

# Differential expression of receptors for Shiga and Cholera toxin is regulated by the cell cycle

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## Summary

Cholera and Shiga toxin bind to the cell surface via glycolipid receptors GM1 and Gb3, respectively. Surprisingly, the majority of Vero cells from a non-synchronized population bind either Cholera or Shiga toxin but not both toxins. The hypothesis that the differential expression of toxin receptors is regulated by the cell cycle was tested. We find that Cholera toxin binds preferentially in G0/G1, with little binding through S-phase to telophase, whereas Shiga toxin binds maximally through G2 to telophase but does not bind during G0/G1 and S-phase. The changes result from the corresponding

changes in Gb3 and GM1 synthesis, not from variations of receptor transport to the cell surface. The changes do not reflect competition of Gb3 and GM1 synthesis for lactosylceramide. Cells as diverse as Vero cells, PC12 cells and astrocytes show the same cell-cycle-dependent regulation of glycosphingolipid receptors, suggesting that this novel phenomenon is based on a conserved regulatory mechanism.

Key words: AB5-toxins, Glycosphingolipid receptors, Cell cycle, Golgi

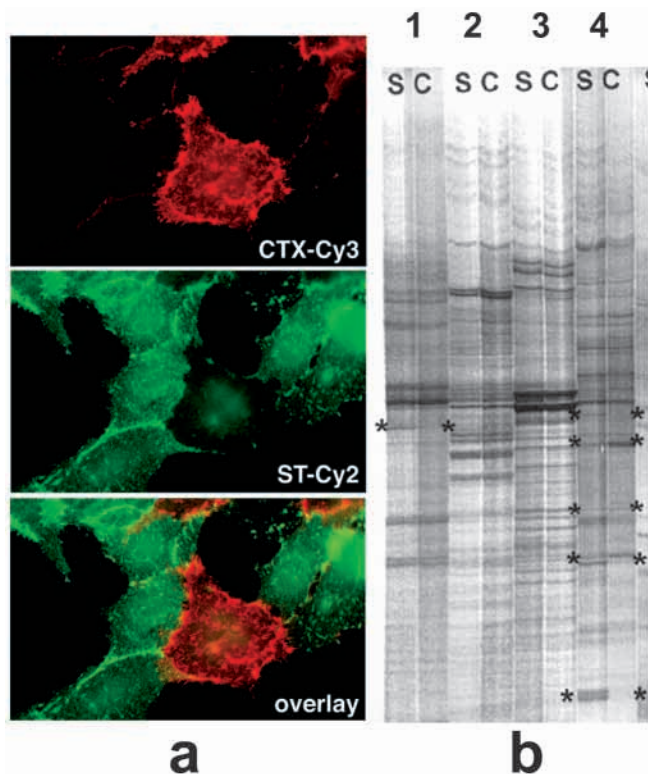
## Introduction

Gangliosides and globosides are classes of glycosphingolipids synthesized from lactosylceramide in the middle and late Golgi. They can serve receptor functions in the plasma membranes of many different cell types. The ganglioside GM1 can act as a receptor for the B subunits of various AB5 toxins like Cholera toxin (CTX) or the heat-stable toxin II from *Escherichia coli* (Van Heyningen, 1974; King and Van Heyningen, 1974; Holmgren et al., 1973; Holmgren et al., 1985; Griffiths et al., 1986; Spangler, 1992) whereas the globotriaoside Gb3 is a receptor for the AB5-toxin Shiga toxin (ST) and some related toxins like Verotoxin (Lindberg et al., 1987; Lingwood et al., 1987). Application of gangliosides or globosides to cells can elicit cellular responses as divergent as signal transduction, cell adhesion or cell growth (Hakomori and Igarashi, 1993). Moreover, the expression of these glycosphingolipids on the surface of the plasma membrane shows strong variations depending on the cell type and the state of the cell.

Cell differentiation affects synthesis and plasma membrane expression of Gb3. Undifferentiated THP-1 cells express significantly more Gb3 on their surface than differentiated cells (Ramegowda et al., 1996), while the situation is opposite in intestinal epithelial cells where differentiated cells express high levels of Gb3 on their surface and undifferentiated cells show an almost complete loss of Gb3 (Jacewicz et al., 1975). In some cells the expression of Gb3 on the cell surface seems to be developmentally regulated. In rabbit intestinal microvilli epithelial cells, the synthesis of Gb3 increases during the third week of life together with an increased sensitivity to the toxic

effects of ST (Mobassaleh et al., 1994). The cellular level of gangliosides also seems to be regulated by differentiation. It decreases in 3T3-L1-adipocytes as they become differentiated. In particular, the level of GM1 drops by 75-80%. These changes could not be observed in the non-differentiating 3T3-C2 cell line (Reed et al., 1980). In colon carcinoma cells (Nojiri et al., 1999) as well as in renal carcinoma cells (Saito et al., 2000), the synthesis of ganglioside GM3, a precursor of GM1, increases following induction of differentiation. The percentage of cultivated MFII-fibroblasts exhibiting high levels of GM3 increases as cells become confluent (Rosner et al., 1990). A similar observation has been made with cultivated Zejdela hepatoma cells (Staedel-Flaig et al., 1987). Interestingly, treatment of a variety of different cell types with butyrate induces synthesis and cell surface expression of GM1 as well as of Gb3 (Fishman and Attikan, 1979), leading to an enhanced binding of CTX as well as of ST and verotoxin. Choi et al. (Choi et al., 1997) have studied the levels of neutral and acidic gangliosides in different phases of the cell cycle in a glioma cell line. Cell-cycle-dependent changes, in particular of GM3, GM2 and GM1, could not be observed, although a decrease in b-series gangliosides was measured during metaphase.

We report here that Vero cells, a green monkey kidney tumor cell line, show cell-cycle-dependent but inverse changes in surface-expressed GM1 and Gb3. During interphase and G1, cells bind almost exclusively CTX; during S-phase binding of both CTX and ST is very low. During G2, binding of ST increases and remains high during metaphase and telophase, whereas the binding of CTX remains low until G0/G1 is reached again. The cell-cycle-dependent changes in the



**Fig. 1.** Differential binding of Shiga and Cholera toxin to Vero cells; differences in gene expression in Shiga versus Cholera toxin binding Vero cells. (a) Differential binding of Cy3-CTX (CTX-Cy3) and Cy2-ST (ST-Cy2) to non-synchronized Vero cells. Cells were incubated simultaneously with the two labeled toxins for 10 minutes at 0°C. Unbound toxins were washed away and the live cells observed by fluorescence microscopy. (b) Differential display comparing ST binding cells (S) and CTX binding cells (C). Non-synchronized Vero cells were labeled simultaneously with Cy2-ST and Cy3-CTX as in (a) and separated by FACS. cDNA was synthesized from RNA extracted from ST and CT binding cells. DNA fragments were produced by PCR using different 5' and 3' primers and analyzed by PAGE. The results of the primer pairs P2/T1, P2/T2, P2/T3, and P2/T4 are shown (see Methods section). Note the differentially amplified cDNA fragments marked by (\*).

expression of GM1 and Gb3 on the cell surface result from corresponding changes of synthesis of GM1 and Gb3, respectively. These observations were confirmed also for other cell types including Vero 317 cells, undifferentiated and differentiated PC12 cells and astrocytes, suggesting that a conserved mechanism is underlying this novel phenomenon.

