Efficient endosome-to-Golgi transport of Shiga toxin is dependent on dynamin and clathrin

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Summary

It has previously been shown that Shiga toxin, despite being bound to a glycolipid receptor, can be efficiently endocytosed from clathrin-coated pits. However, clathrin-independent endocytosis is also responsible for a proportion of the toxin uptake in some cells. After endocytosis the toxin can be transported in retrograde fashion to the Golgi apparatus and the endoplasmic reticulum, and then to the cytosol, where it exerts its toxic effect by inactivating ribosomes. In order to investigate the role of dynamin and clathrin in endosome-to-Golgi transport of Shiga toxin, we have used HeLa dyn^{K44A} and BHK antisense clathrin heavy chain (CHC) cells that, in an inducible manner, express mutant dynamin or CHC antisense RNA, respectively. In these cell lines, one can study the role of dynamin and clathrin on endosome-to-Golgi transport because they, as shown here, still internalize Shiga toxin when dynamin- and clathrin-dependent endocytosis is blocked. Butyric acid has been shown to sensitize A431 cells to Shiga toxin by increasing the proportion of cell-associated toxin that is transported to the Golgi apparatus and the endoplasmic reticulum. Here, we find that, in HeLa and BHK cells also, butyric acid also increased toxin transport to the Golgi apparatus and sensitized the cells to Shiga toxin. We have therefore studied the role of dynamin and clathrin in both untreated and butyric-acid-treated cells by measuring the sulfation of a modified Shiga B fragment. Our results indicate that endosome-to-Golgi transport of Shiga toxin is dependent on functional dynamin in both untreated cells and in cells treated with butyric acid. Interestingly, the regulation of Shiga toxin transport in untreated and butyric-acid-treated cells differs when it comes to the role of clathrin, because only cells that are sensitized to Shiga toxin with butyric acid need functional clathrin for endosome-to-Golgi transport.

Key words: Shiga toxin, Clathrin, Dynamin, Golgi apparatus

Introduction

The bacterial Shiga toxin is produced by *Shigella dysenteriae* and consists of one enzymatically active A moiety and one glycolipid receptor (Gb3) binding B moiety (five subunits). It has previously been shown that Shiga toxin, despite being bound to a glycolipid receptor, can be efficiently endocytosed by clathrin-coated pits (Sandvig and van Deurs, 1996). However, clathrin-independent endocytosis is also responsible for a proportion of the toxin uptake in some cells (Lingwood, 1999; Nichols et al., 2001; Sandvig et al., 2002), indicating that there are cell differences in the handling of the toxin. After endocytosis, a proportion of the toxin can be transported in retrograde fashion to the Golgi apparatus and the endoplasmic reticulum (ER), and then it is translocated to the cytosol, where it exerts its toxic effect by inactivating ribosomes. To monitor transport of Shiga toxin to the Golgi apparatus, we have used a mutated B fragment of Shiga toxin containing a tandem of sulfation sites (Shiga B-Sulf$_2$) (Mallard et al., 1998). This modified Shiga toxin is subjected to sulfation in the Golgi apparatus, a modification that can be quantified using radioactive sulfate.

There is now evidence for several retrograde pathways leading from endosomes to the Golgi apparatus and the ER. One well characterized pathway is the Rab9-dependent transport route from late endosomes to the Golgi apparatus used by the mannose-6-phosphate receptor (M6PR) (Lombardi et al., 1993; Itin et al., 1997; Itin et al., 1999; Nicoziani et al., 2000; Carroll et al., 2001; Miwako et al., 2001). However, Shiga toxin is suggested to be transported directly from early endosomes to the trans-Golgi network (TGN), circumventing the late endocytic pathway (Ghosh et al., 1998; Mallard et al., 1998). Like the plant toxin ricin, Shiga toxin is transported to the Golgi apparatus in a Rab9-independent manner (Iversen et al., 2001; Sandvig et al., 2002). Ricin transport to the Golgi apparatus and, to a lesser extent, Shiga toxin transport differ from the transport of the M6PR by also being regulated by calcium (Lauvrak et al., 2002). Still, Shiga toxin transport to the Golgi apparatus is not identical to that of ricin, based on the findings that endosome-to-Golgi transport of Shiga toxin (Mallard et al., 1998; Wilcke et al., 2000), but not ricin (Iversen et al., 2001), is dependent on Rab11 and also seems to involve clathrin. It is possible that transport of toxin-receptor complexes located in different subdomains could be regulated differently.

We have previously shown that ricin transport to the Golgi apparatus is dependent on functional dynamin (Llorente et al., 1998). In the present study, we were interested in whether Shiga toxin also is transported to the Golgi apparatus in a dynamin-dependent manner. To analyse this, we used HeLa dyn^{K44A} cells, which, in the absence of tetracycline, produce mutant dynamin (Damke et al., 1995b). Furthermore, Shiga toxin has been found to localize with AP1 clathrin coat

components in confocal- and electron-microscopy experiments (Mallard et al., 1998), suggesting that clathrin coats might play a role in Shiga transport. However, endosomal clathrin coats have also been implicated in recycling to the plasma membrane of the transferrin receptor (van Dam and Stoorvogel, 2002). Thus, we also wanted to study the role of clathrin in endosome-to-Golgi transport and intoxication with Shiga toxin; in this context, we have used BHK cells, which, in an inducible manner, express clathrin heavy chain (CHC) antisense RNA. It is possible that the mechanism of toxin transport to the Golgi apparatus could vary depending on the sensitivity of the cell. We have previously shown that butyric acid sensitizes A431 cells to Shiga toxin by increasing the proportion of cell-associated toxin that is transported to the Golgi apparatus and the ER (Sandvig et al., 1992). In the present work, we demonstrate that a similar effect is seen in both the HeLa cells and the BHK cells used. To study whether increased Golgi transport is associated with a change in the mechanism of transport, we have therefore investigated the role of dynamin and clathrin in both untreated and butyric-acid-treated cells. We find that the transport of Shiga toxin from endosomes to the Golgi apparatus is dependent on dynamin in both untreated and butyric-acid-treated cells. Interestingly, Shiga toxin transport seems to be dependent on clathrin only after butyric-acid treatment. Thus, the regulation of Shiga toxin transport in untreated and butyric acid-treated cells seems to differ when it comes to the role of clathrin.

Materials and Methods

Materials

Tetracycline, puromycin, geneticin, butyric acid, transferrin, *n*-octyl-glucopyranoside, 2-mercaptoethanesulfonic acid, sodium salt (MESNa), and Hepes were obtained from Sigma Chemical (St Louis, MO). $Na_2{}^{35}SO_4$ was purchased from Amersham Biosciences (Buckinghamshire, UK). The plasmid construct pcDNA-3 Myc $Rab5^{Q79L}$ was a gift from H. Stenmark (The Norwegian Radium Hospital, Oslo, Norway). Shiga toxin was labelled with Alexa Fluor 488 from Molecular Probes (Leiden, The Netherlands) according to the procedure given by the company. Anti-Myc antibodies were from the 9E10 hybridoma line (Evan et al., 1985). Rabbit anti-Shiga toxin antibodies were obtained by standard immunization.

Cells

The BHK21-tTA cell line (Iversen et al., 2003) was grown in complete Duchenne's modified Eagle's medium (DMEM) (Flow Laboratories, Irvine, UK) supplemented with 7.5% foetal calf serum (FCS), 2 mM L-glutamine, 100 units ml^{-1} penicillin, 100 μg ml^{-1} streptomycin and 200 μg ml^{-1} geneticin. Stable BHK21-tTA/anti-CHC cells were maintained in select DMEM (complete DMEM containing 200 ng ml^{-1} puromycin and 2 μg ml^{-1} tetracycline). To induce CHC antisense RNA expression, tetracycline was removed from the medium. The HeLa cell line tTA-HeLa (Gossen and Bujard, 1992) stably transformed with the cDNA for dyn^{K44A} was grown in DMEM supplemented with 10% FCS, 2 mM L-glutamine, 100 units ml^{-1} penicillin, 100 μg ml^{-1} streptomycin, 400 μg ml^{-1} geneticin, 200 ng ml^{-1} puromycin and 1 μg ml^{-1} tetracycline. This cell line was a gift from S. L. Schmid (The Scripps Research Institute, La Jolla, CA).

