Ricin acts by translocating to the cytosol the enzymatically active toxin A-chain, which inactivates ribosomes. Retrograde intracellular transport and translocation of ricin was studied under conditions that alter the sensitivity of cells to the toxin. For this purpose tyrosine sulfation of mutant A-chain in the Golgi apparatus, glycosylation in the endoplasmic reticulum (ER) and appearance of A-chain in the cytosolic fraction was monitored. Introduction of an ER retrieval signal, a C-terminal KDEL sequence, into the A-chain increased the toxicity and resulted in more efficient glycosylation, indicating enhanced transport from Golgi to ER. Calcium depletion inhibited neither sulfation nor glycosylation but inhibited translocation and toxicity, suggesting that the toxin is translocated to the cytosol by the pathway used by misfolded proteins that are targeted to the proteasomes for degradation. Slightly acidified medium had a similar effect. The proteasome inhibitor, lactacystin, sensitized cells to ricin and increased the amount of ricin A-chain in the cytosol. Anti-Sec61α precipitated sulfated and glycosylated ricin A-chain, suggesting that retrograde toxin translocation involves Sec61p. The data indicate that retrograde translocation across the ER membrane is required for intoxication.

Ricin is a plant toxin that kills eucaryotic cells by inactivating the ribosomes and blocking protein synthesis (1, 2). The toxin is a heterodimer consisting of an A-chain and a B-chain that are linked by a disulfide bond (3, 4). The B-chain has lectin properties and binds to surface structures containing terminal galactose (5). The A-chain is an enzyme that acts on ribosomes by removing an adenine residue from an exposed loop in the 28 S RNA of the large ribosomal subunit without breaking the phosphoribose backbone of the RNA (6, 7). The loop is involved in binding of elongation factors (8, 9), and after toxin treatment, the elongation factors bind poorly, and protein synthesis stops.

Upon binding to the cell surface, the toxin is taken up by endocytosis (10) and a minor fraction (~5%) is transported to the Golgi apparatus (11), and from there it is further transported retrogradely to the endoplasmic reticulum (ER),1 where the A-chain appears to be translocated to the cytosol (12). On the other hand, evidence for translocation from endosomes has also been reported (13, 14).

In attempts to elucidate if translocation from the ER is the main entry route, we have applied a number of conditions that increase or reduce the toxin sensitivity of cells and studied intracellular trafficking and membrane translocation of the toxin. It is an old observation that cells depleted for calcium or kept at moderately acidic conditions are partly protected against ricin (15, 16). On the other hand, treatment with low concentrations of monensin increases the sensitivity of cells to ricin, whereas higher concentrations provide a partial protection (16). In neither case could the changed sensitivity be accounted for by altered rates of endocytosis or altered enzymatic action on ribosomes, and we have therefore here considered the possibility that it could be due to interference with the transport of the toxin from endosomes to the ER or to interference with translocation of the A-chain from ER to cytosol. A mutant ricin A-chain containing a C-terminal KDEL sequence was shown to be more toxic than wild-type ricin (17, 18), and it was proposed that this was due to increased transport to the ER.

In the present work we have taken advantage of a recently developed system where we fused tyrosine sulfation and N-glycosylation signals onto the C-terminal end of ricin A-chain (12). Tyrosine sulfation takes place in the Golgi apparatus and in the trans-Golgi network (19, 20). Therefore, when ricin containing the sulfation signal is added to cells growing in medium containing $^{35}$SO$_4$²⁻, that minority of toxin molecules that is transported to the Golgi apparatus becomes labeled selectively. Furthermore, the addition of glycosylation sites onto the toxin A-chain allows us to follow the labeled toxin to the ER (12). When the toxin reaches this organelle, the A-chain becomes glycosylated, which can easily be monitored by a decreased migration rate in SDS-PAGE.

We here provide evidence that monensin interferes with transport of ricin from the cell surface to the Golgi apparatus, whereas depletion of calcium and reduced pH do not but instead, inhibit translocation of the A-chain across the ER membrane. KDEL-tagged ricin is more efficiently transported from the Golgi apparatus to the ER. Finally, we present evidence that ricin utilizes the ER translocon for retrograde translocation to the cytosol.