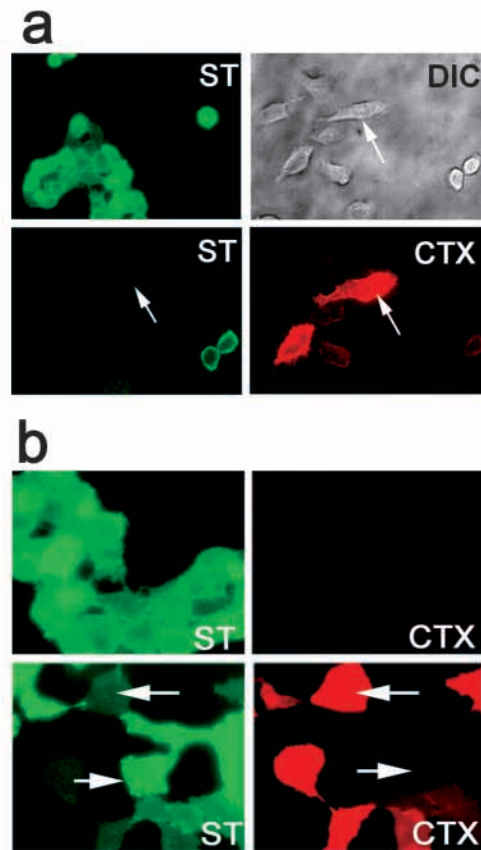
## Materials and Methods

### Cells

Vero and Vero 317 cells were obtained from ATCC. PC12 cells, a rat chromaffin tumor cell line, was a kind gift of Dieter Bruns, this laboratory.

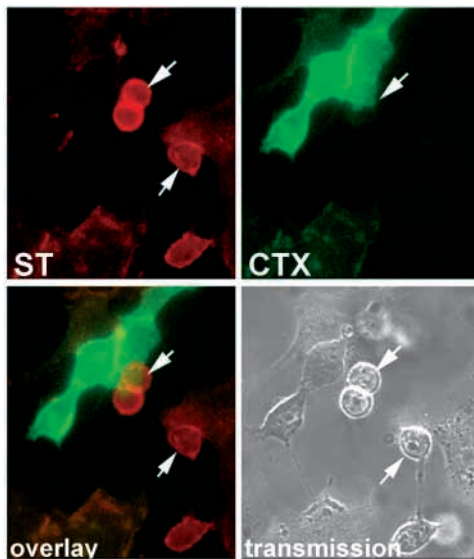
### Toxins and antibodies

CTX-K63 (kindly donated by Mariagrazia Pizza, Siena, Italy) was used throughout the experiments. CTX-K63 has a S<sub>63</sub>K-mutation in the CTX-A subunit that completely abolishes its ADP-ribosylating



**Fig. 2.** Cells that can be detached following colchicine treatment bind preferentially to Shiga toxin, but bind again to Shiga and Cholera toxin after desynchronization. (a) Vero cells detached from the culture dish after colchicine treatment bind to Cy2-ST (upper left panel). Cells remaining attached to the dish are larger, have an extended structure (arrow) and bind only to Cy3-CTX (lower right panel) but not Cy2-ST (lower left panel). DIC (differential interference contrast) of the two lower panels. (b) Cells detached after colchicine treatment were analyzed 30 minutes after detachment for binding of Cy2-ST (upper left panel) and Cy3-CTX (upper right panel). When detached cells were cultivated for another 12 hours, they show again exclusive binding (arrows) of either Cy2-ST (lower left panel) or Cy3-CTX (lower right panel) as seen in non-synchronized cell cultures (compare with Fig. 1a).

activity (Fontana et al., 2000) without affecting its intracellular transport (Majoul et al., 1998). ST was isolated as described previously (Kozlov et al., 1993) (from the Engelhardt Institute of Molecular Biology, Moscow). Both toxins were directly labeled with Cy3- or Cy5-monosuccinimidyl esters or with Cy2-bissuccinimidyl ester according to the Amersham Pharmacia Biotech protocol. Labeled proteins were separated from free chromophores by gel filtration over G-10 and concentrated by Centricon-10 (Amicon™) centrifugation. The labeling stoichiometry was determined from the absorptions at 280 nm (protein), 487 nm (Cy2), 550 nm (Cy3), or 650 nm (Cy5). The molar dye:protein ratios ranged from 0.2 to 1. A polyclonal rabbit anti-cyclin B1 antibody and a mouse monoclonal anti-BrdU-antibody came from Transduction Laboratories (BD Biosciences, Heidelberg, Germany), and a FITC-conjugated mouse monoclonal anti-BrdU-antibody was from Pharmingen (BD Biosciences, Heidelberg, Germany). Mouse monoclonal anti-cyclin B1 antibodies were a kind gift of Jonathan Pines (CRC, Cambridge, UK). A horseradish-peroxidase-conjugated goat anti-rabbit antibody was from Zymed Laboratories (San Francisco, USA).



**Fig. 3.** Dividing cells bind only to Shiga toxin (Cy3-ST). Cells were partially synchronized by a double-thymidine block and analyzed for toxin binding 6 hours later. The relative number of small round-shaped cells binding preferentially to Cy3-ST (upper left panel) is significantly higher than in non-synchronized cultures. Note that the dividing cells (arrows) bind only to ST. The flat, extended, irregularly shaped cells bind only to Cy2-CTX (upper right panel). Lower left panel: overlay of the two upper panels. Lower right panel: digital interference contrast.

#### Cell cultures

Vero, Vero 317 and PC 12 cells, mouse fetal astrocytes and hippocampal neurons of newborn mice were cultivated in DMEM plus 10% FCS supplemented with 100 U/ml penicillin G and 0.1 mg/ml streptomycin. PC12 cells were cultivated on collagen (Vitrogen) or poly-L-lysine (Sigma) coated cover slips; hippocampal neurons were cultured on a feeder layer of fetal mouse astrocytes. For the toxin binding experiments, cells were transferred to DMEM without phenol red, buffered with 10 mM HEPES.