Endocytosis of Shiga toxin and transferrin

Endocytosis of Shiga toxin and transferrin was measured using the ORIGEN Analyzer (IGEN, Rockville, MD) as described previously for cholera toxin (Skretting et al., 1999; Torgersen et al., 2001). Briefly, TAG- and biotin-labelled Shiga toxin and -transferrin were prepared by tagging the proteins with *N*-hydroxysuccinimide ester-activated *tris*(bipyridine)-chelated ruthenium(II) TAG (IGEN) according to the procedure given by the company, and simultaneously biotinylating them with the reducible Sulfo-NHS-SS-Biotin (sulfosuccinimidyl 2-(biotinamido)-ethyl-1,3-dithiopropionate) (Pierce, Rockford, IL). The cells were washed with Hepes medium and incubated with TAG- and biotin-labelled Shiga toxin (5 ng ml^{-1}) or TAG- and biotin-labelled transferrin (50 ng ml^{-1}) in Hepes medium containing 2 mg ml^{-1} bovine serum albumin for different time points at 37°C. The cells were subsequently washed twice with cold buffer (0.14 M NaCl, 2 mM $CaCl_2$, 20 mM Hepes; pH 8.6). Half of the cells were then treated with 0.1 M MESNa in the same buffer at 0°C for 1 hour to remove the SS-linked biotin on the cell-surface-bound toxin; the other half was incubated with buffer alone. Only toxin that is TAG-labelled and still biotinylated is detected in the cell lysate using streptavidin beads (Dynal, Oslo, Norway) and ORIGEN Analyzer. Thus, cells treated with MESNa will represent the amount of endocytosed toxin, whereas untreated cells will represent the total amount of toxin associated with the cells. The cells were then washed twice with cold buffer (0.14 M NaCl, 2 mM $CaCl_2$, 20 mM Hepes; pH 7.0) and lysed (lysis buffer: 0.1 M NaCl, 5 mM $MgCl_2$, 50 mM Hepes, 1% Triton X-100, 60 mM *n*-octyl-glucopyranoside). Then, the lysed cells were centrifuged for 5 minutes at 14,000 rpm in an Eppendorf microcentrifuge to remove the nuclei and the amount of TAG- and biotin-labelled Shiga or transferrin in the cleared lysates were measured using streptavidin beads and the ORIGEN Analyzer.

Measurements of Shiga toxin cytotoxicity

Cells were incubated for 2 hours in Hepes medium lacking leucine and with increasing concentrations of Shiga toxin. The cells were then incubated in Hepes medium containing 2 mCi ml^{-1} [^{3}H]-leucine for 20 minutes at 37°C. Cells were extracted with 5% trichloroacetic acid (TCA) for 10 minutes, followed by a wash (5 minutes) in 5% TCA and subsequently dissolved in 0.1 M KOH. The cell-associated radioactivity was measured.

Sulfation of STxB-$Sulf_2$

The modified version of Shiga toxin B chain containing a tandem of sulfation sites (STxB-$Sulf_2$) (Mallard et al., 1998) was a kind gift from L. Johannes (The Curie Institute, Paris). Cells were washed twice with DMEM without sulfate, and incubated with 0.3 mCi ml^{-1} or 0.6 mCi ml^{-1} $Na_2{}^{35}SO_4$ for 3 hours at 37°C in the same medium but supplemented with 1 mM $CaCl_2$, 2 mM L-glutamine and 1× nonessential amino acids (Life Technologies, Paisley, UK). Then STxB-$Sulf_2$ (2.8 μg ml^{-1}) was added and the incubation was continued for either 15 minutes (cells incubated with 0.6 mCi ml^{-1} $Na_2{}^{35}SO_4$) or 1 hour (cells incubated with 0.3 mCi ml^{-1} $Na_2{}^{35}SO_4$). The cells were washed twice with cold PBS and then lysed [lysis buffer: 0.1 M NaCl, 10 mM Na_2HPO_4, 1 mM EDTA, 1% Triton X-100 and 60 mM *n*-octyl-glucopyranoside, supplemented with a mixture of protease inhibitors (Roche Molecular Biochemicals, Mannheim, Germany), pH 7.4]. The cells were centrifuged for 10 minutes at 5000 rpm in an Eppendorf microcentrifuge to remove the nuclei and the cleared lysate was immunoprecipitated with rabbit anti-Shiga toxin antibodies immobilized on protein-A/Sepharose beads (Amersham Biosciences, Uppsala, Sweden) overnight at 4°C. The beads were then washed twice with PBS containing 0.35% Triton X-100 before the adsorbed material was analysed by sodium-dodecyl-sulfate polyacrylamide-gel electrophoresis (SDS-PAGE) under reducing conditions.

SDS-PAGE

SDS-PAGE was performed in 12% gels as described (Laemmli, 1970).

The gels were fixed in 4% acetic acid and 27% methanol for 30 minutes, and then incubated for 20 minutes in 1 M sodium salicylate, pH 5.8, in 2% glycerol. Kodak XAR-5 films were exposed to the dried gels at –80°C for autoradiography. Signal intensities of the bands were quantified by exposing the gels to PhosphoImager screens and using ImageQuant 5.0 software (Amersham Biosciences, Uppsala, Sweden).

Calculation of endosome-to-Golgi transport of Shiga toxin

Quantification of the signal intensities of the sulfated STxB-Sulf$_2$ bands gives the amount of Shiga toxin B chain that has entered the cells and been transported to the Golgi apparatus. To calculate the effect of expressing mutant dynamin or CHC antisense RNA on the endosome-to-Golgi step alone, the changes in sulfation were corrected for the changes in endocytosis under the different conditions; that is, expression of CHC antisense RNA in the absence of butyric acid reduced the sulfation by 38% (see Fig. 6A) but also reduced the endocytosis by 11% (see Fig. 6B), resulting in only a 30% reduction in endosome-to-Golgi transport (shown in Fig. 6C). Furthermore, to exclude the possibility that the changes in STxB-Sulf$_2$ sulfation for the different conditions were due to any changes in the sulfation machinery rather than a change in Golgi transport, we also analysed the sulfation of endogenous proteins. This was done by counting the amount of ^{35}S-labelled proteins after TCA precipitation of the lysates obtained from the sulfation experiments. Only minor changes in the sulfation of proteins in general were observed for the different conditions (not shown).

Immunofluorescence

Cells grown on coverslips were washed twice with Hepes medium before incubation with Alexa Fluor 488-labelled Shiga toxin (500 ng ml^{-1}) for 20 minutes at 37°C. The cells were subsequently fixed with 3% paraformaldehyde in PBS, permeabilized with 0.1% Triton X-100 and blocked with 5% FCS. The Golgi apparatus was labelled with mouse anti-GM130 antibodies (Transduction Laboratories) followed by CY-5 labelled goat anti-mouse IgG (Jackson Immunoresearch Laboratories, West Grove, PA). Clathrin heavy chain staining was performed by using goat anti-CHC (C-20) antibodies (Santa Cruz Biotechnology) followed by rhodamine-labelled donkey anti-goat IgG (Jackson Immunoresearch Laboratories). When indicated, the cells were transfected with a GTPase-deficient mutant form of Rab5 (Myc-Rab5^{Q79L}) (Raiborg et al., 2001) before incubation with Shiga toxin. Transfected cells were identified using mouse anti-Myc antibodies followed by CY5-labelled donkey anti-mouse IgG (Jackson Immunoresearch Laboratories). The fixed cells were analysed using a LSM 510 Meta confocal microscope (Zeiss, Germany). Pictures were taken of thin single plane sections. The extent of colocalization between Shiga toxin (green channel) and CHC (red channel) was quantified by calculating the ratio between the number of yellow (i.e. colocalization) pixels (fluorescence level between 150 and 255) and the number of green pixels (fluorescence level between 150 and 255) using Adobe Photoshop 7.0. Also, the number of red pixels (fluorescence level between 150 and 255) was calculated.

