Retrograde Transport of KDEL-bearing B-fragment of Shiga Toxin*

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To investigate retrograde transport along the biosynthetic/secretory pathway, we have constructed a recombinant Shiga toxin B-fragment carrying an N-glycosylation site and a KDEL retrieval motif at its carboxyl terminus (B-Glyc-KDEL). After incubation with HeLa cells, B-Glyc-KDEL was progressively glycosylated in the endoplasmic reticulum (ER) and remained stably associated with this compartment. B-fragment with a non-functional KDEL sequence (B-Glyc-KDELGL) was glycosylated with about the same kinetics as B-Glyc-KDEL but localized at steady state to the Golgi apparatus. Morphological studies showed that B-Glyc-KDEL was delivered from the plasma membrane, via endosomes and the cisternae of the Golgi apparatus, to the ER. Moreover, the addition of a sulfation site allowed us to show that B-Glyc-KDEL on transit to the ER entered the Golgi apparatus through the trans-Golgi network. Transport of B-Glyc-KDEL to the ER was slowed down by nocodazole, indicating that microtubules are important for the retrograde pathway. Our results document the existence of a continuous pathway from the plasma membrane to the endoplasmic reticulum via the Golgi apparatus and show that a fully folded exogenous protein arriving in the endoplasmic reticulum via this pathway can undergo N-glycosylation.

The existence of retrograde transport along the biosynthetic/secretory pathway was first demonstrated for a certain class of luminal resident ER proteins that can leave the ER, reach the Golgi apparatus in which they acquire Golgi-specific carbohydrate modifications, and be subsequently retrieved to the ER (1–5). The retrieval of ER-escaped proteins could occur from locations as distal as the trans-Golgi network (TGN) (6). ER resident membrane proteins can also leave the ER and are retrieved from the Golgi apparatus. This process involves basic residues located at the carboxyl- or amino-terminal ends of the proteins (7, 8).

Golgi resident proteins may also undergo retrograde movement. For instance, the medial Golgi protein MG160 is sialylated, suggesting that it cycles between late Golgi/TGN compartments in which sialylation occurs and medial Golgi (9). Retrograde movement has recently been documented for other yeast and mammalian Golgi proteins, such as cis-Golgi mannosyltransferase Och1p, medial/trans-Golgi N-acetylglucosaminyltransferase I, and Golgi-localized Emp47p (10–12).

Other evidence for the existence of a retrograde pathway comes from work on some bacterial toxins that seem to enter the cytosol of higher eukaryotic cells after reaching ER compartments (13). Particularly well studied examples are Shiga toxin from Shigella dysenteriae and Shiga-like toxins (or vero toxins) from Escherichia coli. These toxins are composed of two polypeptidic chains, one of which (A-fragment) carries a dead-endase activity that inhibits protein biosynthesis by acting on the 28 S rRNA, whereas the other subunit (B-fragment) allows the binding of the toxin to target cells (14). Electron microscopic analysis has shown that Shiga toxin can be detected in the ER of T47D cells, of butyric acid-treated A431 cells, and of Daudi cells (15–18). Shiga toxin does not carry a KDEL motif at its carboxyl terminus, and the mechanism of its retrograde transport is currently unknown.

The aim of this study was to investigate further the retrograde transport pathway. For this purpose, we constructed a recombinant B-fragment that was carboxyl-terminally modified by the addition of an N-glycosylation site and the KDEL peptide (B-Glyc-KDEL). We found that a significant part of this chimeric protein was glycosylated after incubation with HeLa cells, indicating that it can be transported to the ER. The presence of the KDEL motif was not critical for retrograde transport per se, but for retention of B-Glyc-KDEL in the ER. A biochemical and morphological analysis allowed us to show that B-Glyc-KDEL passed in a microtubule-dependent fashion from endosomes via the TGN/Golgi apparatus to the ER.

MATERIALS AND METHODS

Cells—HeLa cells were grown in Dulbecco’s modified Eagle medium containing 4.5 g/liter glucose (Life Technologies, Inc.) supplemented with 10% fetal calf serum (Life Technologies, Inc.), 0.01% penicillin/streptomycin, 4 mM glutamine, and 5 mM pyruvate in a 5% CO2 incubator. Mouse hybridoma cells expressing the monoclonal anti-VT1 antibody 13C4 were purchased from American Type Culture Collection (CRL 1794) and kept in culture according to ATCC instructions.