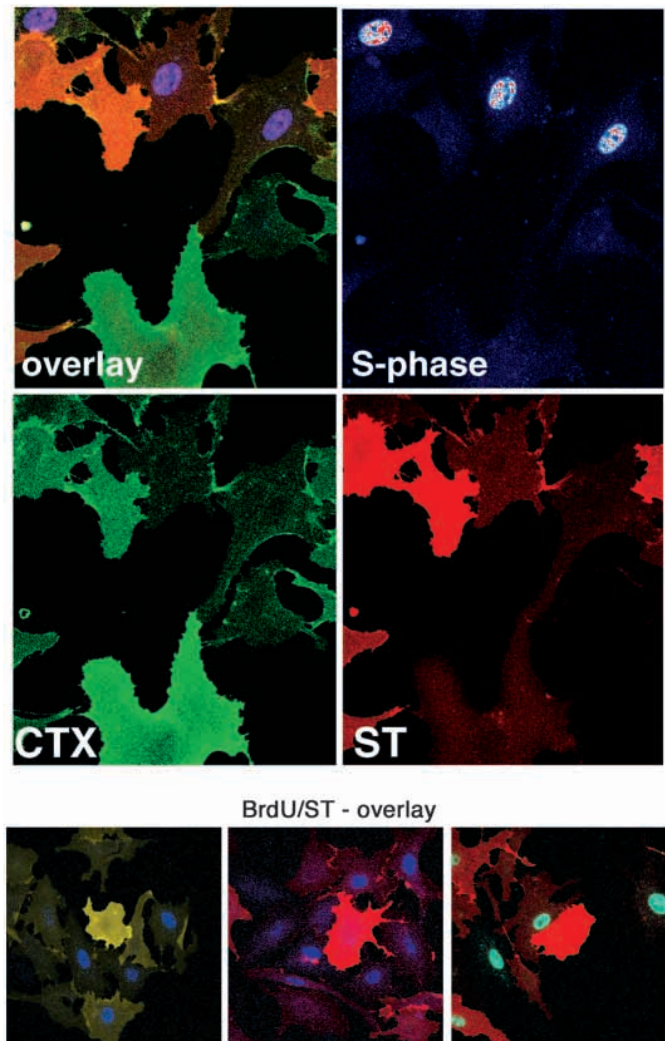
#### Determination of cell cycle phases

S-phase cells were identified by the incorporation of BrdU. To this end, Vero cells were incubated for 30 minutes under standard conditions in the presence of 50–100  $\mu$ M BrdU. Cells were washed with PBS, cooled on ice and exposed to Cy-dye-labeled ST and CTX as below. Cells were fixed with 4% paraformaldehyde and permeabilized with 0.1% saponin. Following blocking with PBS/0.1% fish gelatine, the incorporation of BrdU into DNA was detected by immunofluorescence using either a BrdU-specific monoclonal antibody (1/100) in combination with Cy3- or Cy5-labeled rabbit-anti-mouse secondary antibodies (1/200) or a FITC-conjugated monoclonal anti-BrdU antibody (1/100).

To identify cells in G2 or metaphase, the expression and intracellular distribution of endogenous cyclin B1 was analysed by immunofluorescence using a polyclonal rabbit anti-cyclin B1-antibody or a mouse monoclonal anti-cyclin B1 antibody. For detection Cy-dye labeled secondary antibodies were used. DNA was detected with DAPI dissolved in Moviol.

#### Synchronization and cell cycle arrest

To obtain G0/G1 phase Vero cells, cells were maintained in 0.1% FCS for 36 hours. After addition of serum within 8–10 hours, more than

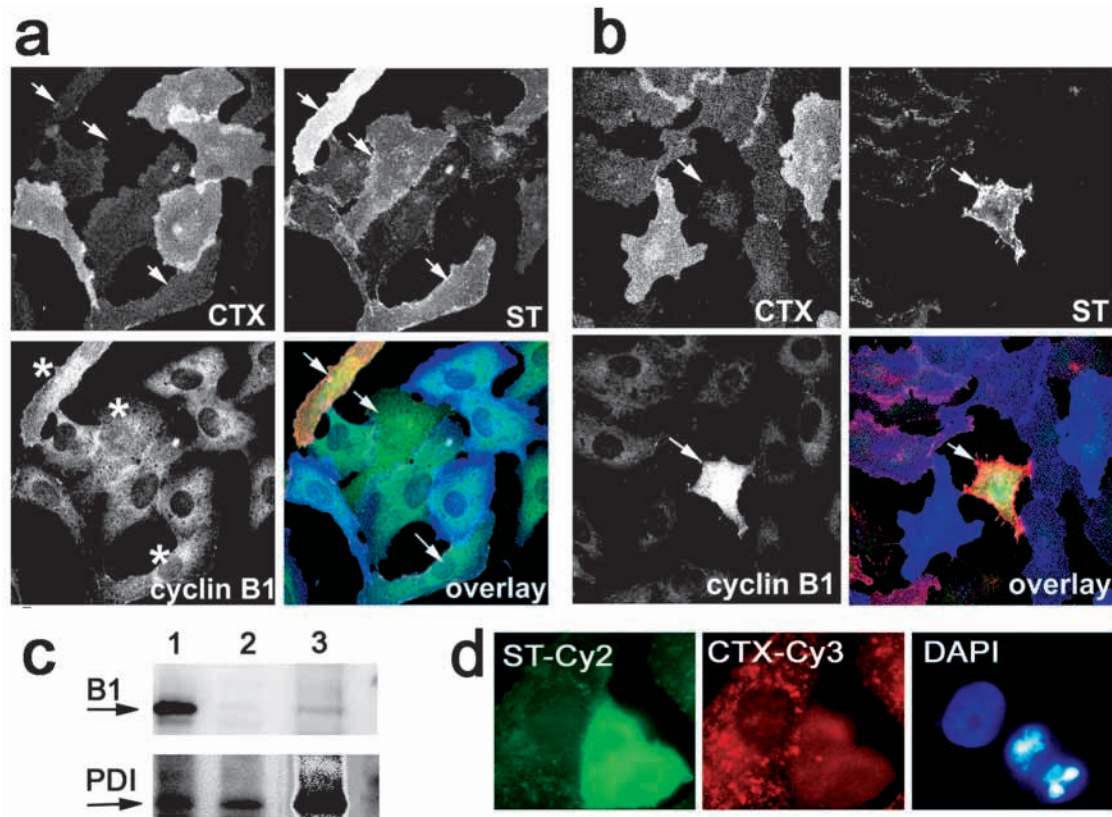


**Fig. 4.** Cells in S-phase exhibit low binding of both toxins. Cells were pretreated with BrdU, exposed to Cy2-CTX and Cy3-ST and analyzed by fluorescence microscopy as in Fig. 1a. Cells were then fixed, permeabilized and analyzed for BrdU incorporation using anti-BrdU-antibodies. Cells strongly stained for BrdU (S-phase) show little or no binding of CTX (CTX) or ST (ST).

30% of Vero cells were in G2 phase. For differentiation of PC12 cells and neurons, 100 ng/ml NGF was added to the culture medium.

