** Mutations in the GlcNAc-TI gene in CHO mutants RK14 and 15B. The protein coding region of the GlcNAc-TI gene was amplified by PCR and sequenced as described under “Experimental Procedures.” The positions of the point mutations in RK14 (a) and 15B cells (b) are shown, together with the deduced amino acid sequences.
By contrast, after toxin treatment (200 ng/ml C2I and 400 ng/ml C2II for 6 h) most actin in wild-type, Lec8, and Lec13 cells was no longer ADP-ribosylated (Fig. 3b–d). C2II. On the other hand, most of the actin from RK14 cells was still modified by C2I, indicating that it has not been ADP-ribosylated before. The slight reduction in the amount of ADP-ribosylated actin compared with untreated RK14 cells is probably due to the presence of cells that regained the ability to synthesize complex N-glycans. Such revertants, which are C2 toxin-sensitive, appeared after several weeks of culture.

**C2 Toxin-resistant RK14 Cells Are Deficient in GlcNAc-TI**—Since both 15B and RK14 cells were resistant to C2 toxin, we next investigated whether they belong to the same (Lec1) complementation group. A phenotypic characterization of 15B and RK14 cells revealed that both exhibit nearly identical sensitivity against the lectins ricin, LCA, and concanavalin A (Table III). A complementation assay using polysialic acid as a marker for expression of complex N-linked carbohydrates (30) showed that RK14 belongs to the 15B/Lec1 complementation group (Fig. 4). While fusions between RK14 and 15B, both of which are negative for polysialic acid, did not correct the phenotype, control fusions with the mutant Lec8 led to reexpression of the marker polysialic acid. Thus, RK14 and 15B cells most probably have a defect in the same gene. This conclusion was confirmed by expressing a cDNA encoding hamster GlcNAc-TI in RK14 cells. RK14 cells stably transfected with pGnT1, encoding hamster GlcNAc-TI, were as sensitive to C2 toxin as CHO wild-type cells (Fig. 5).

Taken together, these results prove that C2 toxin resistance of RK14 cells is a result of GlcNAc-TI deficiency and not of an unrelated genetic defect. The hypothesis of a genetic defect in the GlcNAc-TI gene was confirmed by PCR cloning of the GlcNAc-TI gene from RK14 cells. Sequencing of the PCR product revealed a nonsense mutation (G → A) at nucleotide position 287 resulting in a stop codon instead of Trp-96 (Fig. 6a). To exclude PCR errors, two independent clones were sequenced. We also sequenced the GlcNAc-TI gene from 15B cells and found a missense mutation (A730G), which changes Asn-244 to Ser-244 (Fig. 6b). Notably, Asn-244 is conserved among all GlcNAc-TI genes cloned to date, including that of *C. elegans* and *Arabidopsis thaliana*.

**Inhibition of α-Mannosidase II Partially Inhibits C2 Cytotoxicity**—The next step in N-glycan processing following the condensation of GlcNAc by GlcNAc-TI is the removal of the two terminal mannose residues by α-mannosidase II. To further characterize the carbohydrate structure necessary for C2 toxin uptake, CHO cells were treated with the α-mannosidase II inhibitor swainsonine. Pretreatment with 10 μM swainsonine for 48 h resulted in a weak protection (4-fold increase in resistance) of CHO wild-type cells against C2 toxin (Table II and Fig. 7, a–d). The effect was much stronger in Lec8 cells, where pretreatment with swainsonine resulted in an approximately 16-fold decrease in sensitivity (Table II and Fig. 7, e–h). While treatment of Lec8 cells with 100 ng/ml C2I and 200 ng/ml C2II for 4 h caused rounding up of more than 80%, pretreatment with swainsonine did not result in a significant increase in the number of rounded cells, and there was apparently no breakdown of F-actin filaments (Fig. 7h). In contrast, swainsonine treatment of Lec13 cells affected toxin sensitivity only slightly (Table II). In fact, swainsonine-treated Lec8, Lec13, and wild-type cells were found to be similarly sensitive to C2 toxin. Increasing the toxin concentration (or longer incubations; data not shown) resulted in strong morphological changes, indicating that swainsonine-treated cells are still C2 toxin-sensitive (Fig. 7j).