Antibodies—Monoclonal antibody 13C4 was purified from culture medium on protein A-Sepharose (Pharmacia Biotech Inc.). The monoclonal anti-Lamp-2 antibody H4B4 was purchased from Pharmingen (San Diego); the monoclonal anti-transferrin receptor antibody H88-4 and the polyclonal antibodies anti-signal sequence receptor and antigalectosyltransferase were kindly provided by T. Rowbotham (The Salk Institute, San Diego), T. A. Rapoport (Harvard University, Boston), and E. Berger (Institute of Physiology, University of Zurich, Switzerland), respectively.

Plasmids—To construct a plasmid expressing B-Glyc-KDEL and B-Glyc-KDELGL, a two-step polymerase chain reaction-based strategy...
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For immunoprecipitation, cells were washed twice with ice-cold PBS and columns (Pharmacia).

KDELGL). Incorporated label was removed on PD10 gel filtration columns and eluted as before. The resulting proteins, estimated to contain the N-glycosylation site and the KDEL sequence, composed of oligonucleotides Sulfat5 (5′-GAGGAACCT- GAGATGAGAATTCTGACTCAGAATAGCTC-3′) and Sulfat6 (5′-CUTTCTTCATACATCTAGTT- C3′) were hybridized and ligated at 16 °C for 8 h. Adaptor fragments containing the N-glycosylation site and the KDEL sequence, composed of oligonucleotides Sulfat1 (5′-phosphorylated; 5′-GGGCGGCTCTATCTAGTT-ATTTACTCCT-3′) and Sulfat2 (5′-CTCAGAAGTTAAGTTAGGAT- GC-3′), or of Sulfat3 (5′-GAGCTGAAAAGAAGTATTTGAG-3′) and Sulfat4 (5′-phosphorylated; 5′-AATCTTCATCAAGTTATCTTTT- TTAGA-3′) were ligated overnight at 16 °C. The resulting fragment was further purified by preparative anion exchanger resin and EcoRI restriction fragments containing the cDNA coding for B-Glyc-KDEL (see above). Oligonucleotide-derived sequences were verified by dideoxy sequencing (Pharmacia).

Purification of Recombinant B-fragments—Purification of recombinant B-fragments was essentially done as described (23). After preparation of periplasmic extracts, these were loaded on a QFF column (Pharmacia) and eluted as before. The resulting proteins, estimated to be 95% pure by SDS-polyacrylamide gel electrophoresis, were stored at −80 °C until use.

B-Glyc-Sulf—For iodination, 25 μg (for B-Glyc-KDEL and B-Glyc-KDELGL) or 100 μg (for B-fragment) of purified protein in elution buffer was treated with 200 μCi (B-fragment) or 500 μCi (B-Glyc-KDEL and B-Glyc-KDELGL) iodine (16.9 μCi/μg, Amersham Corp.) on a single IODO-BEAD (Pierce) according to the manufacturer’s instructions. Proteins were labeled to specific activities of about 500 cpm/μg (B-fragment) or 5,000 cpm/μg (B-Glyc-KDEL and B-Glyc-KDELGL). Incorporation of DTAF was performed in the presence of 10 μg/ml tunicamycin (added 1 h before the B-fragments) or 1 mM DMM (Boehringer Mannheim).

DTA fluorophore and Confocal Microscopy—60 μg of recombinant B-fragments in 20 mM HEPES, pH 7.4, 150 mM NaCl, were added to 250 mM NaHCO3 and a 10-fold molar excess of DTAF (Sigma) and incubated by end-over-end rotation for 30 min at room temperature. 0.2 mM N-acetylglucosamine was added, and coupled protein was purified on PD10 columns.

0.7 × 10⁷ HeLa cells grown on 12-mm round glass coverslips, were incubated with 1 μg/ml DTAF-labeled recombinant B-fragments as described above. After the indicated internalization periods, cells were fixed with 3% paraformaldehyde for 10 min, permeabilized with saponin, stained with the indicated primary and secondary antibodies, and mounted, as described previously (26).

Confocal laser scanning microscopy and immunofluorescence analysis were performed using a TCS4D confocal microscope based on a DM 6000 microscope (Leica, Wetzlar, Germany) with an 100× oil immersion objective (NA 1.4). Stained cells were examined using a 63×0.75 numerical aperture oil immersion objective. Double fluorescence acquisitions were performed using the 488 nm and the 568 nm laser lines to excite fluorescein isothiocyanate and Texas Red dyes using a 63 × oil immersion Neofluor objective (NA = 1.4). The fluorescence was selected with appropriate double fluorescence dichroic mirror and band pass filters and measured with blue-green-sensitive and red side-sensitive one photomultipliers.