For synchronization by a double-thymidine block the exponentially growing Vero cells were arrested in G1 by treatment with thymidine (3 mM) at 0.1% FCS. After 12 hours, cells were released from the block by washing with PBS and addition of fresh medium (DMEM, 10% FCS). Cells were grown for another 10 hours and treated a second time with 3 mM thymidine for 12 hours followed by release from the block (as with the first block). Twelve hours after this release, 30–50% of cells revealed a mitotic phenotype and nuclear staining for cyclin B1. Binding of the toxins to the cell monolayer was analyzed 3, 6, 12, and 24 hours after the release from the second thymidine block.

Alternatively, cell monolayers were treated after 12 hours in culture with 5  $\mu$ g/ml colchicine for 2 or 3 hours. Detached cells were sedimented by centrifugation (800 g for 5 minutes), washed with PBS and exposed to Cy-dye-labeled toxins at 0°C while in suspension. The cells were washed again and sedimented by centrifugation. An aliquot of the sedimented cells was plated on a cover slip for fluorescence microscopy.



**Fig. 5.** Cells in G2 (cyclin B1 positive) and anaphase bind preferentially to Shiga toxin. (a,b) Non-synchronized Vero cells were exposed to Cy2-ST and Cy3-CTX and analyzed by fluorescence microscopy as in Fig. 1a. Cells were then fixed, permeabilized and immunostained for cyclin B1 (see Materials and Methods section). (a) and (b) represent different experiments of the same kind. Overlay represents the overlay of CTX, ST and cyclin B1. (c) FACS-separated cells, binding either ST (lane 1) or CTX (lane 2) were extracted and identical amounts of proteins separated by SDS-PAGE. Immunoblots were performed using anti-cyclin-B1- and anti-protein disulfide isomerase antibodies. Lane 3 represents extracts from the same cells but before FACS separation. The amount of protein in lane 3 was about four times that applied in lanes 1 and 2. (d) A cell in anaphase shows a significant binding and uptake of ST but not of CTX. Chromatin staining with DAPI. The cells were fixed about 10 minutes after start of uptake of the toxins. At this time point significant amounts of the toxins have already entered the cells.

#### Binding of Cy-labeled Shiga and Cholera toxin to cell surface receptors

The binding conditions for Shiga and Cholera toxin were similar to those described previously (Majoul et al., 1996). In short, cells were exposed to 0.5  $\mu\text{g/ml}$  each of CTX and/or ST at 0°C for 15 minutes. Unbound toxins were removed by washing at 0°C. The temperature was then increased to 25°C for 5 minutes. Immediately thereafter the live cells were analyzed by fluorescence microscopy. In this way, toxin binding as well as initial toxin uptake could be analyzed.

#### Immunoblotting of cyclin B1 from FACS-separated Vero cells

Non-synchronized Vero cells were exposed to Cy-dye labeled ST and CTX as described above. Cells separated by FACS as described below were lysed on ice in lysis buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 2 mM EDTA, 2 mM EGTA, 1% Triton X-100 with 2  $\mu\text{g/ml}$  leupeptin, 2  $\mu\text{g/ml}$  aprotinin, and 50  $\mu\text{M}$  PMSF). Aliquots from the lysate were heated for 5 minutes in Laemmli sample buffer, resolved by 12% SDS-PAGE and electroblotted onto a PVDF membrane (Immobilon™). The membranes were blocked in 2% dry milk in PBS and incubated with a polyclonal rabbit anti-cyclin B1 antibody (1/1000) followed by chemoluminescence detection with a horseradish-peroxidase-coupled secondary antibody (ECL system, Amersham-Pharmacia, Freiburg). Antibodies were removed by incubation in 0.1 M citric acid, pH 3.3. The blot was then probed a second time with a rabbit anti-

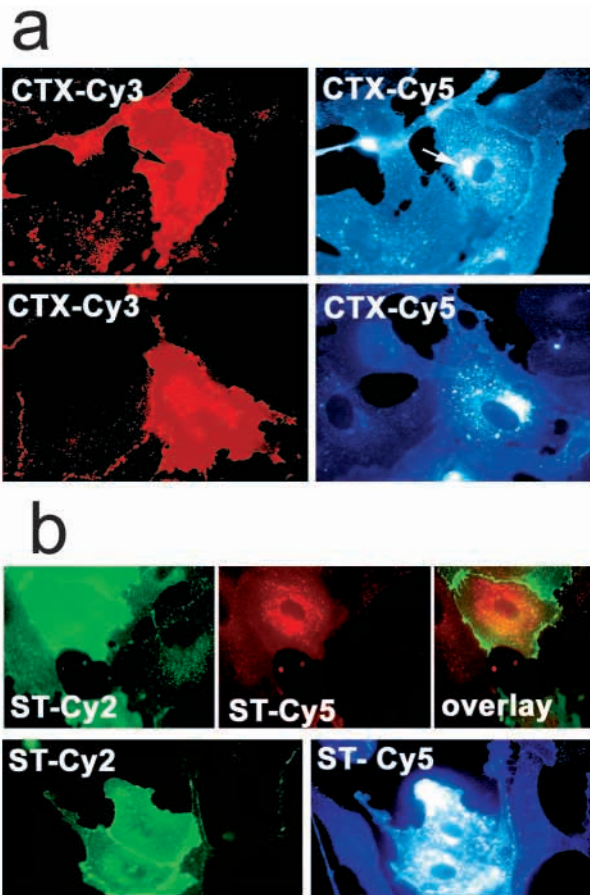
protein disulfide isomerase antibody (1/500) and detected by chemoluminescence using the same secondary horseradish-peroxidase-labeled goat anti-rabbit antibody as above.

#### Analyses of intracellular receptors for Cholera and Shiga toxin

Non-synchronized Vero cells were incubated at 0°C with either Cy3-ST or Cy5-CTX (0.5  $\mu\text{g/ml}$ ) for 20 minutes. Cells were washed at 0°C to remove unbound toxins followed by fixation with 4% paraformaldehyde for 10 minutes. Thereafter cells were exposed to the same concentration of unlabeled toxins at 0°C for 5 minutes to block the remaining toxin receptors on the cell surface; they were then washed and fixed again with 4% paraformaldehyde for 5 minutes. Remaining aldehyde groups were blocked with 50 mM  $\text{NH}_4\text{Cl}$ . Cells were then permeabilized with 0.1% (w/v) saponin in PBS/0.1% fish gelatin and stained with Cy-3 or Cy5-labeled toxins for 20 minutes at room temperature. Unbound toxins were removed by washing with PBS, and cells were analyzed by fluorescence microscopy.