Results

Endocytosis of Shiga toxin in dyn^{K44A} and antisense CHC cells

In order to investigate the involvement of dynamin in endocytosis and intracellular transport of Shiga toxin, we used HeLa dyn^{K44A} cells that, in the absence of tetracycline, produce mutant dynamin that is unable to bind and hydrolyse GTP. Expression of this mutant is known to block the clathrin-dependent uptake of transferrin (Damke et al., 1994). Furthermore, dynamin can be involved in uptake from caveolae and in clathrin- and caveolae-independent endocytic processes (Schnitzer et al., 1996; Oh et al., 1998; Henley et al., 1998; Lamaze et al., 2001). Also, the involvement of clathrin in uptake and intracellular transport of Shiga toxin has been investigated, using a BHK cell line in which CHC antisense RNA is induced in the absence of tetracycline, leading to a block in clathrin-dependent endocytosis as well as inhibiting clathrin function intracellularly (Iversen et al., 2003). Shiga toxin is, despite being bound to a glycolipid receptor, efficiently endocytosed by clathrin-coated pits in some cells (Sandvig and van Deurs, 1996). However, clathrin-independent endocytosis can also be responsible for a fraction of the toxin uptake in some cells (Lingwood, 1999; Nichols et al., 2001; Sandvig et al., 2002).The two cell lines that we use in this study were selected because a large proportion of Shiga toxin is still endocytosed when clathrin-dependent endocytosis is blocked either by expression of mutant dynamin or CHC antisense RNA (Fig. 1A), facilitating studies of intracellular transport

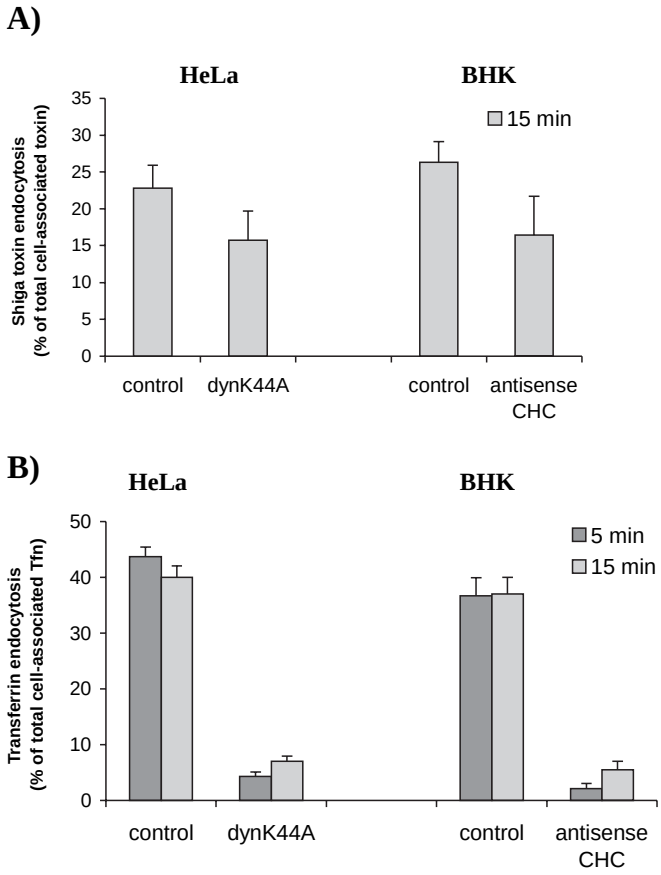

Fig. 1. Shiga toxin and transferrin endocytosis in HeLa and BHK cells. HeLa and BHK cells grown with (control) and without (dyn^{K44A} for HeLa cells or antisense CHC for BHK cells) tetracycline for 48 hours were incubated with TAG- and biotin-labelled (A) Shiga toxin (5 ng ml^{-1}) or (B) transferrin (50 ng ml^{-1}) for 5 or 15 minutes. Half of the cells were then incubated with 0.1 M MESNa for 1 hour at 0°C to remove the SS-linked biotin on the cell surface-bound toxin, before the cells were washed and lysed. The amount of TAG- and biotin-labelled toxin in the lysates was then measured using streptavidin beads and Origen Analyzer, and the degree of endocytosis (as percentage of total cell-associated protein) was calculated. The error bars show the standard deviation between three to eight independent experiments.

steps under these conditions. The extent of reduction in Shiga toxin endocytosis after blocking clathrin-dependent endocytosis varied somewhat between different experiments, probably owing to growth conditions. The amount of total cell-associated toxin was not affected upon expression of mutant dynamin or CHC antisense RNA (data not shown). To make sure that the dynamin- and clathrin-dependent endocytosis was blocked in the absence of tetracycline in our experiments, endocytosis of transferrin was analysed in parallel (Fig. 1B).

Butyric acid sensitizes HeLa and BHK cells to Shiga toxin

It was possible that Shiga toxin transport to the Golgi apparatus occurs by different mechanisms depending on the sensitivity of the cell. We have previously shown that butyric acid sensitizes A431 cells to Shiga toxin by increasing the proportion of cell-associated toxin that is transported to the Golgi apparatus and the ER (Sandvig et al., 1992). In the present study, we therefore first investigated whether butyric acid affected the intracellular routing of Shiga toxin also in HeLa and BHK cells, as earlier observed in A431 cells. Fig. 2 shows that both cell lines were highly sensitized to Shiga toxin when incubated with 2 mM butyric acid for 24 hours. In fact, the increase in the toxic effect of Shiga toxin in butyric-acid-treated cells compared with untreated cells was more than tenfold in both cell lines. This result suggested that butyric acid increases the transport of Shiga toxin to the Golgi apparatus and the ER in these cells. We have previously found that butyric-acid treatment of cells can induce a large increase in Shiga toxin binding sites for the toxin in polarized MDCK cells (Sandvig et al., 1991). However, this was not the case in either the HeLa cells or the BHK cells (data not shown). Also, the difference in endocytic uptake of the toxin (see below) could not explain this strong sensitization.

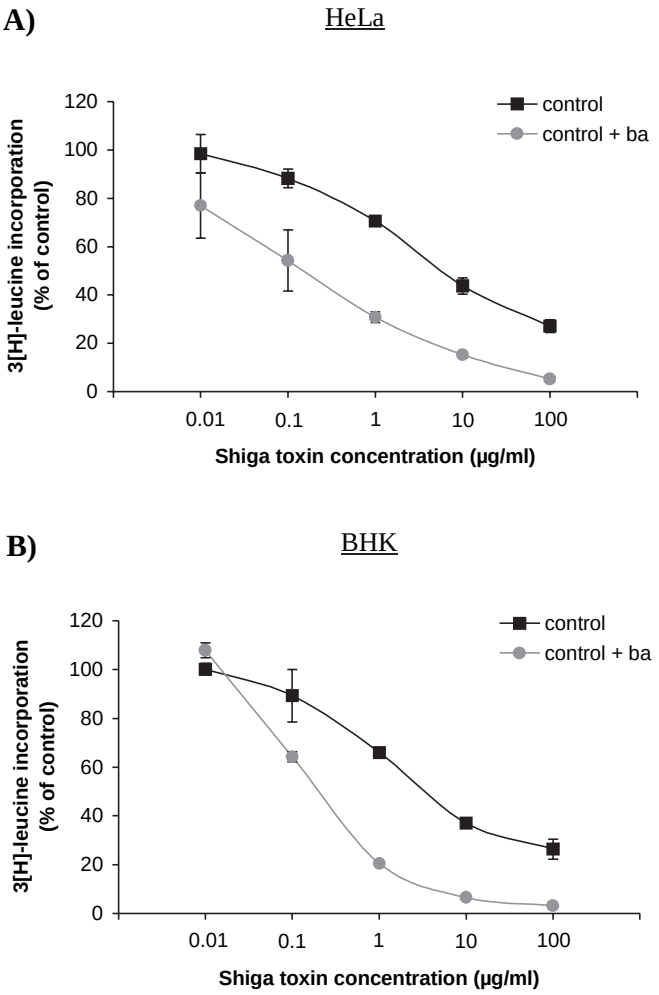

Fig. 2. Butyric acid sensitizes HeLa and BHK cells to Shiga toxin. HeLa (A) and BHK (B) control cells were grown with tetracycline for 48 hours and with or without 2 mM butyric acid (ba) for the last 24 hours. The cells were then incubated with increasing concentrations of Shiga toxin for 2 hours and the protein synthesis measured. The error bars represent deviations between duplicates.

Butyric acid increases Shiga toxin transport to the Golgi apparatus

Consistent with the toxicity experiments, increased transport of Shiga toxin to the Golgi apparatus upon butyric-acid treatment was visualized by immunofluorescence. HeLa and BHK control cells grown for 48 hours in the presence of tetracycline with or without 2 mM butyric acid for the last 24 hours were incubated with Alexa Fluor 488-labelled Shiga toxin for 20 minutes. There was a strong colocalization between Shiga toxin and the Golgi marker GM130 in butyric-acid-treated cells, whereas the toxin staining in untreated cells was more endosomal (Fig. 3). Also, studies in which Shiga toxin transport to the Golgi apparatus was quantified (by measuring the sulfation of a modified Shiga B fragment) revealed increased sulfation (i.e. increased Golgi transport) after treatment with butyric acid (see below).