Immunoelectron Microscopy—B-Glyc-KDEL binding was performed on HeLa cells grown on 50-cm² round tissue culture plates (1 × 10⁷ cells), as described above. Cells were subsequently incubated at 37 °C as indicated, fixed in the culture dishes by adding to the medium an equivalent volume of 4% paraformaldehyde in 0.2 M sodium phosphate buffer, pH 7.4 for 1 h, and then further fixed with a fresh 2% paraformaldehyde solution in 0.1 sodium phosphate buffer for 1 more hour. Cells were collected by careful scraping and processed for cryosectioning according to Kleijmeer et al. (27). The cryosections were retrieved with a 1/1 solution of 2.3 M sucrose and 2% methyl cellulose according to Liou et al. (28).

Immunogold labeling with the monoclonal antibody 13C4 (diluted 1/250) was performed with a rabbit anti-mouse linker antibody (Dako) and protein-A gold conjugate (purchased from Dr. J. W. Slot, Utrecht University). Double labeling experiments were performed as described previously (29) using the rabbit polyclonal anti-galectosyltransferase antibody (O14 affinity-purified serum, diluted 1/100) as first primary antibody and 13C4 as the second one, diluted 1/250.

RESULTS

Shiga Toxin B-fragment with an N-glycosylation site and the KDEL motif is transported to the ER in HeLa cells—Bacterial Shiga toxin is composed of two polypeptide chains, termed A-fragment and B-fragment (14). Previous studies have shown that the whole toxin as well as isolated B-fragment are transported from the plasma membrane to the ER of toxin sensitive cells (15–18). To quantify this transport by monitoring the appearance of Shiga toxin B-fragment in the ER, an N-glycosylation site was added to the carboxyl terminus of the protein (Fig. 1A). The carboxyl terminus of B-fragment was chosen since it sticks out of the globular conformation of the protein (30, 31). N-Glycosylation starts in the ER by the addition of a core oligosaccharyl group from membrane-bounded dolichol to an Asn-Xaa-Ser/Thr acceptor sequence on newly synthesized proteins (32). Further modifications of N-linked oligosaccharides occur only if this basic sugar backbone is present. B-fragment does not carry a KDEL motif (33–35). Therefore, to favor its retention in the ER, the KDEL peptide was appended to the carboxyl terminus of the protein (Fig. 1A). We also constructed a recombinant B-fragment carrying the KDELGL peptide (Fig. 1A), which was previously found to be inactive with respect to KDEL-receptor interaction (6). We will refer in this study to the recombinant B-fragments as B-Glyc-KDEL and B-Glyc-KDELGL (“B” for B-fragment, “Glyc” for periods of time cells were washed three times with PBS and lysed in SDS sample buffer. Samples were run on 10–20% polyacrylamide-SDS gradient gels, analyzed by autoradiography, and quantified with a PhosphorImager (Molecular Dynamics) using the ImageQuant software. In each experiment, the percentage of glycosylated protein was determined.

The treatment with endoglycosidase H and peptide N-glycosidase F was done as described previously (26). In some experiments, internalization of modified B-fragments was performed in the presence of 1 μg/ml tunicamycin (added 1 h before the B-fragments) or 1 mM DMM (Boehringer Mannheim).

For metabolic sulfate labeling 4 × 10⁷ HeLa cells, grown on 12-mm round glass coverslips, were incubated with 1 μg/ml DTAF-labeled recombinant B-fragments as described above. After the indicated internalization periods, cells were fixed with 3% paraformaldehyde for 10 min, permeabilized with saponin, stained with the indicated primary and secondary antibodies, and mounted, as described previously (26).

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It has been shown previously that Shiga toxin binds to the glycolipid Gb₃, which is expressed by toxin-sensitive cells (36) such as HeLa cells. To test whether the carboxy-terminal modifications made on the B-fragment influenced its interaction with cells, we first compared binding to and internalization into HeLa cells of B-Glyc-KDEL and B-Glyc-KDELGL with that of the wild type B-fragment. Recombinant B-fragments were purified from E. coli and iodinated in vitro (see “Materials and Methods”). Unlabeled wild type B-fragment and B-Glyc-KDEL competed equally well with radiolabeled B-Glyc-KDEL for binding to HeLa cells (Fig. 2). Half-maximal competition was observed at 300 nM cold competitor protein. Scatchard analysis showed that the binding constants of wild type B-fragment, B-Glyc-KDEL, and of B-Glyc-KDELGL were comparable (Table I). We calculated that 1–2 × 10⁶ binding sites were present per HeLa cell (Table I), in good agreement with the literature (37). All three proteins were also found to be equally well internalized into HeLa cells (not shown). Altogether, the results indicated that the modifications of the carboxy terminus of B-fragment did not significantly change its binding and uptake properties.