#### Fluorescence and immunofluorescence microscopy and image processing

When binding and uptake of Cy-dye labeled toxins was analyzed in live cells, the cells remained in phenol red free DMEM with 5–7% FCS. Fluorescence microscopy was performed with a Zeiss Axioplan



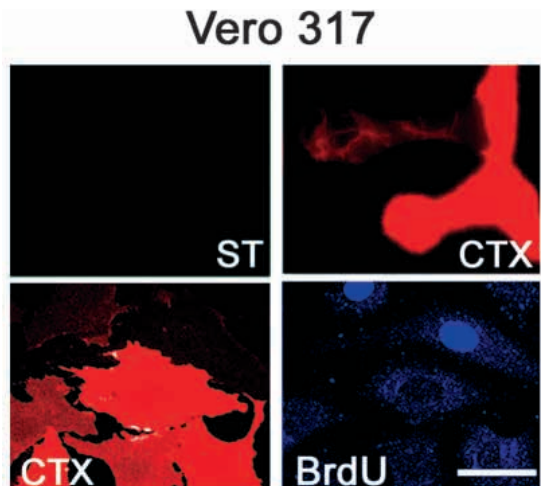
**Fig. 6.** Vero cells that do not bind Shiga or Cholera toxin also lack internal toxin receptors. (a) CTX-binding cells. The left panels (two different experiments) show cells strongly binding Cy3-CTX to the cell surface. The same cells also exhibit a strong binding of Cy5-CTX to internal binding sites after permeabilization, whereas cells that bind little Cy3-CTX to the surface lack internal binding sites for Cy5-CTX. (b) (Upper panels) A cell that strongly binds to Cy2-ST also expresses internal binding sites for Cy5-ST. (Lower panels) Two cells that exhibit strong binding of Cy2-ST to the cell surface also show internal receptors for Cy5-ST after cell permeabilization. Note that surrounding cells that possess almost no internal binding sites fail also to bind ST to the cell surface.

microscope using 63× Plan Achromat and 100× Neofluar objectives and filters for excitation at  $\lambda_{ex}=488$  nm for Cy2 fluorescence and  $\lambda_{ex}=514$  nm for Cy3 fluorescence.

Fixed cells, as used during the analysis of cell cycle markers (see above), were observed using a filter set for AMCA, FITC and Cy3. Simultaneous detection of Cy2- and Cy3-labeled samples was performed with a Zeiss LSM 410 laser scan microscope using laser excitation at 488 nm and 514 nm respectively. Cy5 was detected and laser excitation at 633 nm.

#### Differential display of Cholera and Shiga toxin bound cells

Non-synchronized Vero cells were plated on 1.4 cm culture dishes as described above. After 12 hours, cells were washed with PBS and the dishes transferred to ice. Cells were treated for 20 minutes at 0°C with Cy2-ST and Cy3-CTX (0.2  $\mu\text{g}/\text{ml}$  each). Unbound toxins were removed by washing with ice-cold PBS. Cells were then detached by incubation with PBS containing 10 mM EDTA. Cy2- and Cy3-labeled



**Fig. 7.** Vero 317 cells in S-phase do not bind to Cholera toxin. The experimental conditions used were the same as in Fig. 4. Cells bind CTX (upper right panel) but not ST (upper left panel). Cells in S-phase (BrdU) do not bind Cy3-CTX (lower left panel). Cy3-CTX-binding cells do not incorporate BrdU.

cells were separated using a BD FACS Vantage SE flow cytometry system (BD Biosciences), using filter settings for FITC and TRITC. RNA was extracted from the separated cells according to Chomczynski and Sacchi (Chomczynski and Sacchi, 1987) using the peqGOLD RNA Pure™ reagent and the protocol of the supplier (Peqlab, Erlangen, Germany). cDNA was synthesized using oligo-dT primers and MMLV reverse transcriptase (Delta™ RNA Fingerprinting Kit, Clontech, Heidelberg, Germany). For amplification, ten 5'-primers (P1-P10) and nine 3'-primers (T1-T9) were used (Delta™ RNA Fingerprinting Kit, Clontech, Heidelberg, Germany) together with Advantage Klen *Taq* polymerase in the presence of [ $\alpha$ - $^{32}\text{P}$ ]-ATP. The first cycle was run for 5 minutes at 94°C, 40°C and 72°C; the following two cycles were run for 2 minutes at 94°C and for 5 minutes each at 40°C and 72°C. This was followed by 23 cycles run for 1 minute each at 94°C and 60°C and 2 minutes at 72°C. The PCR products were separated by electrophoresis on denaturing 5% polyacrylamide gels. The gels were blotted onto Whatman 1 paper, vacuum-dried and autoradiographed using Kodak MR film. Differentially amplified cDNA-fragments were cut out from the gel and reamplified under high stringency conditions with the P/T-primer combination used for differential display. The amplified fragments were cloned using the TOPO™-TA cloning kit (Invitrogen), amplified and sequenced. The possibility of contamination by somatic DNA was excluded by comparing the band patterns of non-transcribed RNA-samples with those of RNA-free control samples; both gave identical results. Moreover, using the method described, intron sequences were never detected.

## Results

Vero cells from a non-synchronized population can bind to both Shiga and Cholera toxin

Non-synchronized cells can bind to both toxins (Fig. 1a). However, a closer analysis of binding and internalization of the two toxins on the single cell level revealed that the binding of the two toxins by Vero cells exhibits a heterogeneous pattern. Under steady-state conditions, some cells showed a weak binding of both toxins, whereas most of the cells showed an almost exclusive binding to either CTX or ST (Fig. 1a).

### Transcription is differently regulated in Shiga-toxin- and Cholera-toxin-binding cells

Vero cells were incubated with Cy2-ST and Cy3-CTX and separated by FACS. RNA from the separated cells was extracted, reversed transcribed and analyzed by differential display. The pattern of DNA fragments showed distinct differences between ST- and CTX-binding cells (Fig. 1b). Although the cDNAs corresponding to the RNA fragments have not been identified yet, the different pattern shows clearly that the differences between the two cell populations is not restricted to their differential toxin binding.

### The different toxin binding patterns correspond to different phases of the cell cycle

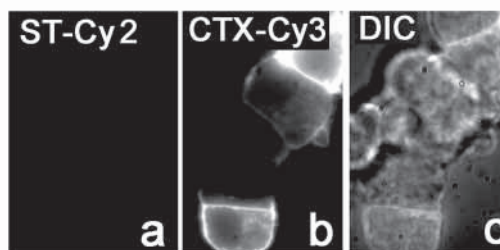
When Vero cells were diluted to the single cell level and recultivated to obtain clonal cells, again a mixture of ST- and CTX-binding cells was obtained after 24-48 hours (data not shown), which indicates that the different toxin binding patterns did not represent genetically different cells but rather different states of clonal cells. This led us to test the hypothesis that the differences in the binding of the two toxins might reflect different phases of the cell cycle. The following experiments were carried out to test this hypothesis.

