Shiga toxin consists of an enzymatically active A-chain and a pentameric binding subunit. The A-chain has a trypsin-sensitive region, and upon cleavage two disulfide bonded fragments, A1 and A2, are generated. To study the role of the disulfide bond, it was eliminated by mutating cysteine 242 to serine. In T47D cells this mutated toxin was more toxic than wild type toxin after a short incubation, whereas after longer incubation times wild type toxin was most toxic. Cells cleaved not only wild type but also mutated A-chain into A1 and A2 fragments. The mutated A-chain was more sensitive than wild type toxin to Pronase, and it was degraded at a higher rate in T47D cells. Subcellular fractionation demonstrated transport of both wild type and mutated toxin to the Golgi apparatus. Brefeldin A, which disrupts the Golgi apparatus, protected not only against Shiga toxin but also against the mutated toxin, indicating involvement of the Golgi apparatus. After prebinding of Shiga(C242S) toxin to wells coated with the Shiga toxin receptor, Gb3, trypsin treatment induced dissociation of A1 from the toxin-receptor complex demonstrating that the presence of the A2 fragment has an inhibitory effect on the enzymatic activity of Shiga toxin (13). The crystal structure of Shiga toxin has been resolved at 2.5-Å resolution (15), and interestingly, it shows that the active site in A1 is physically isolated from the other A-chains. In the present article we demonstrate that the disulfide bond in the A-chain of Shiga toxin is not required for rapid intoxication of cells but seems to be important for toxicity after a long incubation time. The data also suggest that the disulfide bond inhibits dissociation of the A1 fragment from the toxin complex after cleavage and that it inhibits complete degradation of the A-chain both in vitro and in vivo.

EXPERIMENTAL PROCEDURES

Materials—Brefeldin A (BFA) was purchased from Epicentre Technologies (Madison, WI). Pronase, trypsin, Heps, Gb3, N-ethylmaleimide, and PMSF were obtained from Sigma. Na125I was purchased from DuPont (Belgium), and [3H]leucine was purchased from Amersham Int. (Amersham, United Kingdom). Furin was a gift from Dr. Gary Thomas (Vollum Institute, Oregon Health Sciences University, Portland, OR). Shiga toxin was purified as described previously (13) and 125I-labeled as described by Fraker and Speck (16).

Bacteria and Plasmids—Escherichia coli JM109, BHA61 mut.S were used for mutagenesis, transformation, recloning into pUC 19, and for isolation of the Shiga toxin. An EcoRI–BamHI restriction fragment if 1.8 kilobase pairs containing ShlA and ShlB was excised from M13 mp8 and subcloned into pALTER (Promega). After mutagenesis, the fragment containing the genes for A and B subunits and the desired mutation in the A subunit was cloned into pUC 19. All bacterial strains were propagated in Luria broth or Luria broth agar, supplemented with antibiotics when necessary. Restriction endonucleases, T4 DNA ligase, and T4 DNA kinase were purchased from New England Biolabs, Inc. (Beverly, MA). Plasmid DNA was purified by methods described by Maniatis et al. (17).

Site-directed Mutagenesis—The point mutation was introduced in the ShlA gene by means of the Altered Sites System (Promega). Oligonucleotides designed to introduce mutations were synthesized on the basis of the sequence of the shlA as previously published by Kozlov et al. (18). All mutations were confirmed by DNA sequence (19). The oligonucleotides were synthesized on an Applied Biosystems synthesizer and purified with the high pressure liquid chromatography technique. Sequencing primer was 5'-GTTAAAACGGCAAGGCAACAGTCAG-3'; the mutagenesis primer was 5'-CAACTCCGGATCATGATGATGAGAATTCAG-3' (C242S).