Iodinated B-Glyc-KDEL or B-Glyc-KDELGL were first bound for 45 min to HeLa cells on ice. After binding, the cells were incubated for various lengths of time at 37 °C (Fig. 3A). At the end of each incubation period, cells were lysed, and proteins were separated by SDS-polyacrylamide gel electrophoresis. At time 0 (45 min of binding on ice), single fragments migrating with an apparent molecular mass of 9.5 kDa and corresponding to the expected size of B-Glyc-KDEL and B-Glyc-KDELGL were observed (Fig. 3A). B-Glyc-KDEL migrated slightly faster than B-Glyc-KDELGL (Fig. 3A), consistent with the fact that B-Glyc-KDELGL is two amino acids longer than B-Glyc-KDEL. Internalization of B-Glyc-KDEL led to the appearance of further bands (Fig. 3A, left). After 1, 2, or 4 h, one species (Fig. 3A, triangle) and after 15 h, two species (Fig. 3A, triangle and asterisk) with a lower electrophoretic mobility than the original B-Glyc-KDEL were observed. As shown in Fig. 3B, their appearance was strongly inhibited by treatment of cells with tunicamycin, an inhibitor of N-glycosylation. In addition, these bands were lost after treatment of samples with endoglycosidase H or peptide N-glycosidase F, two N-glycosylation-specific glycosidases (Fig. 3B). These observations suggest that the upper bands represent N-glycosylated B-Glyc-KDEL. The fact that they remained endoglycosidase H-sensitive indicated that B-Glyc-KDEL reached the ER and then stayed associated with it. The appearance of the second species of N-glycosylated B-Glyc-KDEL (Fig. 3A, asterisk) was inhibited by DMM (Fig. 3C), an inhibitor of mannosidase I, indicating that this species resulted from the trimming of the primary glycosylation product of B-Glyc-KDEL (Fig. 3A, triangle) by cis-Golgi-specific mannosidase activity. B-Glyc-KDEL thus cycles between ER and cis-Golgi compartments, as described for other resident ER proteins (1, 5). The same results on glycosylation of B-Glyc-KDEL were obtained in A431 cells and Vero cells (not shown).

The incubation of HeLa cells with B-Glyc-KDELGL gave a different pattern. Multiple bands with a lower electrophoretic mobility than unmodified B-Glyc-KDELGL were observed, giving the appearance of a smear (Fig. 3A, open bracket). Some of them were found to be endoglycosidase H-resistant (not shown). In addition, the appearance of the upper bands was inhibited by DMM (Fig. 3C). These observations suggest that B-Glyc-KDELGL was also transported to the ER in HeLa cells where it was glycosylated. In contrast to B-Glyc-KDEL, B-Glyc-KDELGL seemed to be able to move in the anterograde direction up to cis-/medial Golgi and acquire complex carbohydrates (endoglycosidase H resistance). The unequal behavior of B-Glyc-KDEL and B-Glyc-KDELGL was also observed in morphological studies (see below).

In addition to slower migrating glycosylation products, faster migrating bands were observed after incubation of B-Glyc-KDEL or B-Glyc-KDELGL with cells (Fig. 3A, circles). These proteins were insensitive to tunicamycin, endoglycosidase H, and peptide N-glycosidase F treatment (Fig. 3B) and could represent degradation products. In this respect, it is interesting to note that even after 15 h of incubation in cells, less than 3% of internalized B-Glyc-KDEL and B-Glyc-KDELGL became trichloroacetic acid-soluble (not shown). This indicates that if the lower bands are degradation products, these remain stably associated with cells.

To quantify the arrival of modified B-fragments in the ER, autoradiographs were scanned using a PhosphorImager (Fig.
To allow an accurate quantification of the glycosylated bands, especially for B-Glyc-KDELGL, internalization of modified B-fragments was performed in the presence of DMM. Glycosylation of B-Glyc-KDEL increased linearly with incubation time and reached 22% of the total cell-associated protein after a 15-h incubation (Fig. 3D). Similar results were obtained for cells incubated without DMM (not shown). Interestingly, glycosylation of B-Glyc-KDELGL was only slightly less efficient than that of B-Glyc-KDEL (Fig. 3D). In addition, the kinetics of glycosylation was not markedly modified. This indicates that the functional KDEL motif is important for the retention of the protein within the ER but not for its retrograde transport.