Cultured cells in metaphase bind weakly to cover slips and can, therefore, be detached by colchicine treatment and shaking, whereas cells in interphase or G1 remain on the glass under the same condition. Therefore, Vero cells were treated with colchicine as described in the Methods section, and detached cells and cells that remained bound to the cover slip were analyzed for ST- and CTX-binding. The detached and replated cells had a round-shaped appearance and bound exclusively to ST (Fig. 2a), whereas most of the cells remaining on the cover slip were larger, flat and irregularly shaped and bound only to CTX (Fig. 2a). Of 150 detached and replated cells, all showed a strong binding of ST, whereas only three of these cells exhibited a significant binding of CTX. On the other hand, of the 50 large, flat, irregularly shaped cells that had remained on the coverslip after colchicine treatment and shaking, all bound strongly to CTX, and none of these cells exhibited a significant binding of ST. When the detached cells, which bound almost exclusively to ST, were reseeded, cultured in the presence of serum for 12 hours and then analyzed for toxin binding, the normal distribution between CTX- and ST-binding cells was obtained (Fig. 2b). These results indicate that Vero cells in interphase or G1 bind preferentially to CTX, whereas cells in metaphase or anaphase bind mainly to ST.

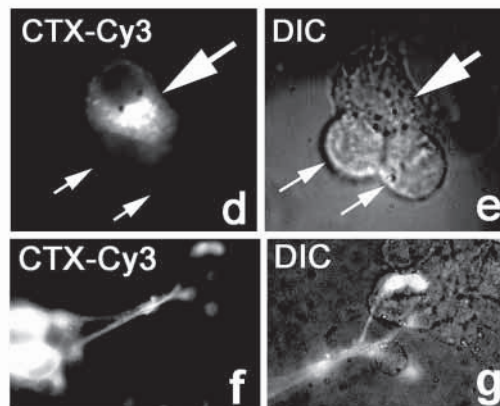
To further substantiate this conclusion, Vero cells were partially synchronized by a double thymidine block. Twelve hours after release from the block, 30-50% of the cells were in metaphase or anaphase (Fig. 3). These cells bound almost exclusively to ST (Fig. 3, upper left panel), whereas cells in interphase or G1, which were characterized by their strong adhesion and their flat and extended appearance, bound only to CTX (Fig. 3, upper right panel).

In order to obtain a more detailed picture, we correlated the binding of the two toxins with the incorporation of BrdU on one hand and the expression and intracellular distribution of cyclin B1 on the other. Strong incorporation of BrdU is an indicator of S-phase, whereas an increased expression and an enhanced nuclear uptake of cyclin B1 is an indicator for G2

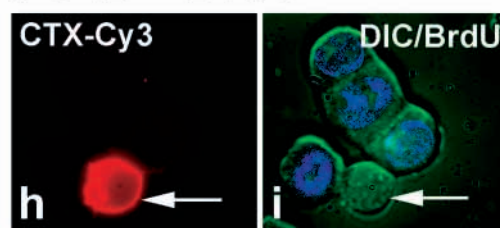
## PC12



## PC12 + NGF



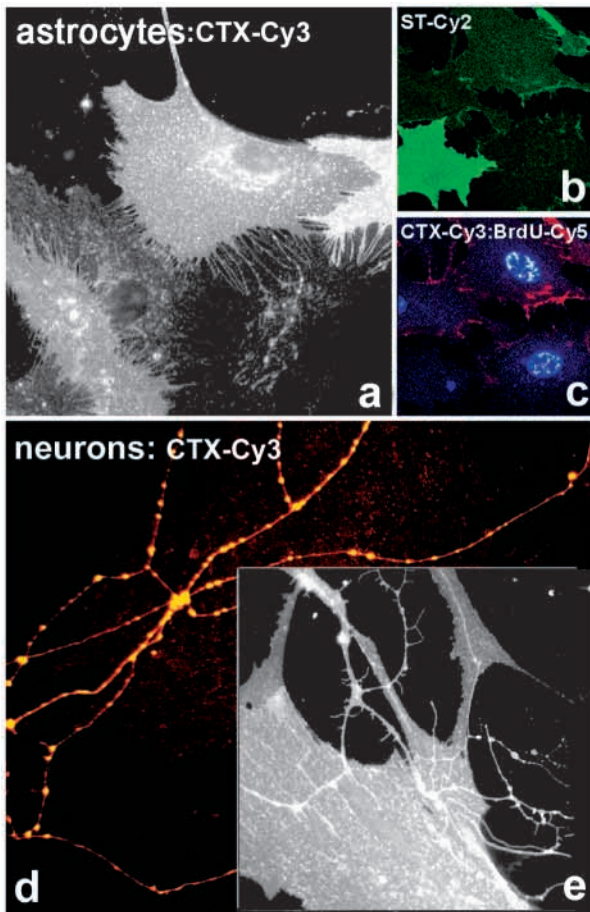
## PC12 + BrdU



**Fig. 8.** PC12 cells also show cell-cycle-dependent expression of receptors for CTX. (a-c) represent identical cells. No binding of Cy2-ST is observed. Cy3-CTX binds almost exclusively to the large peripheral cells; (c) differential interference contrast picture. (d-g) NGF-differentiated cells show increased binding and uptake of Cy3-CTX (d) and (f), whereas undifferentiated cells characterized by their round shape and lack of axonal protrusions (small arrows in (d) and (e)) show little CTX-binding. (h) and (i) PC12 cells in S-phase as characterized by BrdU incorporation (i) do not bind CTX (h).

and metaphase (Kakino et al., 1996; Jin et al., 1998). As depicted in Fig. 4, cells in S-phase exhibited only low binding of both toxins, indicating that the expression of receptors for both toxins on the cell surface strongly decreases during S-phase. All of the 50 cells showing a strong incorporation of BrdU exhibited either no or a very low binding to CTX and ST. When the degree of toxin binding was quantified using a 7 degree scale (ranging from 1 equals no detectable binding to 7 equals very strong binding) the mean binding of CTX was  $2.3 \pm 1.1$  and the mean binding of ST was  $2.2 \pm 1.3$ . As the binding of CTX remained low through S-phase, G2, metaphase and anaphase (see below) it is clear that some BrdU-negative cells show a low degree of CTX-binding.