Endosome-to-Golgi transport of Shiga B is dependent on dynamin

We have previously found that expression of mutant dynamin inhibits the transport of ricin to the Golgi apparatus and the ER (Llorente et al., 1998). To investigate whether dynamin is also involved in endosome-to-Golgi transport of Shiga toxin, HeLa cells incubated with (control) or without (dyn^{K44A}) tetracycline for 48 hours, with or without butyric acid for the last 24 hours, were incubated with a modified Shiga B fragment containing a tandem of sulfation sites (STxB-$Sulf_2$). As seen in Fig. 4A, the sulfation of STxB-$Sulf_2$ was almost blocked in cells expressing mutant dynamin in both untreated cells and in cells treated with butyric acid. There were only minor changes in the sulfation of cellular proteins (data not shown). Furthermore, butyric acid increased the sulfation of STxB-$Sulf_2$ to a large extent, and relatively large amounts of Shiga toxin had reached the TGN in these cells already after 15 minutes. This increased sulfation is consistent with the observed increase in sensitivity to Shiga toxin after butyric-acid treatment (Fig. 2A) and the confocal studies (Fig. 3).

Fig. 4A shows the effect of expressing mutant dynamin on the amount of STxB-$Sulf_2$ that has first entered the cells and then been transported further to the TGN, where it is sulfated. To calculate the effect of expressing mutant dynamin on the endosome-to-Golgi step alone, the changes in sulfation were corrected for the actual amount of Shiga toxin that was

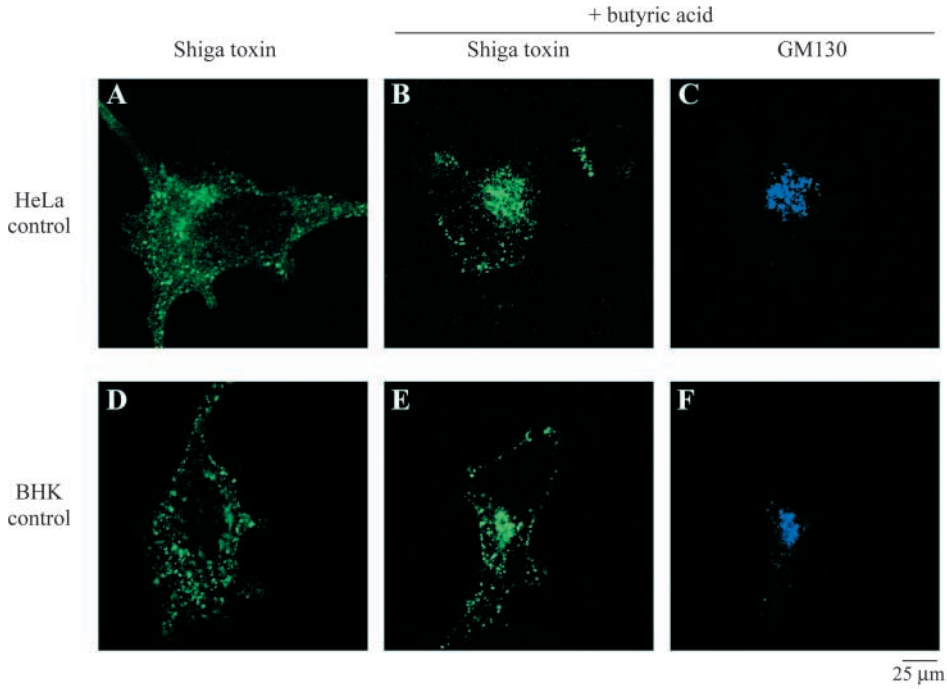

Fig. 3. Effect of butyric acid on transport of Shiga toxin to the Golgi apparatus. HeLa (A-C) and BHK (D-F) control cells grown with tetracycline for 48 hours and with (B,C,E,F) or without (A,D) 2 mM butyric acid for the last 24 hours were incubated with 1 µg ml^{-1} Alexa Fluor 488-labelled Shiga toxin for 20 minutes. The Golgi apparatus was labelled with mouse anti-GM130 antibodies followed by CY-5 labelled goat anti-mouse IgG (C,F).

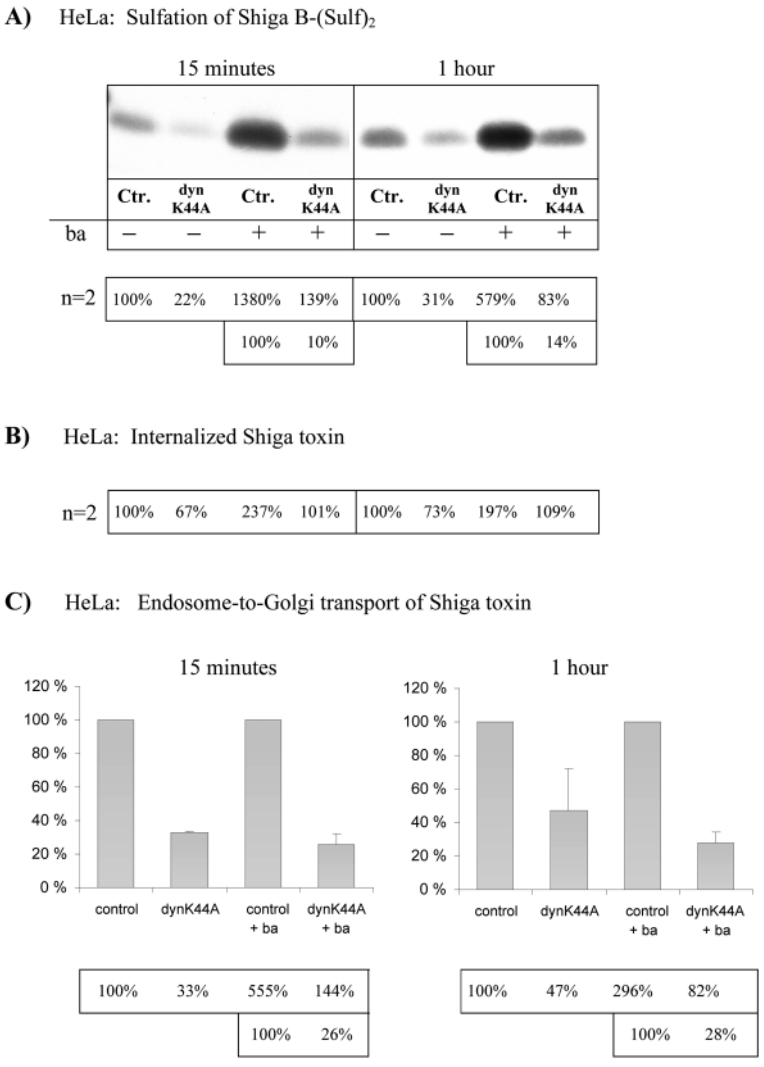

Fig. 4. Endosome-to-Golgi transport of Shiga toxin in HeLa cells. HeLa cells were grown with (control) and without (dyn^{K44A}) tetracycline for 48 hours and with and without 2 mM butyric acid (ba) for the last 24 hours. (A) Sulfation of STxB-Sulf$_2$ was analysed by incubating the cells with radioactive sulfate for 3 hours at 37°C before STxB-Sulf$_2$ (2.8 µg ml^{-1}) was added for 15 minutes or 1 hour. The cells were subsequently washed, lysed and immunoprecipitated with rabbit anti-Shiga toxin antibodies. The adsorbed material was analysed by 12% SDS-PAGE before autoradiography. Quantified average signal intensities (as percentage of the value in control cells) from two independent experiments are shown (A, bottom). As shown, butyric acid strongly increased the sulfation and expression of mutant dynamin gave a strong reduction. (B) In parallel, the internalization of Shiga toxin for the different conditions in (A) was analysed by incubating the cells with TAG- and biotin-labelled Shiga toxin (5 ng ml^{-1}) for 15 minutes or 1 hour. The SS-linked biotin on the cell-surface-bound toxin was then removed by incubating the cells with 0.1 M MESNa for 1 hour at 0°C. Subsequently, the cells were washed and lysed, and the amounts of TAG- and biotin-labelled Shiga toxin in the lysates were measured using streptavidin beads and an Origen Analyzer. Average values from these two experiments are shown as the percentage of the value in control cells. As shown, butyric-acid treatment almost doubled the amount of endocytosed toxin. (C) The effect of expressing mutant dynamin on the endosome-to-Golgi transport of Shiga toxin (shown as percentage of the value in control cells) was calculated by correcting the average signal intensities in (A) for the amount of Shiga toxin that was internalized in (B). The error bars show the standard error of the mean from the two experiments.

internalized under the different conditions (Fig. 4B). The calculations reveal that endosome-to-Golgi transport of Shiga toxin is reduced by at least 70% in cells expressing mutant dynamin, in both untreated cells and cells treated with butyric acid (Fig. 4C). Thus, in both untreated and butyric-acid-treated cells, transport of Shiga toxin to the Golgi apparatus seems to be dependent on functional dynamin. Furthermore, butyric acid increased the proportion of internalized toxin that is transported to the Golgi apparatus by more than five times after 15 minutes incubation with the toxin (Fig. 4C, bottom). However, this effect seems to be smaller after longer incubation times: only a threefold increase was observed after 1 hour.