B-Glyc-KDEL and B-Glyc-KDELGL Are Transported to the ER via the Golgi Apparatus—To characterize the intracellular pathway followed by B-Glyc-KDEL or B-Glyc-KDELGL from the plasma membrane to the ER, recombinant proteins were covalently linked to the fluorophore DTAF. After 15 min at 37 °C, the bulk of B-Glyc-KDEL was found associated with the plasma membrane and endosomes, as shown by its colocalization with the transferrin receptor (Fig. 4, A and B). After 45 min, the protein became detectable in the Golgi apparatus...

Fig. 3. B-Glyc-KDEL and B-Glyc-KDELGL are glycosylated after incubation with HeLa cells. Panel A, 50 nM recombinant B-Glyc-KDEL and B-Glyc-KDELGL were incubated with HeLa cells for 45 min on ice (time 0). After washing, cells were incubated at 37 °C for various lengths of time. At the end of each incubation period, cells were solubilized in sample buffer, and lysates were analyzed on 10–20% polyacrylamide-SDS gradient gels, followed by autoradiography. Equal quantities of B-Glyc-KDEL or B-Glyc-KDELGL were loaded on gels in each case. A triangle indicates the primary glycosylation product of B-Glyc-KDEL, a star the secondary glycosylation product, and a circle a putative degradation product of B-Glyc-KDEL or B-Glyc-KDELGL. An open bracket marks the positions of the multiple glycosylation products of B-Glyc-KDELGL. Panel B, B-Glyc-KDEL was bound to and internalized into HeLa cells as described in panel A. After 15 h of incubation in the presence or absence of 1 μg/ml tunicamycin, cells were lysed, B-fragment was immunoprecipitated, and the immunoprecipitates were mock treated (CLT) or incubated in the presence of endoglycosidase H or peptide N-glycosidase F. Panel C, cells were incubated with modified B-fragments in the presence or absence of 1 mM DMM for 15 h before being treated as described in panel A. Panel D, the percentage of glycosylated B-Glyc-KDEL (○) and B-Glyc-KDELGL (□) was calculated after various incubation times in the presence of DMM. The means (± S.E.) of three to five independent experiments are shown. Linear regression analysis gave y = 1.6 + 1.4x, r = 0.99 for B-Glyc-KDEL (solid line) and y = 1.2 + 1.0x, r = 0.99 for B-Glyc-KDELGL (broken line).

Fig. 4. Colocalization of B-Glyc-KDEL and B-Glyc-KDELGL with various marker proteins during internalization into HeLa cells. DTAF-labeled B-Glyc-KDEL (panels A, C, and E) or B-Glyc-KDELGL (panel G) was bound to HeLa cells as described in Fig. 3. After 15 min (panels A and B), 45 min (panels C and D), 4 h (panels E and F), or 15 h (panels G and H) at 37 °C, cells were fixed and labeled with antibodies against transferrin receptor (panel B), Rab6 (panel D), and signal sequence receptor (panels F and H). Single representative optical slices obtained by confocal microscopy are shown.
B-Glyc-KDEL retrograde transport was analyzed further on cryosections by immunoelectron microscopy. After 30 min at 37 °C, the protein was detected in coated pits and vesicles (Fig. 6A, see insets), in structures resembling the TGN, and in cis-ternae of the Golgi apparatus (Fig. 6A). At this time, some B-Glyc-KDEL was already detectable in the ER (not shown). The presence of B-Glyc-KDEL in coated pits and vesicles suggests that the protein entered the cells through the clathrin-dependent pathway, as documented previously for Shiga toxin (41, 42). After 2 h at 37 °C, the ER became heavily labeled (Fig. 6B). To establish further the presence of B-Glyc-KDEL within the Golgi apparatus, thin cryosections were double labeled for B-Glyc-KDEL and galactosyltransferase, an enzyme resident of the late Golgi/TGN compartments (43). Both proteins were detected on the same membrane profiles in Golgi stacks and in the TGN region (Fig. 6C, arrows). Since B-Glyc-KDEL behaves as a resident protein of the ER, once having arrived in this compartment (see above), the presence of the protein in the cisternae of the Golgi apparatus suggests that at least a fraction of internalized B-Glyc-KDEL traverses the Golgi apparatus before reaching the ER.