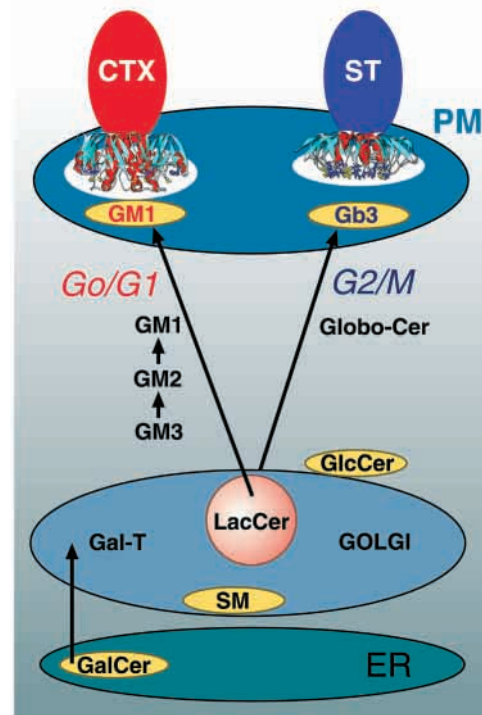
When cells move from S-phase into G2 and metaphase, the



**Fig. 9.** Differential binding of Cy2-labeled Shiga toxin and Cy3-labeled Cholera toxin to fetal mouse astrocytes and binding of Cy3-labeled CTX to hippocampal neurons. (a) Binding of Cy3-CTX to astrocytes. Binding conditions were the same as in Fig. 1a. During inspection of the live cells under the fluorescence microscope, some CTX is taken up and accumulates in the Golgi. (b,c) Astrocytes that exhibit significant BrdU incorporation (blue-white nuclei in (c)) show only weak binding of ST (b) or CTX (c). (d,e) Binding and uptake of Cy3-CTX by hippocampal neurons. All axons and dendrites exhibit a strong binding of the toxin. In (e), several neuronal cells on top of a Cy3-CTX-binding astrocyte are depicted.

expression of cyclin B1 increases together with an enhanced translocation into the nucleus (Fig. 5a,b). This shift was associated with an increase in the binding of ST, whereas the binding of CTX remained as low as during S-phase (Fig. 5a,b). Out of the 60 cells that gave a strong nuclear signal for cyclin B1, all showed a strong binding of ST, whereas binding of CTX to these cells was either undetectable or very low. Those cells in which significant amounts of cyclin B1 were detected in the nucleus, showed a very strong binding of ST. This was further substantiated by estimating the amount of cyclin B1 from immunoblots from ST- and CTX-positive cells after their separation by FACS (Fig. 5c). Clearly, the cells preferentially binding to ST expressed significantly more cyclin B1 than the CTX-positive cells.

In summary, we find that cells in interphase and G1 bind almost exclusively to CT. Cells in S-phase exhibit a decreased binding of both toxins, whereas the transition from S-phase to the G2, metaphase and anaphase is associated with a loss of



**Fig. 10.** A schematic presentation of cell-cycle-dependent differential synthesis of receptors for Cholera and Shiga toxin.

binding of CTX and an increased binding of ST (Fig. 5d). Upon transition into G1, binding of CTX reappears, whereas the ability of the cells to bind to ST decreases or disappears.

#### Decreased toxin binding results from decreased receptor biosynthesis

As binding of the two toxins to the plasma membrane is determined by the density of receptor molecules in the membrane, we conclude that the cell-cycle-dependent differential binding of the two toxins reflects cell-cycle-dependent expression of GM1 and Gb3 on the plasma membrane. This could result from reduced or absent synthesis of toxin receptors or from a block of receptor transport from the place of synthesis to the plasma membrane. Therefore, we analyzed the expression of intracellular receptors in permeabilized Vero cells. Unpermeabilized cells showing strong binding to CTX also exhibited significant binding of this toxin to intracellular sites (Fig. 6a). The same holds for cells showing strong surface binding of ST. Cells that exhibited no or little binding of either toxin to the cell surface were also devoid of intracellular toxin binding sites for the corresponding toxin after cell permeabilization (Fig. 6a,b). This allows for the conclusion that changes in the binding of either toxin results from the corresponding changes in synthesis of the glycolipid receptors.

#### Cell-cycle-dependent binding of Cholera toxin to Vero 317 cells

Both the ganglioside GM<sub>1</sub> and the globoside Gb<sub>3</sub> are synthesized from the same precursor, namely lactosyl

ceramide. We, therefore, explored the possibility that an increased synthesis of Gb3 from lactosylceramide during late G2 and M-phase might have competed for the synthesis of GM3 or GA2, precursors of GM1 (Fig. 10). This hypothesis was tested in Vero 317 cells, which are unable to bind ST (Pudymaitis et al., 1991) while still binding CTX (Fig. 7). As these cells also show a decreased or absent binding of ST after permeabilization of the cells in comparison to normal Vero cells (data not shown), the decreased surface binding of ST by Vero 317 cells indicates not simply a disturbed translocation of Gb3 to the plasma membrane but a decreased synthesis of Gb3. Therefore, if the decreased binding of CTX during S-phase, G2 and M-phase in normal Vero cells results from a competition for lactosylceramide owing to increased Gb3 synthesis, such a decrease should not occur in Vero 317 cells. We analyzed, therefore, the cell-cycle-dependent binding of CTX in Vero 317 cells.

Similar to normal Vero cells, Vero 317 cells show CTX binding in flat, extended cells of the G0/G1-type (Fig. 7). Vero 317 cells in S-phase, which are characterized by a high incorporation of BrdU, bound little or no CTX (Fig. 7). From this we conclude that the cell-cycle-dependent changes in expression of the CTX-receptor GM1 on the cell surface does not result from a cell-cycle-dependent competition for lactosylceramide. This conclusion is supported by data obtained from PC12 cells and from astrocytes (see below).

#### Cell-cycle-dependent binding of CTX to PC12 cells, primary cultured hippocampal neurons and astrocytes

We next addressed the question of whether the cell-cycle-dependent expression of toxin receptors is restricted to Vero and Vero 317 cells or whether it occurs also in completely different cell types.

To this end, we performed experiments with PC12 cells, a neuro-endocrine cell line. PC12 cells bind CTX but not ST (Fig. 8a,c). But not all PC12 cells showed strong CTX binding; many cells exhibited weak or no CTX binding. In particular PC12 cells in S-phase, as indicated by a high incorporation of BrdU, did not bind CTX (Fig. 8h,i). In order to achieve some kind of synchronization, we treated PC12 cells with NGF, as this growth factor is known to induce cellular differentiation and arrest of the cells in G0/G1 (Hughes et al., 2000). Indeed, all cells showing signs of differentiation (flat, extended shape, development of axonal protrusion or axons) bound CTX (Fig. 8d,g). This indicates that in PC12 cells, CTX binding is regulated by the cell cycle in a similar way as in Vero and Vero 317 cells. This conclusion was further supported by our observation that primary cultured hippocampal neuronal cells, which exist only in interphase, uniformly exhibited a strong binding to CTX (Fig. 9d,e).

As the hippocampal neuronal cells were cultivated on top of a feeder layer of astrocytes, we have also explored the binding of the two toxins to these cells. Astrocytes bound ST as well as CTX, but, similar to Vero cells, cells exhibiting a strong binding of ST did not bind CTX and vice versa (Fig. 9a,c). Furthermore, astrocytes in S-phase, as indicated by a strong incorporation of BrdU, showed little binding of both toxins (Fig. 9b,c). Thus, astrocytes show the same cell-cycle-dependent differential expression of receptors for ST and CTX as Vero cells.