Cell Culture—HEp-2 cells (from epidermoid carcinoma) were obtained from ATCC (Rockville, MD). HEp-2 cells were grown in Dulbecco's modified Eagle's medium supplemented with 5% serum. T47D cells (from human breast carcinoma) were obtained from the Fibiger Labo-
corary, Denmark. T47D cells were grown in RPMI medium supplemented with insulin (8 mg/ml) and 10% serum. LoVo cells transfected with mouse furin (LoVo-fur) and control vector (LoVoneo) were a gift from Dr. E. Mekada (Kurume University, Kurume, Fukuoka, Japan). LoVo cells were grown in Ham’s F-12 supplemented with 10% serum. Establishment of these cell lines is described elsewhere (20).

Assay of Shiga Toxin Cytotoxicity—Cells were transferred to 24-wells plates at a density of 3 × 10⁵ cells/well 2 or 3 days prior to the experiments. The cells were incubated with increasing amounts of toxin for the indicated period. Then the cells were incubated for 10 min in Hepes medium with 1 μCi/ml [3H]leucine and no unlabeled leucine. The medium was removed, and the cells were washed 2 times in ice-cold 0.1 M KOH, and the radioactivity was measured.

Cleavage of Shiga Toxin by Cultured Cells—Cells were seeded out with a density of 6 × 10⁵ cells/well in 12-well plates 3 days prior to the experiments. The cells were washed in Hepes medium, and then 125I-labeled toxin was added (100 ng/ml) in Hepes medium at 37 °C, and the incubation was continued for the indicated period. Then the cells were washed three times with phosphate-buffered saline and lysed in 1% Triton (1% Triton X-100, 20 mM Hepes, 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, pH 7.4) on ice for 30 min. The cell lysate was transferred to spin tubes, nuclei were removed by centrifugation, and proteins were precipitated for 30 min on ice in the presence of 5% trichloroacetic acid. After centrifugation the pellet was washed in ether, dissolved in sample buffer with or without 6% (v/v) 2-mercaptoethanol, and then subjected to SDS-PAGE.

In Vitro Cleavage of Shiga Toxin and Shiga-(C242S) Toxin by Purified Furin—The cleavage was performed in a reaction volume of 25 μl containing 5 μM CaCl₂, 1 μM 2-mercaptoethanol, 100 mM buffer (sodium acetate, pH 5.0; MES, pH 5.5–7.5), 10 ng of 125I-Shiga toxin, and 3 ng of purified furin. The reaction mixture was incubated for 3 h at 37 °C, and the reaction was stopped by adding SDS sample buffer with 2-mercaptoethanol. The samples were boiled and subjected to SDS-PAGE.

Trypsin Cleavage of Wild Type Shiga Toxin and Shiga-(C242S) Toxin Bound to Gb₃—Wells in a 24-well plate were coated with Gb₃, at a concentration of 200 μg/ml in 100 μl of Hepes medium for 30 min at 37 °C and then washed 3 times with Hepes medium, before 100 μl of Hepes medium containing 600 ng/ml 125I-labeled toxin was added. The toxin was allowed to bind for 30 min at room temperature, and unbound toxin was removed by washing 3 times with 100 μl of Hepes medium. Then 100 μl of Hepes medium at different pH values (pH 5.5, 6.0, 6.5, 7.0) containing 10 μg/ml trypsin was added to each well and incubated for 2 min at room temperature. The supernatant was transferred to an Eppendorf tube on ice containing a final concentration of 5 μM PMSF and sample buffer; the wells were placed on ice and washed once with Hepes medium, and sample buffer with 2-mercaptoethanol and 5 μM PMSF was added. The wells were scraped with a plastic pipette, and the contents were transferred to Eppendorf tubes. The samples were boiled and subjected to SDS-PAGE and autoradiography.

Trypsin Sensitivity of Wild Type Shiga Toxin and Shiga-(C242S) Toxin—125I-Labeled toxin at a concentration of 800 ng/ml in Hepes medium was treated with increasing concentrations of trypsin (0, 0.2, 1, 5, and 10 μg/ml) for 2 min at room temperature. The reaction was stopped by addition of sample buffer containing 2-mercaptoethanol and a final concentration of 5 μM PMSF. The samples were immediately boiled and then subjected to SDS-PAGE and autoradiography.