**EXPERIMENTAL PROCEDURES**

Reagents and Buffers — Na$_2$$^{35}$SO$_4$ was from Amersham Pharmacia Biotech. Hepes medium: bicarbonate-free Eagle’s minimum essential medium buffered with 20 mM Hepes to pH 7.4. PBS: 140 mM NaCl, 10 mM Na$_2$HPO$_4$, pH 7.2. Lysis buffer: PBS, pH 7.2, containing 1 mM EDTA, 1% Triton X-100, 200 units/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride. Rabbit anti-ricin was obtained by standard immunization. Rabbit antibodies against the C terminus of Sec61p was a gift from Olsnes@radium.uio.no.

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5. The abbreviations used are: ER, endoplasmic reticulum; SLO, streptolysin O; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline.
RESULTS

Toxicity of Modified Ricin—Ricin A-chain was modified to contain a C-terminal tyrosine sulfation signal without ricin-A-sulf1) and with (ricin A-sulf2) three partly overlapping N-glycosylation sites. In one case (ricin A-sulf2-KDEL) a C-terminal KDEL sequence was added as well (Fig. 1A). The constructs were expressed as fusion proteins with malto-binding protein to allow their purification on an amylose column. Subsequently, the maltose-binding protein was cleaved off with factor Xa, a site that had been inserted between the two proteins.

The modified A-chains were reconstituted with ricin B-chain to form holotoxins. In the following the reconstituted toxins will be referred to as wild-type ricin, ricin-sulf1, ricin-sulf2, and ricin-sulf2-KDEL. When increasing concentrations of the reconstituted toxins were added to Vero and U2OS cells and the ability of the cells to incorporate \(^{3}H\)leucine into trichloroacetic acid-precipitable material was measured after 4 h, there was in all cases a dose-dependent reduction in incorporated radioactivity that did not differ much from that obtained with wild-type ricin (Fig. 1, A and B). Ricin-sulf2-KDEL was somewhat more toxic than the parent ricin-sulf2, particularly in U2OS cells. Incubation of cells for shorter times (1 or 2 h) gave similar results (data not shown). This demonstrates that the alterations introduced into ricin A-chain did not strongly interfere with the toxic effect.

Sulfation and Glycosylation of Modified Ricin—When ricin-sulf-2 was added to cells in the presence of \(\text{Na}_2\text{SO}_4\), two labeled bands were observed (Fig. 2A, lane 1) in accordance with earlier data (12). The lower band represents the glycosylated form of ricin-A-sulf2, and the upper band represents the glycosylated form (gA). When a C-terminal KDEL sequence was added (ricin-sulf2-KDEL), the amount of glycosylated protein was considerably reduced (lane 2), suggesting that the KDEL sequence increased transport of the protein to the ER.

The presence of brefeldin A, which disrupts the Golgi apparatus, completely prevented sulfation of ricin-A-sulf2-KDEL (Fig. 2B, lane 2) as we have earlier found with ricin-A-sulf-2 (12). Tunicamycin prevented the appearance of the heavier band (lane 3), confirming that this band represents the glycosylated form of ricin-A-sulf2-KDEL.
FIG. 2. Sulfation and glycosylation of mutant ricin A-chain in U2OS cells in the absence and presence of inhibitors. A, U2OS cells were preincubated with Na\(^{35}\)SO\(_4\) (100 \(\mu\)Ci/ml) in DMEM without sulfate for 4 h. Then 200 ng/ml reconstituted ricin-sulf2 or ricin-sulf2-KDEL were added, and the cells were incubated for 4 h more. Subsequently, the cells were washed twice with PBS and lysed, and the clarified lysate was treated with immobilized anti-ricin antibodies. The adsorbed material was analyzed by SDS-PAGE under reducing conditions followed by fluorography. B, U2OS cells were preincubated with Na\(^{35}\)SO\(_4\) for 4 h with and without 2 \(\mu\)g/ml brefeldin A or with 1 \(\mu\)M tunicamycin for the last 30 min. Then ricin-sulf2-KDEL was added, and the cells were incubated for 4 h more and subsequently treated as above.