**B-Glyc-KDEL with a Sulfation Site Is First Sulfated in the TGN and Then Glycosylated in the ER**—To analyze in further detail the intracellular pathway followed by B-Glyc-KDEL, a sulfation site was introduced between the N-glycosylation site and the KDEL peptide (B-Glyc-Sulf-KDEL). After 2-h incubation at 37 °C, the ER became heavily labeled (Fig. 7, lane 1). This band became visible after 30 min (not shown). Further incubation (10 h) led to the appearance of an additional band of lower electrophoretic mobility, corresponding to the glycosylation product (Fig. 7, lane 2, arrow).

To determine whether the behavior of the sulfated B-Glyc-Sulf-KDEL represents the behavior of the bulk of cell-associated B-fragment, we estimated in the same experiment the fraction of sulfated and iodinated B-Glyc-Sulf-KDEL (representing the pool of cell-associated protein) which became glycosylated. After a 10-h incubation, 12 and 17% of sulfated B-Glyc-Sulf-KDEL and then metabolically labeled with [35S]sulfate. After a 2-h incubation at 37 °C, a species corresponding to sulfated B-Glyc-Sulf-KDEL was detected (Fig. 7, lane 1). This band became visible after 30 min (not shown). Further incubation (10 h) led to the appearance of an additional band of lower electrophoretic mobility, corresponding to the glycosylation product (Fig. 7, lane 2, arrow).

In agreement with the literature, we noticed that the intensity of B-Glyc-KDEL labeling showed some heterogeneity from cell to cell in the same population (17, 18). Next to cells that were brightly labeled (Fig. 5A, large arrow) we also detected cells that had internalized few B-Glyc-KDEL (Fig. 5A, small arrows). However, we estimated that about 70% of the population showed the same labeling intensity.

The appearance of putative degradation products (Fig. 3A, circles) led us to test for B-Glyc-KDEL transport to lysosomal compartments. We compared after 30 min (not shown) or 60 min at 37 °C the localization of B-Glyc-KDEL with that of the lysosomal-associated protein Lamp-2. As shown in Fig. 5, C and D, B-Glyc-KDEL was largely excluded from Lamp-2-positive structures. To exclude the possibility that the fluorophore DTAF was inactivated upon entry into lysosomes, similar experiments were performed with internalized B-Glyc-KDEL, which was detected with a specific monoclonal antibody, and again no colocalization with Lamp-2 was observed (not shown).

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To determine whether the behavior of the sulfated B-Glyc-Sulf-KDEL represents the behavior of the bulk of cell-associated B-fragment, we estimated in the same experiment the fraction of sulfated and iodinated B-Glyc-Sulf-KDEL (representing the pool of cell-associated protein) which became glycosylated. After a 10-h incubation, 12 and 17% of sulfated B-Glyc-Sulf-KDEL (Fig. 7, lane 2) and iodinated B-Glyc-Sulf-KDEL (Fig. 7, lane 4) were found to be glycosylated, respectively. This indicates that about two-thirds of B-Glyc-Sulf-KDEL passes through the TGN before reaching the ER. Without ruling out the possibility that a fraction of B-Glyc-Sulf-KDEL is transported to the ER without passing through the TGN, the difference in glycosylation efficiency between iodinated and sulfated B-Glyc-Sulf-KDEL could be explained by the fact that not all B-Glyc-Sulf-KDEL in transit through the TGN is sulfated. Alternatively, sulfated B-Glyc-Sulf-KDEL might be a poorer substrate for oligosaccharyltransferase activity than iodinated B-Glyc-Sulf-KDEL.

**Retrograde Transport of B-Glyc-KDEL Is Slowed Down by Nocodazole**—We further characterized the intracellular transport of B-Glyc-KDEL by pharmacological means using nocodazole, which inhibits the polymerization of microtubules. Recent studies suggest that microtubules play a role in retrograde transport between the Golgi apparatus and the ER (3, 20, 45). To test the involvement of microtubules in retrograde transport of B-Glyc-KDEL, internalization of the protein into HeLa cells and intracellular transport to the ER were performed in the presence or absence of nocodazole. Immunofluorescence studies revealed that B-Glyc-KDEL was able to reach the dispersed cisternae of the Golgi apparatus in nocodazole-treated cells where it colocalized with Rab6, a GTPase associated with Golgi and TGN membranes (38, 39) (Fig. 4, C and D). After 4 h at 37 °C, B-Glyc-KDEL displayed a typical ER staining and colocalized with the signal sequence receptor protein (Fig. 4, E and F).