Pronase Sensitivity of Wild Type Shiga Toxin and Shiga-(C242S) Toxin—125I-Labeled toxin at a concentration of 800 ng/ml in a reaction volume of 25 μl of Hepes medium was incubated with increasing concentration of Pronase (0, 1, 3, 10, 30, and 100 μg/ml) for 10 min at room temperature. The reaction was stopped by adding sample buffer containing 2-mercaptoethanol and a final concentration of 5 μM PMSF. The samples were immediately boiled and then subjected to SDS-PAGE and autoradiography.

Acrylamide Gel Electrophoresis—Electrophoresis was carried out as described by Laemmli (21). After electrophoresis the gels were fixed for 30 min in 4% trichloroacetic acid and 27% ethanol. For autoradiography, the bands corresponding to A and A₁ of Shiga toxin were quantified by densitometry (model 3000A, Molecular Dynamics).

Subcellular Fractionation—The cells were fractionated essentially as earlier described (6). Proteins in fractions corresponding to the Golgi zone, lead zone, and endosomal/lysosomal zone were precipitated with 5% trichloroacetic acid, dissolved in sample buffer containing 2-mercaptoethanol, and analyzed by SDS-PAGE and autoradiography.

RESULTS

Toxicity of a Mutated Shiga Toxin (C242S) Lacking the Disulfide Bond in the A-chain—Shiga toxin A-chain contains 2 cysteine residues, cysteine 242 and cysteine 261, linked by an internal disulfide bond. The polypeptide loop between the 2 cysteines contains the sequence Arg-Val-Ala-Arg which is recognized and nicked by trypsin or furin (13, 14), thereby separating the A-chain into A₁ and A₂ fragments. To study the role of the disulfide bond in toxin entry into cells, we constructed a mutated Shiga toxin referred to as Shiga(C242S) toxin where cysteine 242 has been mutated to a serine (Fig. 1). This mutated toxin can no longer form a disulfide bond. To test whether the disulfide bond is required for intoxication of cells, we measured the ability of Shiga(C242S) toxin to intoxicate T47D cells, which are highly sensitive to Shiga toxin. Interestingly, the result showed that Shiga(C242S) toxin was 5–10 times more toxic than wild type toxin when protein synthesis was measured after a 50-min incubation period with toxin continuously present in the medium (Fig. 2A), whereas after 3 h, wild type toxin was 5–10 times more toxic than the mutated toxin (Fig. 2B). After incubation with toxin for 5 h or overnight the difference was almost 100-fold (data not shown). In HEp-2 cells, wild type toxin and Shiga(C242S) toxin were equally toxic after 50 min incubation, whereas after 3 h incubation wild type toxin exhibited a 20–30 times higher toxic activity than the mutated toxin (data not shown). To test whether cleavage by furin was responsible for the comparatively low toxicity of the mutated toxin after long incubation times, we tested the effect of the toxins on LoVo cells, which do not contain functional furin (22). Also in LoVo cells wild type toxin was around 30 times more toxic than the mutated toxin after 3 h incubation with cells, and the difference was 100-fold when cells were exposed to toxins overnight (data not shown).

The lack of increase in toxic effect of the mutated toxin after long incubation times with cells could be due to inactivation of the toxin molecules by serum proteases in the medium. To test this possibility wild type toxin and Shiga(C242S) toxin were incubated in serum-containing medium (without cells) overnight at 37 °C, and then the ability of the toxins to reduce protein synthesis in a short time experiment was measured. The data showed that overnight exposure to medium with serum did not reduce the toxic effect of the mutated molecule (data not shown). To further investigate the reduced toxicity of the mutated toxin after long incubation times with cells, toxin was prebound to the cells at 0 °C, then unbound toxin was washed away, and the incubation was continued for 1.5, 5, or 18 h. The cytotoxicity of the wild type toxin increased up to 5 h, whereas the toxic effect of the mutated toxin did not increase after 90 min (Fig. 3). Similar results were also obtained on HEp-2 cells (data not shown). These results suggest that the cells inactivate the mutated toxin more rapidly than the wild type toxin.