Effect of Monensin on Ricin Toxicity—When low concentrations of monensin (0.01–0.1 \(\mu\)M) were added to the culture medium, the cells were approximately 3-fold more sensitive to ricin than in the absence of the compound (Fig. 3, A and B). In the presence of higher concentrations (1–10 \(\mu\)M monensin), the sensitivity to the toxin was somewhat reduced. This is in accordance with earlier observations (16).

Monensin affects the structure of the Golgi apparatus, which swells up at higher concentrations (23). To study if the altered sensitivity was due to altered transport to or altered function of the Golgi apparatus, we measured the ability of ricin-sulf2 to be sulfated and glycosylated in the presence of monensin. The results in Fig. 3, C and D, demonstrate that at the lower concentrations of monensin there was a moderate increase in labeling of the toxin with sulfate, whereas at the higher concentrations, the labeling was considerably reduced. This indicates that low concentrations of monensin increase the transport of toxin to the Golgi apparatus, whereas higher concentrations either inhibit the transport or reduce the labeling due to swelling of the Golgi apparatus. On the other hand, the relative fraction of labeled toxin that became glycosylated was not affected, as the relation between the two labeled bands was approximately the same at the different concentrations of monensin (Fig. 3, E and F). The data therefore indicate that monensin interferes either with the transport of toxin to the Golgi apparatus or with the sulfate labeling but not with the further retrograde transport to the ER.

The electrophoresis was carried out under nonreducing conditions, and in Fig. 3C the gel was run to also visualize the separated chains to check to what extent cell-induced reduction of the toxin A- and B-chains had taken place. Such cell-mediated reduction could be a requirement for translocation to the cytosol. The data demonstrate that monensin did not strongly influence the relative amount of toxin that was reduced by the cells.

Altogether, there is good correlation between the toxic effect on cells and labeling of the toxin and subsequent glycosylation. The findings are therefore consistent with the possibility that transport through the Golgi apparatus to the ER is necessary for intoxication.

Ability of Low pH of the Medium to Protect Cells against Ricin—When the cell culture medium is slightly acidified under conditions that do not inhibit endocytosis from coated pits (24), the cells are partly protected against ricin (16). The data in Fig. 4, A and B, demonstrate that this is also the case with ricin-A-sulf2 and ricin-sulf2-KDEL.

Experiments using radioactive sulfate showed that there was no reduction in the labeling and glycosylation of the protein at low pH (Fig. 4C). In fact, in some cases there was rather an increased labeling of the toxin. In Vero cells the KDEL construct was less labeled than the parent ricin-sulf2, but there was no difference between the two pH values with respect to sulfate labeling and glycosylation. It therefore appears that incubation at slightly acidic pH provides protection against ricin by a mechanism occurring after the toxin has reached the ER.

To test if the altered sensitivity to ricin in cells incubated at pH 6.5 was due to interference with the translocation of the A-chain to the cytosol, we treated the cells with SLO to selectively permeabilize the surface membrane (12, 25, 26) and release soluble proteins from the cytosol into the buffer. Due to technical problems in carrying out SLO experiments with...
Fig. 4. Ability of acidic medium to protect against ricin intoxication. A and B, Heps medium adjusted to pH 6.5 or 7.5 was added to Vero (A) and U20S cells (B). Subsequently, increasing amounts of ricin mutants were added, and the cells were incubated for 4 h. Protein synthesis was then measured during the next 20 min as incorporation of [3H]leucine. C, U20S and Vero cells were preincubated with Na$_2$-sylated A-chain. As demonstrated in Fig. 5, cells were incubated with Na$_2$-sylated A-chain. D, the pH of the medium was adjusted to 6.5 or 7.5. Ricin mutants as indicated were added, and the cells were incubated for 4 h more. Finally, the cells were lysed, and the immunoprecipitated ricin was analyzed by SDS-PAGE and fluorography.