#### Discussion

We show here that glycosphingolipid receptors specific for two different AB5 toxins exhibit cell-cycle-dependent changes in their expression on the cell surface. The expression of the ST receptor globotriaoside Gb3 is low during interphase and G1, as well as during S-phase. It strongly increases during G2 and metaphase. In contrast, the highest expression of the CTX receptor GM1 takes place during interphase and G1, decreases during S-phase and stays low during G2, metaphase and telophase. One practical aspect of these results is that they offer a fast and straightforward method for assessing cell-cycle phases at the single cell level in non-synchronized cells.

A decreased expression of these glycolipids on the cell surface could reflect either their decreased net synthesis or an inhibition of their translocation from the Golgi to the plasma membrane. An enhanced degradation of the glycolipids or their increased endocytosis could also be responsible. We observed that cells that did not bind ST or CTX on the outer plasma membrane had also lost the corresponding binding sites inside the cell. This argues strongly for an increased net synthesis of Gb3 or GM1 in cells exhibiting an increased binding of ST or CTX, respectively. Using verotoxin, a Shiga-like toxin, in experiments with Vero cells, Pudymaitis and Lingwood (Pudymaitis and Lingwood, 1992) observed a maximum toxicity at the G1/S-phase boundary but could not detect changes in the cellular Gb3 content or in the incorporation of [<sup>14</sup>C]-galactose into Gb3. In these experiments, the whole population of cultured cells was analyzed at different time points after a double-thymidine block. Thus, a correlation between surface expression of Gb3 and parameters of the cell cycle could not be examined at the single cell level. It is our experience that Vero cells after a double-thymidine block start within a few hours to desynchronize. Therefore, the cell-cycle-dependent expression of Gb3 and GM1 can be reliably correlated only by analyzing single cells. Interestingly Pudymaitis et al. (Pudymaitis et al., 1991) observed that in verotoxin-resistant Vero cells the decreased toxin binding was paralleled by a decreased cellular Gb3 content and a strong reduction of the activity of UDP-galactose:lactosylceramide- $\alpha$ -galactosyltransferase, which converts lactosylceramide into Gb3 (Kojima et al., 2000; Keusch et al., 2000). As we found here an inverse relationship between the binding of CTX and that of ST we considered the possibility that the biosynthetic reactions leading to GM1 and Gb3 respectively might compete for the common precursor lactosylceramide and that a cell-cycle-dependent change in the activity of  $\alpha$ -galactosyltransferase-activity might be responsible for the observed changes. However, as shown here by the experiments with the ST-resistant Vero317-cell line, the cell-cycle-dependent changes in the expression of GM1 on the cell surface occurred in the same way as in normal, ST-sensitive cells. These data were confirmed by our experiments with PC12 cells.

At the moment we can only speculate on the biochemical mechanism underlying the cell-cycle-dependent expression of GM1 on one hand and Gb3 on the other. These mechanisms must act downstream of the synthesis of lactosylceramide as this metabolite is a common precursor for both types of glycosphingolipids. Synthesis of Gb3 may then be regulated by the cell-cycle-dependent expression of the highly specific  $\alpha$ -galactosyltransferase (Kojima et al., 2000; Keusch et al., 2000).



The biosynthesis of GM1 may involve several steps. An UDP-N-acetyl-galactosyl transferase converts lactosylceramide to GA2, and GM3 to GM2, both precursors of the CTX-receptor GM1. It has been reported in a hybrid rat glioma-neuroblastoma cell line, that the activity of this enzyme reaches a maximum during S- and G2-phase (Scheideler et al., 1984). This is not in line with our observation that the expression of CTX receptors on the cell surface reaches a minimum during S- and G2-phase.

Another possibility would be a cell-cycle-dependent regulation of sialyltransferase I, which catalyzes the conversion of lactosylceramide to GM3, and of GA2 to GA1, both precursors for GM1. The activity of this enzyme reaches its maximum during telophase and G1 and is elevated in contact-inhibited cultured cells (Burczak et al., 1984). Another mechanism has also to be considered, namely regulation by breakdown. Scheideler et al. (Scheideler et al., 1984) observed, in a synchronized hybrid rat glioma-neuroblastoma cell line, a strong increase in the activities of N-acetyl- $\beta$ -hexosaminidase and of  $\beta$ -galactosidase 18-20 hours after release of the cells from a block in G1. This would fit our observation of a very low binding of CTX in S- and G2-phase. As the activities of the glycosphingolipid-degrading enzymes undergo a complex regulation by sphingolipid activator proteins (SAP-A to SAP-D and the GM2 activator) (for a review, see Fürst and Sandhoff, 1992) it seems possible that these regulatory proteins may also be involved in regulating the net amount of the AB5 receptors and their expression on the cell surface.

The question is does cell-cycle-dependent expression of Gb3 and GM1 represent an epiphenomenon or does it play a regulatory role in the progression of the cell cycle. Physiological ligands for Gb3 or GM1 have not been reported so far. However, effects of glycosphingolipids on growth-related processes have been described. In bovine aortic endothelial cells GM1 or GM2 inhibit bFGF-induced mitogenesis by interfering with bFGF binding, whereas GM3 is synergistic with bFGF (Slevin et al., 1999). In T-lymphocytes gangliosides inhibit the interaction of IL-2 with its plasma membrane receptor, which was explained by a direct interaction of IL-2 with the gangliosides (Chu et al., 1993). In the fetal cell lines, CH I and CH II gangliosides inhibit [<sup>3</sup>H]-thymidine incorporation into DNA (Icard-Liepkalns et al., 1982). In keratinocytes, GM3, but not GM1 or GD1a, stimulates proliferation (Paller et al., 1993), which contrasts with the situation in K562 leukemia cells, where GM3 inhibits growth, while GM1, GM2 or GD1a have no effect (Nakamura et al., 1991). In lung adenocarcinoma cells, GM3 inhibits the proliferative action of EGF, most probably by activation of a EGF-receptor-directed phosphotyrosine phosphatase (Suarez Pestana et al., 1997). Inhibition by gangliosides of T-cell proliferation seems also to result from the activation of a protein phosphatase, in this case resulting in a diminished phosphorylation of the retinoblastoma protein (Irani, 1998). In PC12 cells, GM1 potentiates the action of NGF by tight binding to the NGF receptor, thus enhancing its tyrosine kinase activity and dimerization (Mutoh et al., 1995). In the absence of NGF, GM1 did not have any effect. On the other hand, the mere overexpression of GD3, GD1b and GT1b induced dimerization and a long-lasting activation of the NGF-receptor TrkA with subsequent activation of the Ras/MEK/ERK pathway (Fukumoto et al., 2000). Under these conditions the

sensitivity towards NGF was lost but could be restored by the addition of GM1 (Mutoh et al., 1995).

It thus appears that glycosphingolipids are involved in the tuning of complex processes like growth and differentiation, which are tightly linked to cell cycle activity. Further progress will require a knowledge of the physiological ligands for the glycosphingolipid receptors on the plasma membrane.

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