Butyric acid increases dynamin-dependent endocytosis in HeLa cells and clathrin-dependent endocytosis in BHK cells

Interestingly, butyric acid also stimulates the endocytic uptake of Shiga toxin in HeLa control cells: 45% of total cell-associated toxin was endocytosed in butyric-acid-treated cells compared with 29% in untreated cells during a 15 minute incubation (Fig. 5A). By contrast, Shiga toxin endocytosis in BHK control cells was essentially unchanged upon butyric-acid treatment. The increased uptake of Shiga toxin in HeLa cells seems to be dynamin dependent because it was not observed in cells expressing mutant dynamin (dyn^{K44A}). In fact, the proportion of Shiga toxin endocytosis that was dynamin dependent was increased from 12% in untreated cells to 49% in cells treated with butyric acid [calculated from the difference between control cells and dyn^{K44A} cells, and (control + ba) and (dyn^{K44A} + ba), respectively, in Fig. 5A]. Even though butyric-acid treatment did not elevate Shiga toxin endocytosis in BHK control cells, the proportion of toxin uptake that was clathrin dependent was indeed increased in these cells after butyric-acid treatment, from 19% to 43% (Fig. 5A). To verify that the dynamin-dependent endocytosis was also blocked in butyric-acid-treated cells, we analysed the transferrin endocytosis under the same conditions (Fig. 5B).

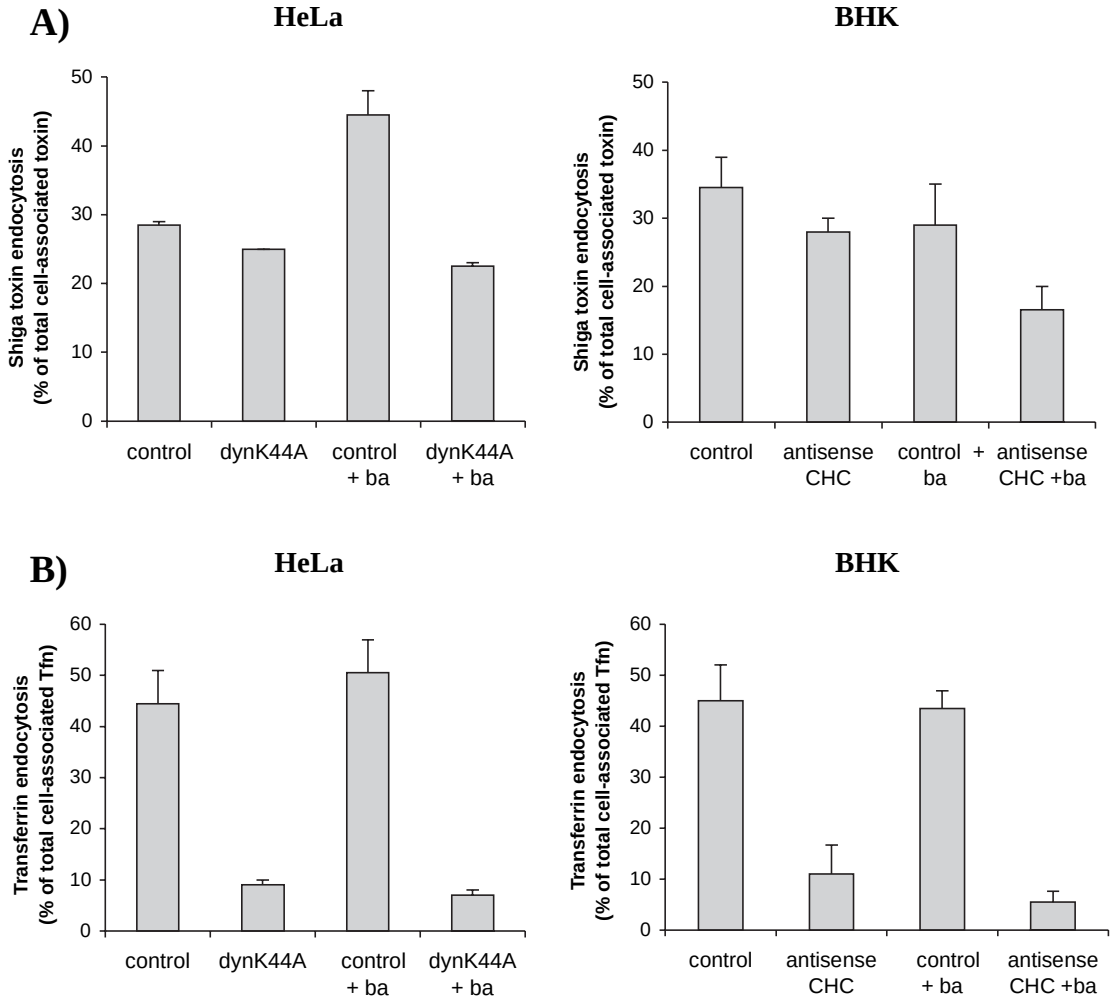

Fig. 5. Butyric acid increases the proportion of Shiga toxin endocytosed in a dynamin- and clathrin-dependent manner. HeLa and BHK control cells were grown with tetracycline for 48 hours and with and without 2 mM butyric acid (ba) for the last 24 hours. The cells were then incubated with TAG- and biotin-labelled Shiga toxin (5 ng ml^{-1}) (A) or transferrin (50 ng ml^{-1}) (B) for 15 minutes. Half of the cells were then incubated with 0.1 M MESNa for 1 hour at 0°C to remove the SS-linked biotin on the cell-surface-bound toxin before the cells were washed and lysed. The amounts of TAG- and biotin-labelled toxin in the lysates were then measured using streptavidin beads and an Origen Analyzer, and the degree of endocytosis (as the percentage of total cell-associated protein) was calculated. The error bars represent deviations between two independent experiments.

Endosome-to-Golgi transport of Shiga B becomes clathrin dependent upon butyric-acid treatment

To investigate whether clathrin plays a role in endosome-to-Golgi transport of Shiga toxin, we incubated BHK cells, grown with (control) or without (antisense CHC) tetracycline for 48 hours, and with or without butyric acid for 24 hours, with STxB-Sulf$_2$. In Fig. 6A, the sulfation of STxB-Sulf$_2$ is shown and, after correcting for the amount of Shiga toxin internalized under the different conditions (Fig. 6B), we find that the endosome-to-Golgi transport of Shiga toxin was only reduced by 30% and 11% by expression of CHC antisense RNA after incubation for 15 minutes and 1 hour, respectively (Fig. 6C). Interestingly, the reduction in transport in antisense CHC cells was much stronger upon butyric-acid treatment. After incubation with the toxin for 15 minutes or 1 hour, the transport was reduced by 80% and 75%, respectively, compared with 30% and 11% in untreated cells. The increased proportion of cell-associated toxin that is transported to the Golgi apparatus upon butyric-acid treatment was also apparent in these cells, consistent with the observed increase in sensitivity to Shiga toxin after butyric-acid treatment (Fig. 2B) and the confocal studies (Fig. 3).