U20S cells, the experiments were performed only with Vero cells. After centrifugation, we then analyzed the buffer and the cellular pellet for labeled material. In a previous paper it was shown that mainly the glycosylated form of ricin-A-sulf2 was found in the cytosol after SLO treatment (12). In accordance with this, the data in Fig. 4C demonstrate that mainly glycosylated ricin-A-sulf2 was recovered from the cytosol of cells kept at pH 7.5. In cells incubated at pH 6.5 very little material was found in the cytosol, indicating that the translocation from the ER lumen to the cytosol was inhibited. A brief exposure to medium with neutral pH was, however, sufficient to allow the A-chain to appear in the cytosolic fraction (data not shown).

Effect of Ca$^{2+}$-Depletion of Cells on Translocation of Mutant Ricin A-chain—When cells were briefly treated with EGTA and then incubated in Ca$^{2+}$-free medium, they were found to be less sensitive to wild-type ricin than cells kept in normal medium (15). As demonstrated in Fig. 5, A and B, this is also the case with ricin-sulf2 and ricin-sulf2-KDEL. When cells were incubated with mutated ricin in the presence of $^{35}$SO$_4^{2-}$, two labeled bands were obtained corresponding to glycosylated and nonglycosylated A-chain both with and without Ca$^{2+}$ depletion (Fig. 5C). This indicates that depletion of Ca$^{2+}$ does not inhibit transport of toxin to the ER.

Translocation of misfolded proteins to the cytosol for degragation requires normal Ca$^{2+}$ concentration in the ER (27). In cells depleted for Ca$^{2+}$, the concentration of Ca$^{2+}$ in the ER is reduced (28). To investigate if the depletion of Ca$^{2+}$ inhibits the translocation to the cytosol of ricin, we permeabilized the cells with SLO and analyzed the cytosol. In the Ca$^{2+}$-depleted cells, the translocation from the ER to the cytosol was strongly inhibited (Fig. 5D). Also in this case a short exposure of the cells to normal medium containing Ca$^{2+}$ was sufficient to let the A-chain appear in the cytosolic fraction (data not shown).

Sensitization of Cells to Ricin by the Proteasome Inhibitor Lactacystin—It has been reported that misfolded proteins that...
are translocated retrograde from the ER to the cytosol become ubiquitinated by a membrane-bound enzyme and thereby targeted for degradation by proteasomes in the cytosol (29–31).

To study if ricin A-chain is partially degraded by proteasomes, we tested if an inhibitor of proteasomes, lactacystin, is able to sensitize cells to ricin. The data in Fig. 6, A and B, demonstrate that in the presence of lactacystin, the cells were approximately 3-fold more sensitive to ricin than in the absence of the inhibitor.

As shown in Fig. 6C, the amount of sulfate-labeled and glycosylated A-chain in the whole cells was essentially the same with and without lactacystin. On the other hand, the amount of A-chain present in the cytosolic fraction after SLO treatment of the cells was approximately 3-fold higher when the activity of the proteasomes was inhibited by lactacystin (Fig. 6D). The data therefore indicate that although a fraction of ricin A-chain translocated to the cytosol is able to escape degradation, a considerable part is degraded by the proteasomes.

Additive Sensitization of Cells by Lactacystin and Ca\(^{2+}\) —To study if lactacystin and calcium sensitize the cells by acting at the same or different steps in the intoxication process, we tested the sensitivity of cells in the presence of each component as well as their combination. The data in Fig. 7 demonstrate that compared with Ca\(^{2+}\)-depleted cells, calcium and lactacystin sensitized the cells to wild-type ricin to approximately the same extent. The combination of the two conditions sensitized the cells to an extent corresponding to an additive effect of the two conditions. The data therefore indicate that lactacystin and calcium sensitize the cells to ricin by two independent mechanisms.