Proteolytic Processing of the Wild Type Toxin and Shiga-(C242S) Toxin—Since Shiga(C242S) toxin was less toxic than...
wild type toxin after long incubation times with cells (Fig. 3), we tested whether Shiga(C242S) toxin was processed differently than wild type toxin. 125I-Labeled toxin was prebound to T47D cells at 0 °C, then the cells were washed, and the incubation was continued at 37 °C for the indicated period of time and analyzed by SDS-PAGE under nonreducing (Fig. 4) and reducing conditions (data not shown), similar to the experiment in Fig. 3. The result showed that both wild type and mutated Shiga toxin A-chain were processed efficiently to A1 and A2 fragments. Densitometric measurements of the full-length A and A1 bands after a 1-h incubation showed that 55% of the wild type A-chain and 47% of the mutated A-chain was processed to A1 (Fig. 6, lanes 3, 4). In contrast, the intensity of the A bands of the mutated toxin to the A1 fragment occurred at the same period (lanes 3 and 4).

The difference in the rates of degradation could be due to different sensitivity of the molecules to proteolytic enzymes and possibly also to different intracellular sorting. We therefore studied the sensitivity of the wild type and the mutated toxin to trypsin and Pronase treatment. Wild type and mutated A-chain were equally sensitive to cleavage by trypsin which under the condition used induced processing to A1 and A2 fragments (Fig. 5). Similarly, processing of the wild type toxin and the mutated toxin to the A1 fragment occurred at the same concentration of Pronase. After treatment with 1 μg/ml Pronase, 20% of the wild type and 22% of the mutated toxin were processed to A1 (Fig. 6, lanes 2 and 8), and after treatment with 3 μg/ml Pronase 53% of wild type and 48% of the mutated toxin were processed (lanes 3 and 9). In contrast, wild type Shiga toxin was more resistant to complete degradation by Pronase than the mutated toxin. In the Pronase sensitivity experiment (Fig. 6), the A and A1 bands were quantified by densitometry. The result showed that the intensity of the A bands of wild type toxin decreased to 55% when the toxin molecules were treated with 100 μg/ml Pronase for 10 min at room temperature. At lower concentrations of Pronase there was no degradation of the wild type A-chain, only processing to form the A1 fragment. In contrast, the intensity of the A bands of the mutated toxin decreased to 64% at 10 μg/ml Pronase. At higher concentra-

FIG. 3. Ability of prebound toxins to inhibit protein synthesis in T47D cells. Increasing concentrations of Shiga toxin and Shiga(C242S) toxin were added to cells that were subsequently kept on ice for 30 min. The cells were washed and then incubated for 1.5, 5.5, or 18 h at 37 °C. The ability of the cells to incorporate [3H]leucine was measured at the end of the incubation, as described under “Experimental Procedures.” •, wild type Shiga toxin; ▲, Shiga(C242S) toxin.

FIG. 4. Degradation of the A-chain of Shiga(C242S) toxin and wild type Shiga toxin in T47D cells. 125I-Labeled toxins were added to the cells in Hepes medium at 0 °C. After 30 min, the cells were washed, new medium was added, and the cells were incubated for 1 h or 5 h at 37 °C. At the indicated time the cells were washed and lysed in the presence of 5 mM N-ethylmaleimide, and electrophoresis (SDS-PAGE) was carried out under nonreducing condition. Lane 1, molecular mass standards; lanes 2–4, wild type toxin; lanes 5–7, Shiga(C242S) toxin. Lanes 2 and 5 contain wild type toxin and Shiga(C242S) toxin, respectively, which had not been incubated with cells.
and A2 fragments (14). To examine whether Shiga(C242S) toxin was cleaved by furin as efficiently and under the same conditions as the wild type toxin, we compared cleavage in vitro at different pH values. As shown in Fig. 7, both wild type toxin and the mutated toxin were cleaved to the same extent with a pH optimum at 5.5.