Increased colocalization between Shiga toxin and CHC after butyric-acid treatment

The sulfation experiment shows that expression of CHC antisense RNA in butyric-acid-treated BHK cells blocks the Shiga toxin transport from endosomes to the Golgi apparatus, whereas the transport is only slightly reduced in untreated cells. This suggests that the toxin is more dependent on a pathway that involves clathrin in butyric-acid-treated cells than in untreated cells. We therefore performed confocal microscopy experiments to analyse the colocalization between Shiga toxin and CHC in untreated and butyric-acid-treated cells. Thus, HeLa and BHK control cells grown for 48 hours in the presence of tetracycline and with or without 2 mM butyric acid for the last 24 hours were incubated with Alexa Fluor 488-labelled Shiga toxin for 20 minutes. As shown in Fig. 7, the colocalization between Shiga toxin and CHC is quite low for both cell lines in both untreated and butyric-acid-treated cells. Thus, the figure images show no striking difference in the degree of colocalization upon butyric-acid treatment. However, quantification of the extent of colocalization (as the percentage of the total amount of toxin) from eight or nine cells using Adobe Photoshop 7.0 software revealed that the proportion of Shiga toxin colocalized with

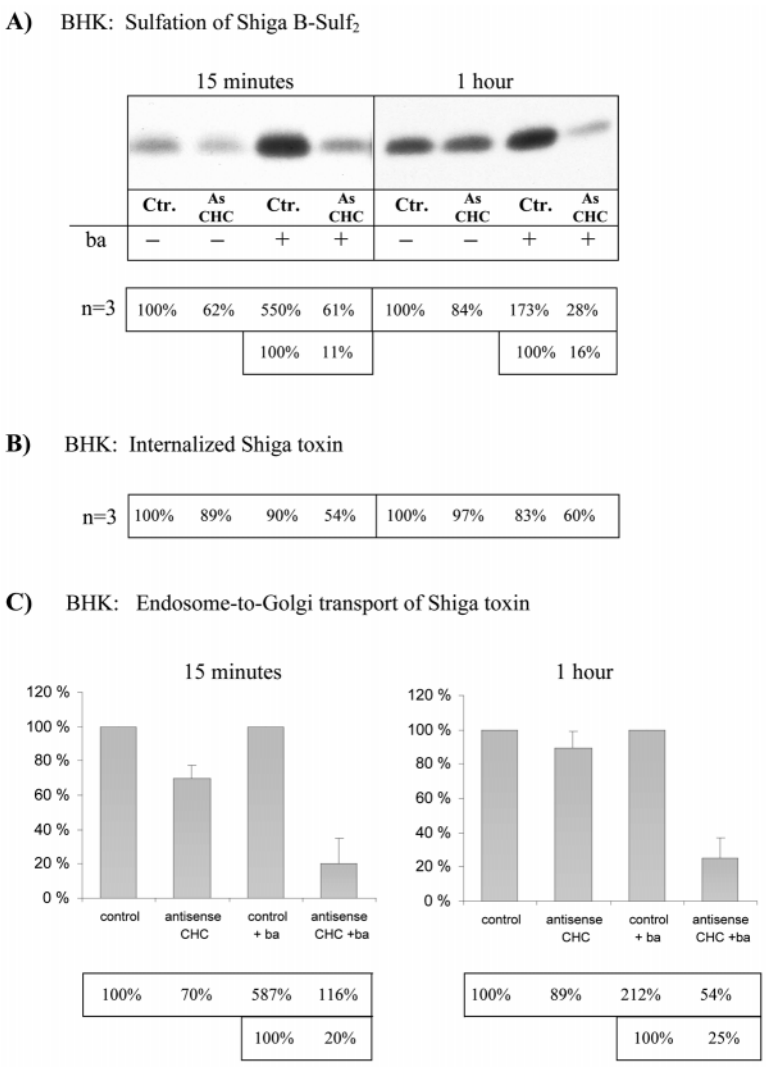

Fig. 6. Endosome-to-Golgi transport of Shiga toxin in BHK cells. BHK cells were grown with (control) and without [antisense (As) CHC] tetracycline for 48 hours and with and without 2 mM butyric acid (ba) for the last 24 hours. (A) Sulfation of STxB-Sulf$_2$ was analysed by incubating the cells with radioactive sulfate for 3 hours at 37°C before STxB-Sulf$_2$ (2.8 μg ml^{-1}) was added for 15 minutes or 1 hour. The cells were subsequently washed, lysed and immunoprecipitated with rabbit anti-Shiga-toxin antibodies. The adsorbed material was analysed by 12% SDS-PAGE before autoradiography. Quantified average signal intensities from three independent experiments are shown in the lower panel of (A) (as the percentage of the value in control cells). As shown, butyric acid increased the sulfation strongly, and expression of CHC antisense RNA gave a strong reduction in butyric-acid-treated cells but not in untreated cells. (B) In parallel, the internalization of Shiga toxin for the different conditions in (A) was analysed by incubating the cells with TAG- and biotin-labelled Shiga toxin (5 ng ml^{-1}) for 15 minutes or 1 hour. The SS-linked biotin on the cell-surface-bound toxin was then removed by incubating the cells with 0.1 M MeSNa for 1 hour at 0°C. Subsequently, the cells were washed and lysed, and the amounts of TAG- and biotin-labelled Shiga toxin in the lysates were measured using streptavidin beads and an Origen Analyzer. Average values from these three experiments are shown as the percentage of the value in control cells. As shown, butyric acid increased the proportion of Shiga toxin endocytosed in a clathrin-dependent manner. (C) The effect of expressing CHC antisense RNA on the endosome-to-Golgi transport of Shiga toxin (shown as the percentage of the value in control cells) was calculated by correcting the average signal intensities in (A) for the amount of Shiga toxin that was internalized in (B). The error bars show the standard error of the mean from the three experiments.

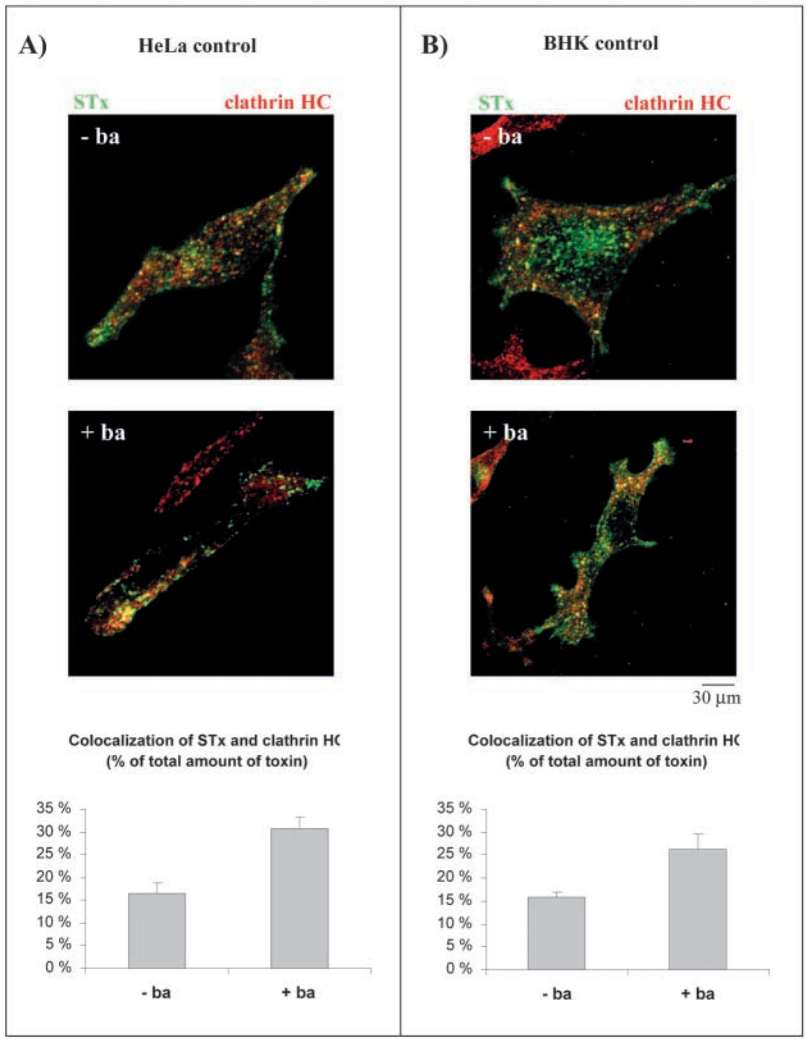

Fig. 7. Localization of Shiga toxin and clathrin heavy chain in K44A and BHK control cells. HeLa (A) and BHK (B) control cells grown with tetracycline for 48 hours and with (+ ba) or without (– ba) 2 mM butyric acid for the last 24 hours were incubated with 1 µg ml^{-1} Alexa Fluor 488-labelled Shiga toxin (STx) for 20 minutes. CHC staining was performed by using goat anti-CHC antibodies followed by rhodamine-labelled donkey anti-goat IgG. Quantification of the extent of colocalization between STx and CHC (as the percentage of the total amount of toxin) from eight or nine cells is shown in (A,B, bottom). The error bars represent the standard error of the mean.