**Immunoprecipitation of Ricin-A-sulf2 with Anti-Sec61a Antibodies** —Several recent studies have provided evidence that misfolded ER proteins destined for degradation are first transported to the cytosol in a Sec61p-dependent manner before being degraded by proteasomes (32–35). To test if ricin also interacts with the Sec61p translocon, we first incubated cells with \(^{35}\)SO\(_4\)\(^{-}\) and ricin-sulf1 (Fig. 8, panel A) or ricin-sulf2 (Fig. 8, panel B). After a 4-h incubation, we lysed the cells and subjected the lysate to sequential immunoprecipitation. In a first round we used an irrelevant antibody (anti-HSP90 (Fig. 8A) or anti-human immunodeficiency virus-Tat (Fig. 8B)) coupled to protein A-Sepharose beads. Subsequently, we incubated the same lysate with antibodies against Sec61a coupled to protein A-Sepharose. Finally, the lysate was incubated with immobilized anti-ricin antibodies. As shown in Fig. 8, A and B, no ricin was precipitated with the control antibodies, whereas a small amount of sulfated and glycosylated ricin was precipitated with anti-Sec61a. Control experiments demonstrated that the antibodies used did not precipitate \(^{125}\)I-labeled ricin as such (data not shown).

Interestingly, in the material immunoprecipitated with anti-Sec61a in Fig. 8, panel B, the relation between the glycosylated and the nonglycosylated form of ricin A-chain was 1.6 and 1.9, whereas in the immunoprecipitate with anti-ricin antibodies, the relation was only 0.9. Clearly, the glycosylated form of the A-chain is preferentially associated with Sec61p. The data indicate that ricin interacts with the Sec61p complex in the ER. When compared with the total amount of labeled ricin in the cells, the amount immunoprecipitated with anti-Sec61a was found to be 0.93% in the case of Fig. 8A and 0.58 and 0.63% in the case of Fig. 8B.

When the cells were kept in the absence of Ca\(^{2+}\), where
translocation to the cytosol is inhibited, we were not able to immunoprecipitate more ricin A-chain with anti-Sec61a than from cells kept under normal conditions (data not shown). This indicates that in calcium-depleted cells the inhibition of translocation is at a step before the A-chain interacts with Sec61p.

**DISCUSSION**

Previously we provided direct evidence that ricin is transported retrograde through the Golgi apparatus to the ER and subsequently translocated to the cytosol (12), but we did not exclude the possibility that there could be other translocation routes that could be more efficient. It has been reported that ricin can be translocated from endosomes (13, 14) and that transport through the Golgi apparatus may not be required (36). In the present paper we have investigated in detail the effect on transport and translocation of different conditions that decrease or enhance the sensitivity of cells to the toxin. Under these conditions the toxic effect correlated with transport to the Golgi apparatus and translocation from the ER lumen to the cytosol.

Certain toxins have a C-terminal KDEL or KDEL-like sequence (37, 38), which is known to act as an ER retrieval signal. It has been proposed that the KDEL system could facilitate transport of toxins to the ER (39). Wild-type ricin does not contain a KDEL sequence, but addition of this sequence to the C terminus of the isolated A-chain of ricin increased its toxicity to a varying extent in different cells (18, 40). To check the effect of the KDEL sequence on the transport of ricin to the ER, we added this sequence to the C terminus of ricin-A-sulf2. Ricin-sulf2-KDEL was more efficiently transported to the ER in U20S cells than the parent ricin-sulf2, and it was more toxic. Interestingly, ricin-sulf2-KDEL was labeled very weakly in Vero cells. Possibly, in these cells the mutant leaves the Golgi apparatus quickly such that sulfation is incomplete. Ricin-sulf2-KDEL was more extensively glycosylated in U20S cells at lower pH, indicating more efficient transport to the ER, possibly due to a higher affinity to the KDEL receptor at lower pH (41).

Monensin, which is a carboxylate ionophore, prevents at higher concentrations acidification of intracellular compartments (23), thereby inhibiting the degradation of ricin by lysosomes. Also, endosomes and the Golgi apparatus swell in the presence of the drug, and the transport to and through the Golgi stacks is inhibited. The effect of submicromolar concentrations of monensin is less well understood. At lower concentrations of monensin, there was increased labeling of the toxin and increased toxicity. This supports earlier findings that transport of the toxin to and through the Golgi apparatus is required for intoxication (42, 43).