Furin appears to cleave endocytosed wild type toxin most likely in the TGN and/or in endosomes (13, 14). We therefore studied the processing of Shiga(C242S) in LoVo cells, both in a stable transfected cell line expressing furin (LoVo/fur) and in a control cell transfected with the vector alone (LoVo/neo). The cells were incubated with 125I-labeled toxin, and toxin processing was then analyzed by SDS-PAGE and autoradiography. In LoVo/fur cells both wild type A-chain and the mutated A-chain were cleaved efficiently into A1 and A2 fragments, and after a 4-h incubation most of the cell-associated toxin molecules were cleaved (Fig. 8a, lanes 1–3). In the A-chain of wild type Shiga toxin after cleavage, we used 125I-labeled toxin bound to wells coated with Gb3, the receptor for Shiga toxin. The toxin did not bind to wells not coated with Gb3 (data not shown). After binding, unbound toxin was washed away, and trypsin (10 μg/ml in Heps medium) was added for 2 min at room temperature to cleave the toxin. Then the supernatant was removed, and SDS sample buffer was added to the well to solubilize the receptor-bound toxin molecules. Proteins both in the supernatant and bound to the plastic were subjected to SDS-PAGE and autoradiography. The experiment was performed at pH 5.5–7.0 to see if pH affected the release of A1 after cleavage. The low pH was used to mimic the low pH in endosomes or the TGN. The results showed that upon trypsin treatment the A1 fragment of wild type toxin was released efficiently, but as shown earlier (14), furin, which had not been added, could not be detected in the autoradiogram.

Intracellular Trafficking of the Toxin Molecules—It has been shown previously that Shiga toxin is transported to the Golgi apparatus (7, 23), and BFA, which disrupts the Golgi apparatus in several cell lines (23–32), protects cells against Shiga toxin (13). This suggests that transport to the Golgi apparatus is required for intoxication. To investigate whether Shiga(C242S) toxin follows the same endocytic pathway to the cytosol, we tested the effect of BFA also on intoxication with this.
molecule. We found that BFA also protected against Shiga-(C242S) toxin (Fig. 10). Furthermore, subcellular fractionation of cells incubated with 125I-labeled toxin showed that approximately 6% of both wild type toxin and Shiga(C242S) toxin were transported to the Golgi apparatus during 1 h (data not shown). Importantly, as shown in Fig. 11, uncleaved Shiga(C242S) toxin A-chain reaches the Golgi apparatus (lane 1).

DISCUSSION

We have introduced a mutation in Shiga toxin A-chain, C242S, to eliminate the disulfide bond in the A-chain, to study the role of the disulfide bond for toxin entry into cells, and for stabilization of the A-chain. Our data show that the disulfide bond is not strictly required for intoxication of cells but is important for efficient intoxication of cells after long incubation times. The results also indicate that the disulfide bond inhibits degradation of the A-chain and inhibits dissociation of the A1 fragment from the toxin-receptor complex after cleavage.

Shiga toxin A-chain is easily cleaved by furin both in vitro and in vivo separating the A-chain into A1 and A2 fragments connected by a disulfide bond (14). In Shiga toxin furin recognizes the sequence Arg-Val-Ala-Arg (14), which is a recognition motif for the membrane-anchored protease furin (33). However, cleavage by furin might also be dependent on protein conformation, and it was not clear whether the enzyme would cleave the mutated molecule under the same conditions as the wild type toxin. As shown here, a soluble form of furin cleaved both the mutated and wild type toxin with the same pH optimum. Furthermore, Shiga(C242S) toxin A-chain was also efficiently cleaved in LoVo cells transfected with furin, whereas in control cells, which do not express functional furin (22), the cleavage was strongly reduced. This demonstrates that furin is able to cleave the mutated toxin also in vitro. Earlier studies have shown that in LoVo cells not transfected with furin (14) wild type toxin is cleaved slowly into A1 and A2 fragments and that an inhibitor of the cytosolic protease calpain both inhibited cleavage and protected LoVo cells against Shiga toxin. Therefore, a protease other than furin, possibly calpain, may cleave and activate Shiga toxin in cells with low furin activity. Calpain has been reported to recognize several different amino acid sequences at the cleavage site, suggesting that other characteristics such as amino acids at a distance from the cleavage site or the protein conformation may be important (34, 35). Thus, the reduced cleavage of the mutated toxin compared with wild type toxin in LoVo cells could be due to lack of recognition of the mutated toxin by calpain.