CHC was almost doubled in butyric-acid treated HeLa control cells compared with untreated cells, from 16.6% to 30.8% (Fig. 7A, bottom). In BHK control cells, this colocalization increased from 15.9% to 26.2% upon butyric-acid treatment (Fig. 7B, bottom). For both cell lines, the amount of clathrin was also quantified and there seemed to be more membrane-associated clathrin in the butyric-acid-treated cells (data not shown). The relatively low degree of colocalization that we observe in our experiments is in agreement with earlier electron microscopy studies showing that 9.7% of total internalized Shiga B localizes with the AP1-type clathrin coat component γ-adaptin (Mallard et al., 1998). From the experiments shown in Fig. 7, it is difficult to distinguish between early endosomes and other cellular compartments, such as late endosomes and the recycling compartment. Thus, the observed increase in colocalization only indicates that Shiga toxin transport in general seems to be more clathrin-dependent after butyric-acid treatment; it does not implicate the clathrin dependency in any specific transport step. To analyse the involvement of clathrin in the transport step specifically from early endosomes, we transfected HeLa and BHK control cells grown in the presence of tetracycline with a GTPase-deficient mutant form of Rab5 (Myc-Rab5^{Q79L}), which has been shown to increase membrane fusion, resulting in the formation of enlarged early endosomes (Raiborg et al., 2001). The cells were further grown with or without butyric acid for 24 hours, before incubation with Alexa Fluor 488-labelled Shiga toxin for 20 minutes. Fig. 8 shows transfected cells containing enlarged endosomes and quantification of the extent of colocalization between Shiga toxin and CHC (as the percentage of the total amount of toxin on the enlarged endosome) of 6-11 endosomes from four to six transfected cells revealed a twofold increase in the proportion of Shiga toxin colocalized with CHC in butyric-acid-treated cells compared with untreated cells, from 16.9% to 36.0% in HeLa control cells (Fig. 8A, graph) and from 16.1% to 35.8% in BHK control cells (Fig. 8B, graph). Also, in these experiments, quantifications suggested that butyric acid increased the amount of clathrin on the enlarged endosomes (data not shown).

Intoxication with Shiga toxin is dependent on dynamin and clathrin

We further investigated the effect of expressing mutant dynamin or CHC antisense RNA on the ability of Shiga toxin to inhibit protein synthesis. Cells grown with (control) or without (dyn^{K44A} or antisense CHC) tetracycline for 48 hours and with or without butyric acid for the last 24 hours were incubated with increasing toxin concentrations for 2 hours. As shown in Fig. 9A, expression of mutant dynamin protected untreated as well as butyric-acid-treated HeLa cells against Shiga toxin. A protection against the toxin was also observed in BHK cells expressing CHC antisense RNA (Fig. 9B). In agreement with studies of sulfation of Shiga B-Sulf$_2$, this protective effect was increased upon butyric-acid treatment. Consistent with the sulfation experiments, Fig. 9 also demonstrates that the sensitivity to Shiga toxin in both cell lines was strongly increased (about ten times) in cells treated with butyric acid compared with untreated cells.

Discussion

The main finding in the present article is that Shiga toxin transport from endosomes to the Golgi apparatus is inhibited by dominant negative mutant dynamin, and that the requirement for clathrin at the endosomal level seems to be dependent on the efficiency of Golgi transport.

Studies of retrograde transport have revealed that there are

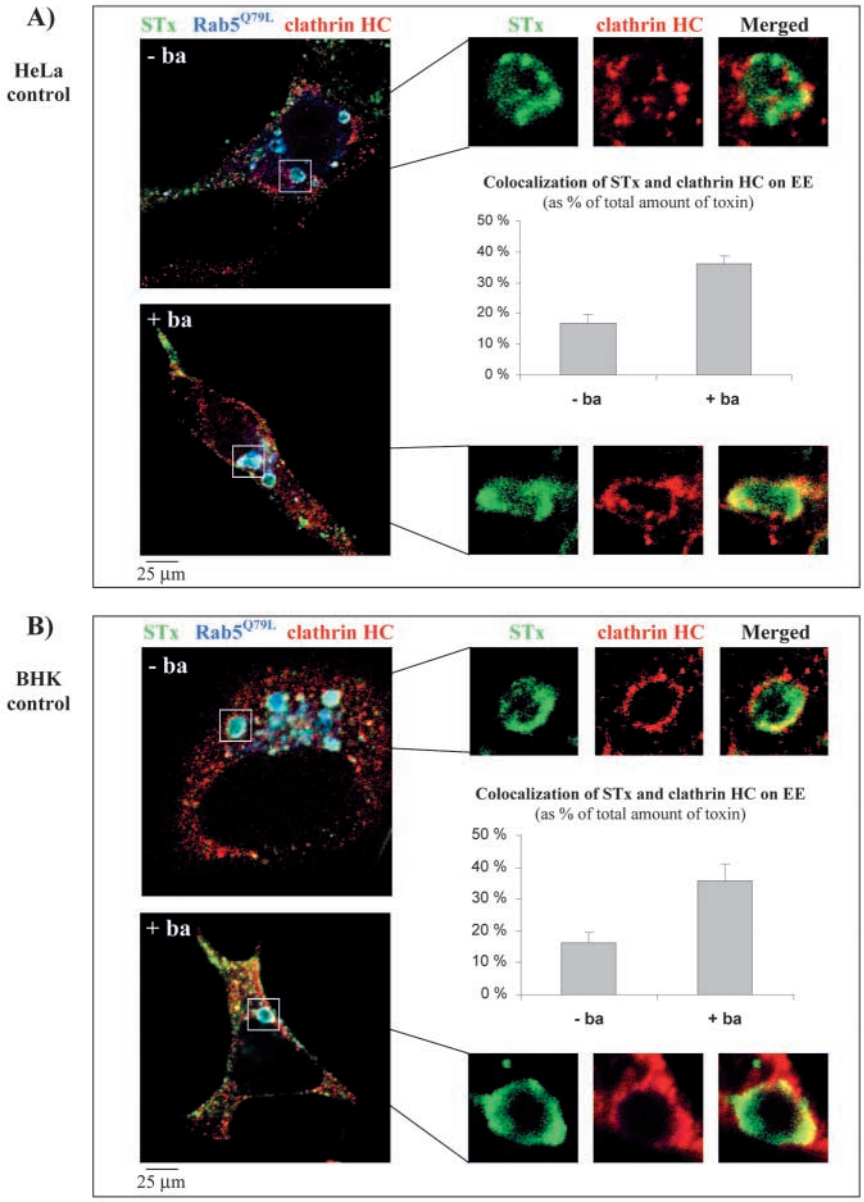

Fig. 8. Localization of Shiga toxin and CHC in Myc-Rab5^{Q79L}-transfected cells. HeLa (A) and BHK (B) control cells grown in the presence of tetracycline were transfected with a GTPase-deficient mutant form of Rab5 (Myc-Rab5^{Q79L}) to induce the formation of enlarged early endosomes (EE). The cells were further grown with (+ ba) or without (– ba) butyric acid for 24 hours before incubation with 1 µg ml^{-1} Alexa Fluor 488-labelled Shiga toxin (STx) for 20 minutes. CHC staining was performed by using goat anti-CHC antibodies followed by rhodamine-labelled donkey anti-goat IgG. The enlarged endosomes in transfected cells were identified with mouse anti-Myc antibodies followed by CY5-labelled donkey anti-mouse IgG. The white squares to the left are shown in enlarged versions to the right (green and red channels alone and the merged picture). The graphs show quantification of the extent of colocalization between STx and CHC on EE (as the percentage of the total amount of toxin) of 6-11 endosomes from four to six cells. The error bars represent the standard error of the mean.