Earlier it was demonstrated that the toxicity of ricin is reduced in cells depleted for calcium, incubated at slightly reduced pH or in the presence of high concentrations of monensin, although the binding of ricin to cells and endocytosis of the toxin was not altered under these conditions (15, 16). Also, the rate of inactivation of ribosomes in a cell-free system was not inhibited.

When cells are kept in Ca\(^{2+}\)-free medium, the concentration of Ca\(^{2+}\) in the ER rapidly decreases from 500–600 \(\mu\)M to about 150 \(\mu\)M (28). It is known that calcium plays an important role in the translocation of proteins across the ER membrane (44). The majority of chaperones, like BiP, protein disulfide isomerase, GRP94, calnexin, and calreticulin, which could assist ricin during translocation, are calcium-dependent (45, 46). BiP is supposed to close the luminal end of the Sec61p translocation channel (47) and would therefore have to be removed before ricin A-chain could enter the pore. Reduced calcium concentration could interfere with this process. Importantly, a decrease in the Ca\(^{2+}\) concentration in the ER of yeast due to a mutated Ca\(^{2+}\) pump resulted in an inability of the cells to degrade misfolded carboxypeptidase Y (27), a process occurring by the proteasomes after retrograde transport of the protein to the cytosol (48).

A moderate reduction in pH of the medium (from 7.5 to 6.5) was also found to strongly reduce the translocation of ricin A-chain from the ER to the cytosol. The reason for this is not clear. A reduction in extracellular pH will only moderately reduce the pH of the cytosol due to the correcting effect of the Na\(^+\)/H\(^+\) exchanger (49), which is operative in the bicarbonate-free Hepes medium used. The pH in the ER is close to that in the cytosol (50), and it would be expected to be slightly lowered by the transfer of the cells from the regular medium to the pH 6.5 medium. The question of whether the slightly reduced pH in the ER or that in the cytosol is the reason for the reduced translocation efficiency remains to be elucidated.

It is known that certain proteins may be transported retrograde from the ER to the cytosol. Wirz et al. (30) provided evidence that this retrograde transport is mediated by the Sec61p channel. The HCMV gene US2 product bound to newly synthesized major histocompatibility complex class I heavy chains and the complex formed was rapidly transported from the ER to the cytosol, deglycosylated by N-glycanase, and subsequently degraded by the proteasomes (51). Also, it was demonstrated in yeast that misfolded secretory proteins in the ER are exported via the Sec61p channel to the cytosol for degradation (48, 52). Using two cold-sensitive mutants of Sec61p at a temperature permissive for protein import, membranes were deficient for export of misfolded secretory proteins, which remained associated with Sec61p. In the presence of ATP and cytosol, the misfolded secretory protein was released from the ER to the cytosol, and increased toxicity. This supports earlier findings that the toxicity of ricin is reduced in cells depleted for calcium, incubated at slightly reduced pH or in the presence of high concentrations of monensin, although the binding of ricin to cells and endocytosis of the toxin was not altered under these conditions (15, 16). Also, the rate of inactivation of ribosomes in a cell-free system was not inhibited.

We have found here that the toxin interacts with Sec61p. Compared with the total amount of labeled ricin in the cells, only a small fraction (0.6–0.9\%) is associated with Sec61p. The reason for this could be that only a small fraction of the toxin
A-chain is in the process of translocating at any time or that the association is weak and that only a minor part does not dissociate during the immunoprecipitation. In the absence of Ca\(^{2+}\) the translocation is inhibited. However, this inhibition appears to be at some step before the translocation, since we were not able to immunoprecipitate more ricin A-chain with anti-Sec61α under these conditions.

A protein bound to the ER membrane, Cue1p, is a component of a ubiquitin system that interacts with the soluble Ubc7p enzyme at the ER surface. In the absence of Cue1p, the degradation substrates remained inside the ER, indicating that an enzyme at the ER surface. In the absence of Cue1p, the degradation substrates remained inside the ER, indicating that a ubiquitin system that interacts with the soluble Ubc7p enzyme at the ER surface. In the absence of Cue1p, the degradation substrates remained inside the ER, indicating that a ubiquitin system that interacts with the soluble Ubc7p

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