The disulfide bond in the A-chain of Shiga toxin is not strictly required for intoxication of cells. Interestingly, Shiga (C242S) toxin intoxicated T47D cells more efficiently than wild type toxin after short incubation times. However, after longer incubation times wild type toxin was more toxic than the mutated toxin after short incubation times. These surprising results could be due to different intracellular sorting of the two toxins. However, brefeldin A protected T47D cells not only against wild type toxin (13) but also against the mutated toxin in a short incubation time with cells. These surprising results could be due to different intracellular sorting of the two toxins. However, brefeldin A protected T47D cells not only against wild type toxin (13) but also against the mutated toxin in a short incubation time with cells. Furthermore, subcellular fractionation of cells showed that both wild type and mutated toxin were transported to the Golgi apparatus to the same extent. The results therefore indicate that the pathway used for intoxication of the cells is the same for the two toxins.

Cell-associated Shiga(C242S) toxin clearly intoxicates cells
less efficiently than wild type toxin in long incubation times. This is not due to inactivation of Shiga (C242S) toxin by serum proteases in the incubation medium, but seems to be due to cell-mediated inactivation only. In fact, studies in cells showed that the degradation rate of the mutated A-chain was higher than that of the wild type A-chain. Also, the mutated A-chain was more sensitive to Pronase treatment than wild type A-chain. These data suggest that the normal resistance of the wild type toxin to proteases (2) is dependent on the formation of the disulfide bond of the A-chain. Thus, the reduced toxicity of the mutated toxin compared with wild type toxin after long incubation times can be explained by increased cellular degradation of the mutated toxin. We can only speculate on the reason for the strong toxic effect of the mutated toxin on T47D cells after short incubation times. It is possible that the slow reduction rate of the disulfide bond that occurs in the A-chain of the wild type toxin (Ref. 13, also shown in Fig. 4) will delay the action of the toxin sufficiently so that the mutated toxin despite the higher degradation rate can be more toxic than wild type toxin in a short incubation time with cells. This might especially occur in cells like T47D cells where retrograde transport of Shiga toxin to the endoplasmic reticulum may be especially efficient (13).

It appears from the crystal structure of Shiga toxin that the active site cleft in A1 is blocked by a segment of the A2 fragment (15). However, as shown here, possible interactions between the A1 and A2 fragments of Shiga toxin are not sufficiently strong to inhibit the dissociation of the A1 fragment from the toxin-receptor complex of Shiga(C242S) toxin. When the toxin molecule was prebound to a well coated with the receptor for Shiga toxin, cleavage induced rapid dissociation of the A1 fragment from the toxin-receptor complex. In accordance with this, trypsin-nicked mutated toxin was less toxic to T47D cells than unnicked toxin. In contrast to Shiga toxin, the A1 fragment of cholera toxin does not seem to dissociate easily from the toxin complex after reduction of the A-chain (36).

Shiga(C242S) toxin is, similar to the wild type toxin (14), most likely cleaved in the TGN and/or in endosomes, and the A1 fragment may dissociate from the toxin-receptor complex at this location and therefore not intoxicate cells efficiently. Subcellular fractionation of cells showed that uncleaved Shiga(C242S) toxin is able to reach the Golgi apparatus, suggesting that the A-chain can be further transported to the location in the cells where it is translocated to the cytosol, most likely in the endoplasmic reticulum (8–10). Since the mutated toxin is also less toxic than wild type toxin in LoVo cells, which do not process the mutated A-chain into A1 and A2 fragments, the data suggest that increased degradation is more important than furin-induced dissociation of the A1-chain for the decreased ability to intoxicate cells. The data support the view that the disulfide bond has an important role in keeping the A-chain assembled after cleavage and that it protects the A1 fragment from degradation.