several pathways leading from endosomes to the Golgi apparatus. Much is known about the Rab9-dependent transport of the M6PR from late endosomes to the Golgi apparatus. However, the mechanism behind the transport of Shiga toxin is less clear. Earlier studies suggest that the toxin is, in contrast to the M6PR, transported directly from early endosomes to the TGN, circumventing the late endocytic pathway (Ghosh et al., 1998; Mallard et al., 1998). Furthermore, Rab11 has been reported to be involved in this transport, suggesting that Shiga toxin might pass through the recycling compartment on its way to the TGN (Wilcke et al., 2000). In this study, we demonstrate that endosome-to-Golgi transport of Shiga toxin is also dependent on functional dynamin. Sulfation experiments using STxB-Sulf$_2$ revealed that the transport of Shiga toxin was almost blocked in cells expressing mutant dynamin. Because the toxic effect of Shiga toxin varies between different cell lines, it was possible that toxin transport to the Golgi apparatus occurred by different mechanisms in cells that exhibit different sensitivity to the toxin. Therefore, we also investigated the effect of abolishing dynamin function in HeLa cells sensitized by treatment with butyric acid. As shown here, and as previously demonstrated for A431 cells, butyric acid increases the proportion of cell-associated toxin that is transported to the Golgi apparatus and the ER (Sandvig et al., 1992). A block in endosome-to-Golgi transport was also observed in butyric-acid-treated HeLa dyn^{K44A} cells, suggesting that dynamin also plays a role in the efficient transport of Shiga toxin in highly sensitive cells. Dynamin has also been implicated in the transport of ricin (Llorente et al., 1998), as well as the M6PR (Nicoziani et al., 2000), to the Golgi apparatus. However, the type of coat (if any) involved in the dynamin-dependent vesicle formation does not seem to be the same for the different pathways. Previous findings suggest that ricin travels to the Golgi apparatus independently of clathrin (Iversen et al., 2001). Furthermore, endosome-to-Golgi transport of the M6PR seems to involve AP-1 (Meyer et al., 2000) but not clathrin (Draper et al., 1990; Hinners and Tooze, 2003). By contrast, Shiga toxin might be transported to the Golgi apparatus in a clathrin-dependent manner, because it has been found to localize with clathrin and AP-1 by confocal- and electron-microscopy experiments (Mallard et al., 1998). In the present study, we have investigated the role of clathrin in endosome-to-Golgi transport of Shiga toxin using BHK cells that, in an inducible manner, express CHC antisense RNA. We find that expression of CHC antisense RNA inhibits the efficient endosome-to-Golgi transport of Shiga toxin in butyric-acid-treated cells by at least 75-80%. Interestingly, the regulation of Shiga toxin transport in untreated and butyric-acid-treated cells differs when it comes to the role of clathrin, because only a slight reduction in transport is observed without butyric acid in cells expressing CHC antisense RNA.

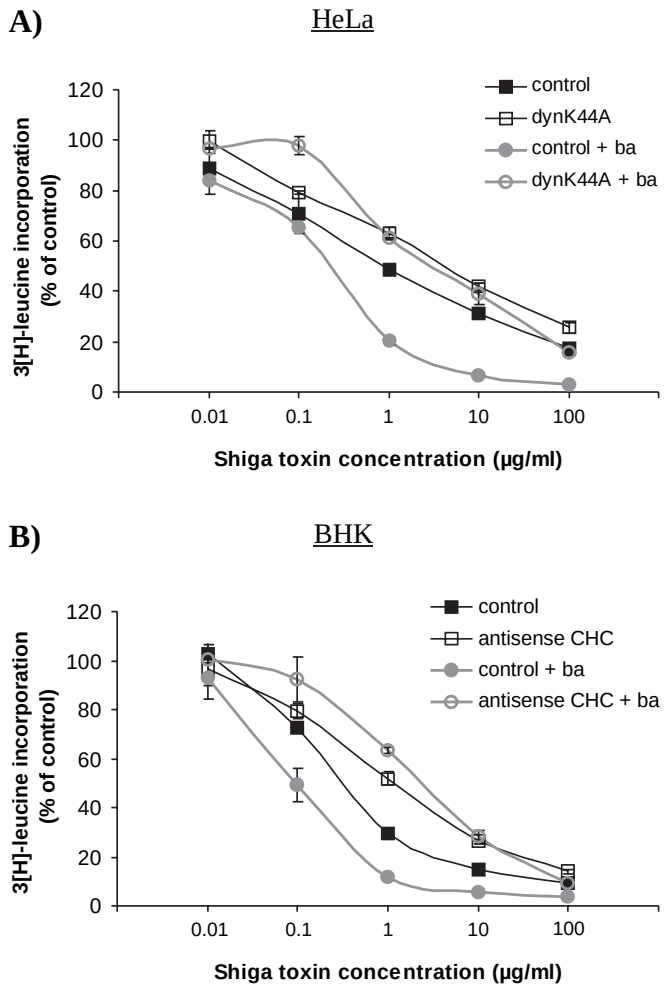

Fig. 9. Cytotoxicity of Shiga toxin in HeLa and BHK cells. HeLa (A) and BHK (B) cells were grown with (control) and without (dyn^{K44A} for HeLa cells or antisense CHC for BHK cells) tetracycline for 48 hours and with and without 2 mM butyric acid (ba) for the last 24 hours. The cells were then incubated with increasing concentrations of Shiga toxin for 2 hours and protein synthesis measured. The error bars represent deviations between duplicates.

We have earlier found that Shiga toxin can induce movement of toxin-Gb3 complexes to clathrin-coated pits (Sandvig et al., 1989). The intracellular transport of Shiga toxin is dependent on the length of the fatty acid found in Gb3. Cells of high sensitivity tend to synthesize Gb3 with C16 and C18 fatty acid chains, and both very short and very long fatty acids will reduce transport of Shiga toxin to the Golgi apparatus (Lingwood, 1999). We have previously demonstrated by mass spectrometry that there is a change in the length of the fatty acid of Gb3 upon butyric-acid treatment (Sandvig et al., 1994). The change in Gb3 upon butyric-acid treatment might increase the transport of Shiga-toxin/Gb3 complexes to clathrin-coated pits. This might explain the increase in the proportion of dynamin-dependent endocytosis in dyn^{K44A} cells upon butyric-acid treatment from 12% to 49% and the increase in the proportion of clathrin-dependent endocytosis in antisense CHC cells from 19% to 43%. Furthermore, we have also found that the change in lipid composition of Gb3 by butyric acid is essential for the elevated proportion of cell-associated toxin that is transported to the Golgi apparatus and for the increased sensitivity (Sandvig et al., 1994; Sandvig et al., 1996). An explanation for the different regulation of Shiga toxin transport in cells that exhibit different sensitivity to the toxin could be that, also on the endosomal level, a larger proportion of Gb3 with bound Shiga toxin becomes associated with clathrin-coated regions after butyric-acid treatment and that the transport thereby becomes clathrin dependent. Importantly, confocal microscopy experiments support this idea: there was a twofold increase in the proportion of Shiga toxin colocalized with CHC after butyric-acid treatment compared with untreated cells [both at the level of early endosomes (enlarged by Myc-$Rab5^{Q79L}$-expression) and when quantifying the colocalization from all cellular compartments]. The increased proportion of Shiga toxin localized with clathrin might also be due, at least partly, to there being more clathrin at the membrane upon butyric-acid treatment. Furthermore, our findings suggest that the endosome-to-Golgi transport involving clathrin-dependent vesicle formation is much more efficient than clathrin-independent Shiga toxin transport, as seen by the fivefold increase in Shiga B-$Sulf_2$ sulfation (and no increase in toxin binding sites) after butyric-acid treatment. The increased transport of Shiga toxin to the Golgi apparatus upon butyric-acid treatment was also visualized by immunofluorescence.

Receptor-mediated endocytosis was, for several years, thought to occur only through clathrin-coated pits but there are other endocytic mechanisms (for review, see Sandvig et al., 2002; Conner and Schmid, 2003). For instance, the plant toxin ricin can be internalized by clathrin- and dynamin-independent mechanisms (Llorente et al., 1998; Iversen et al., 2001), whereas the interleukin-2 receptor has been shown to be internalized through a clathrin-independent pathway that requires dynamin (Lamaze et al., 2001). Also the Shiga-toxin receptor Gb3 seems to be internalized by both clathrin-dependent and clathrin-independent mechanisms (Sandvig and van Deurs, 1996; Lingwood, 1999; Nichols et al., 2001; Sandvig et al., 2002). However, the extent to which the two mechanisms contribute to the toxin uptake seems to vary between different cell lines. Interestingly, in contrast to the internalization of the interleukin-2 receptor, our data suggest that the clathrin-independent endocytosis of Shiga toxin also can be independent of dynamin, because dyn^{K44A} cells expressing mutant dynamin take up almost as much toxin as control cells. It is important to realize that the high degree of dynamin- and clathrin-independent endocytosis of Shiga toxin in the HeLa dyn^{K44A} cells and the BHK antisense CHC cells that we use in this study might not reflect the amount of toxin normally taken up by this mechanism in HeLa and BHK cells. Cells expressing mutant dynamin or CHC antisense RNA might compensate for the lack of clathrin-dependent endocytosis by upregulating other endocytic mechanisms (Damke et al., 1995a; Llorente et al., 1998). However, the use of these cell lines has, in the present study, facilitated investigations of endosome-to-Golgi transport.

In conclusion, we demonstrate that endosome-to-Golgi transport of Shiga toxin is dependent on dynamin in both untreated and butyric-acid-treated cells. Interestingly, only the efficient transport in butyric-acid-treated cells seems to be dependent on clathrin. Thus, the transport mechanism seems

to differ when it comes to the role of clathrin, depending on the transport efficiency and the sensitivity of the cell to the toxin.

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