In contrast to B-Glyc-KDEL, B-Glyc-KDELGL was mainly localized in the Golgi region after incubation of cells for 1 h, 4 h (not shown), or 15 h at 37 °C (Fig. 4, G and H). The same result was obtained for DTAF-labeled wild type B-fragment (not shown), which is in good agreement with previously published experiments on B-fragment transport to the Golgi apparatus (40). However, a fraction of B-Glyc-KDELGL was present in the ER, as documented by colocalization with signal sequence receptor protein (Fig. 4, G and H). These data are consistent with the hypothesis that B-Glyc-KDELGL is transported to the ER and that the protein can then move in the anterograde direction up to the Golgi apparatus (see above; Fig. 3A).

In agreement with the literature, we noticed that the intensity of B-Glyc-KDEL labeling showed some heterogeneity from cell to cell in the same population (17, 18). Next to cells that were brightly labeled (Fig. 5A, large arrow) we also detected cells that had internalized few B-Glyc-KDEL (Fig. 5A, small arrows). However, we estimated that about 70% of the population showed the same labeling intensity.

The appearance of putative degradation products (Fig. 3A, circles) led us to test for B-Glyc-KDEL transport to lysosomal compartments. We compared after 30 min (not shown) or 60 min at 37 °C the localization of B-Glyc-KDEL with that of the lysosomal-associated protein Lamp-2. As shown in Fig. 5, C and D, B-Glyc-KDEL was largely excluded from Lamp-2-positive structures. To exclude the possibility that the fluorophore DTAF was inactivated upon entry into lysosomes, similar experiments were performed with internalized B-Glyc-KDEL, which was detected with a specific monoclonal antibody, and again no colocalization with Lamp-2 was observed (not shown).
However, glycosylation of B-Glyc-KDEL was inhibited in the presence of nocodazole. After a 2-h incubation at 37 °C in the presence of the drug, the glycosylation level reached 56% (± 2.8%; n = 3) of that observed under control conditions and 73.5% (± 0.7%; n = 3) after 4 h (Fig. 8E). Interestingly, if nocodazole was added after only 1 h of incubation at 37 °C, and incubation was continued for another 3 h in the continued presence of the drug, glycosylation was not significantly affected (Fig. 8E). This observation suggests that a large fraction of B-Glyc-KDEL has passed nocodazole-sensitive transport steps after 1 h. Since immunofluorescence studies suggest that a large fraction of B-Glyc-KDEL has left the Golgi apparatus by this time (Fig. 8, C and D), it is likely that nocodazole inhibits either intra-Golgi or Golgi-to-ER transport of B-Glyc-KDEL.

DISCUSSION

In the present study Shiga toxin B-fragment was carboxyl-terminally modified by the addition of an N-glycosylation site and the KDEL peptide (protein termed B-Glyc-KDEL). B-Glyc-KDEL arrival in the ER of HeLa cells was monitored in a quantitative way through its modification by N-glycosylation. Biochemical and morphological experiments indicated that B-Glyc-KDEL became detectable in the ER as early as 30–60 min after internalization. This figure is consistent with previous studies on Shiga toxin which showed the appearance of the toxin in the ER after an equivalent incubation period (17, 18), and with recent studies on cholera toxin A-subunit which was detected in the ER 30–90 min after internalization (20).

On the other hand, even 2 h after incubation at 37 °C some B-Glyc-KDEL was still detectable, on cryosections, in the Golgi apparatus and the TGN. It seems that a fraction of B-Glyc-KDEL is transported rapidly to the ER, whereas the remainder follows during further incubation. It has been proposed that sorting in the retrograde pathway is a process whose efficiency is increased by repetitive application of sorting information in succeeding compartments (46). Such a mechanism could explain our results.