**DISCUSSION**

RK14 cells have been isolated from mutagenized CHO cells by selection for C2 toxin resistance and were found to be deficient in a functional receptor for this toxin (14). We show here that the inability of RK14 cells to bind C2II is the result of a mutation in the GlcNAc-TI gene, which prevents the cells from producing N-linked complex and hybrid carbohydrates. Correcting this defect by transfecting a cDNA encoding an active GlcNAc-TI made the cells C2-sensitive again. Furthermore, independent mutants of the same complementation group (15B, ldlD.Lec1) are also C2-resistant. Thus, the possibility can be excluded that a secondary genetic defect is responsible for
C2 resistance in RK14 cells, showing that binding of C2II depends on the presence of N-linked complex or hybrid carbohydrates. Since synthesis of glycolipids is unaffected in GlcNAc-TI-deficient cells, our results also indicate that glycolipids do not serve as receptors for C2 toxin. Treatment of CHO cells with the α-mannosidase II inhibitor swainsonine reduced the toxin sensitivity but did not abolish toxin binding completely. The only difference in the N-glycan structures of RK14 or 15B cells and swainsonine-treated Lec8 cells is the absence of the β1,2-linked GlcNAc to the α-1,3-linked mannose of the core structure (Fig. 8) in the former cell lines (note that CHO cells did not express the bisecting enzyme N-acetylglucosaminyltransferase III (31). Our results therefore provide strong evidence that this GlcNAc residue is an essential determinant of the C2 toxin receptor.

Partial inhibition of the cytotoxic effect by swainsonine suggests that complex carbohydrates are more efficient in C2II binding than hybrid structures. Alternatively, the two terminal mannose residues of the GlcNAc1-Manα-GlcNAc2 hybrid structure might interfere with toxin binding. However, the observation that Lec8 and Lec2 cells are highly C2-sensitive clearly demonstrate that galactose and sialic acid are not required for toxin binding. In contrast, the higher sensitivity to C2 toxin of Lec2 and Lec8 cells suggests that sialic acid partially blocks the interaction of the toxin with its receptor. Masking of cell surface receptor structures by sialic acid is not uncommon due to their exposed position and negative charge (32). In line with our results, Sugii and Kozaki (25) showed that hemagglutination by C2II is not inhibited by sialic acid and that neuraminidase-treated erythrocytes are more sensitive to the hemolytic activity of C2II.

Fucose-lacking Lec13 cells are slightly less sensitive to C2 toxin. One possible explanation is that fucose is part of the receptor structure. Alternatively, the fucose deficiency of Lec13 cells might reduce the cell surface expression level of the receptor. At present, it is not possible to decide between these possibilities. However, a direct involvement of fucose in toxin binding would be in agreement with observations by Sugii and Kozaki (25) showing that hemagglutination by C2II is not inhibited by sialic acid and that neuraminidase-treated erythrocytes are more sensitive to the hemolytic activity of C2II.

C2II exhibits significant sequence homology to the binding components of other actin ADP-ribosylating toxins, namely C. difficile CD196 toxin (11), C. perfringens iota toxin (35), and C. spiroforme toxin (9). In addition, sequence comparison showed that anthrax protective antigen is structurally related to C2II. Considering the fact that anthrax toxin protective antigen binds its receptor via the C terminus (36), it seems reasonable to assume that the other toxins mentioned above recognize their receptors also by their C termini. Interestingly, sequence similarity between C2II and the other toxins is completely absent from the C terminus. Thus, it seems very likely that the C2-related toxins have different structural requirements for binding. At least this holds true for iota toxin, which is able to enter C2 toxin-resistant RK14 cells (14).

At present, it remains unclear whether GlcNAc-ylated N-glycans are sufficient for C2II binding or whether C2II binding requires also the presence of a specific receptor protein. A C2-specific receptor has to be ubiquitously expressed, because no naturally C2 toxin-resistant cell line has been found, and even erythrocytes from different species are agglutinated by C2II (25). C2 toxin-resistant CHO mutants expressing complex carbohydrates have not been found in the screen reported by Fritz et al. (14), which might be due to the very high frequency of GlcNAc-TI-deficient CHO mutants obtained after chemical mutagenesis (34).

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