It has been shown previously that a peptide carrying an N-glycosylation site and the KDEL sequence can be transported from the TGN to the ER where it is glycosylated (6). We show here that a fully folded protein arriving by the retrograde transport route can equally undergo glycosylation. However, glycosylation of B-Glyc-KDEL was found to be a relatively inefficient process that increased progressively with incubation time. N-Glycosylation normally occurs cotranslationally, and nascent proteins are presented to oligosaccharyltransferase activity while they are still in an unfolded state (32, 47). Fully folded B-Glyc-KDEL may be a relatively poor substrate with respect to these criteria. In addition, retrograde transport may target B-Glyc-KDEL to subcompartments of the ER which do not contain oligosaccharyltransferase activity. Diffusion of B-fragment in the ER might then be slowed by interaction with...
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and Dapparatus (KDEL remains associated with the dispersed cisternae of the Golgi and the Golgi apparatus and only thereafter in the ER. Our that the bulk of B-Glyc-KDEL was first detected in endosomes the secretory pathway (48). By confocal microscopy we found here the plasma membrane to the ER, before regaining access to the based on studies on caveolin which might transfer directly from pits and detected in endosomes, the Golgi apparatus, and the ER (17, 18, 41, 42). It was thus assumed that Shiga toxin passes to the ER via the Golgi apparatus. However, the exist-
ence of the drug (column 1) or presence (panels A and B) of 10 μM nocodazole. Cells were then labeled with anti-Rab6 antibody (panels B and D). In control cells (panels C and D), a part of B-Glyc-KDEL appears to have already entered the ER. In nocodazole-treated cells (panels A and B), B-Glyc-KDEL remains associated with the dispersed cisternae of the Golgi apparatus (arrows). Panel E, HeLa cells were incubated with 50 μM B-Glyc-KDEL as described in Fig. 3. Internalization was allowed to proceed for 2 h (column 1) or 4 h (column 2) in the presence of 10 μM nocodazole. Alternatively, cells were first incubated for 60 min in the absence of nocodazole followed by a 3-h incubation period in the presence of the drug (column 3). Results are expressed as percentage of glycosylation signal (means ± S.E.; n = 3) in each experimental condition compared with mock-treated control cells.

its receptor Gb3.

Sandvig and co-workers have shown by electron microscopic studies that Shiga toxin can be internalized via clathrin-coated pits and detected in endosomes, the Golgi apparatus, and the ER (17, 18, 41, 42). It was thus assumed that Shiga toxin passes to the ER via the Golgi apparatus. However, the existence of an alternative transport pathway has been suggested based on studies on caveolin which might transfer directly from the plasma membrane to the ER, before regaining access to the secretory pathway (48). By confocal microscopy we found here that the bulk of B-Glyc-KDEL was first detected in endosomes and the Golgi apparatus and only thereafter in the ER. Our immunoelectron microscopy studies also showed B-Glyc-KDEL in the Golgi apparatus on transit to the ER. These data thus suggest the existence of a continuous retrograde transport route leading from the plasma membrane via endosomes, the TGN, and the Golgi apparatus to the ER. In addition, B-Glyc-KDEL, with a sulfation site was first sulfated in the TGN and only subsequently glycosylated, an observation that is also consistent with transport via the TGN to the ER.

Using Shiga toxin coupled to horseradish peroxidase, it has been shown that an important fraction of this protein is transported to lysosomes (41, 42). We have observed in our experiments a fragment that may correspond to a degradation product of B-Glyc-KDEL (Fig. 3A, circle). However, no obvious colocalization between B-Glyc-KDEL and the lysosomal marker Lamp-2 was detected. These data are consistent with a recent report on intracellular transport of verotoxin-1 B-fragment (100% similar to Shiga toxin B-fragment) in which no accumulation of B-fragment in lysosomal structures has been detected (40). Interestingly, we found that BFA, which was shown to have only limited effects on transport to lysosomes (49–52), not only completely abolished glycosylation of B-Glyc-KDEL, but also strongly inhibited the appearance of the putative degradation product of B-Glyc-KDEL. In addition, a very low amount of B-Glyc-KDEL-associated radioactivity became trichloroacetic acid-soluble after 15 h of incubation with cells. Further experiments are under way to determine at what point B-Glyc-KDEL degradation may occur.

An interesting result of this study is that B-fragment bearing a nonfunctional KDEL sequence was glycosylated with about the same kinetics as KDEL-bearing B-fragment. At steady state, B-Glyc-KDELGL localized to the Golgi apparatus, as does wild type B-fragment (40). This indicates that the KDEL motif is not important per se for retrograde transport of B-Glyc-KDEL, but for retention of the protein in the ER. The mechanism of Shiga toxin retrograde transport is not yet understood, but one might hypothesize the existence of a yet unknown retrograde transport signal in the sequence of the B-fragment. Alternatively, its retrograde transport may be achieved in association with another protein.

Finally, our study shows that it is possible to quantify B-Glyc-KDEL transport from the plasma membrane to the TGN (by sulfation) and to the ER (by glycosylation). B-Glyc-KDEL thus has the potential to become a model molecule for the dissection of the plasma membrane to TGN and Golgi to ER transport routes.

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Note Added in Proof—Using a similar strategy to that described in this paper, it was recently shown that sequence modified ricin is also first sulfated in the TGN and then glycosylated in the ER (Rapak, A., Falnes, P. O., and Olnes, S. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 3783–3788).

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