Addition of an Endoplasmic Reticulum Retrieval Sequence to Ricin A Chain Significantly Increases Its Cytotoxicity to Mammalian Cells*

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An Escherichia coli expression system was used to produce recombinant ricin A chain (RTA) and RTA modified either by the addition of a carboxyl-terminal endoplasmic reticulum retrieval sequence Lys-Asp-Glu-Leu (RTAKDEL) or a nonfunctional analogue Lys-Asp-Glu-Ala (RTAKDEA). These RTA molecules can enter mammalian cells by fluid phase endocytosis. RTAKDEL was significantly more cytotoxic than either RTA or RTAKDEA to both Vero cells and HeLa cells (250- and 10-fold, respectively), despite the fact that all these RTA molecules had comparable enzymatic activities. This difference did not result from KDEL-mediated binding of RTAKDEL to the cell surface. Enhanced cytotoxicity could be correlated with an increased level of ribosome inactivation, measured as the RTA-catalyzed depurination of 28 S ribosomal RNA. These results indicate that the added KDEL sequence facilitated RTA entry into the cytosol. We propose that interaction with the intracellular KDEL receptor promotes retrograde transport of the toxin to the endoplasmic reticulum, where translocation of RTA into the cytosol occurs.

With the exception of DT, which translocates from acidified endosomes (6), the site of toxin entry into the cytosol is not known. In the case of PE and ricin, it has recently been proposed that these toxins must travel the entire secretory pathway in reverse to reach the ER lumen before translocation can occur (6, 7).

The key finding supporting this proposal was reported by Pastan and his colleagues (8) who found that several amino acid residues at the carboxyl terminus of PE were essential for cytotoxicity although they were not required for cell binding or ADP-ribosylation activity. The carboxyl terminus of PE is Arg-Glu-Asp-Leu (REDL). The terminal lysine residue could be deleted without effect, but deletion of 2 or more residues drastically reduced cytotoxicity because the mutant forms of PE were unable to reach the cytosol. The PE sequence REDL is strikingly similar to the ER retrieval sequence KDEL (9), and replacing REDL in PE with KDEL produced a mutant toxin even more cytotoxic than native PE (10).

A speculative interpretation of these data is that PE interacts with the KDEL receptor during cell entry, possibly to facilitate the later stages of its journey from the cell surface to the ER lumen. If this is indeed the case, how then might ricin reach the ER lumen since neither RTA nor RTB contains a carboxyl-terminal KDEL or related sequence? We have shown that RTB galactose binding activity plays a role not only in cell surface attachment but also intracellularly for ricin cytotoxicity (11). Ricin containing mutant RTB no longer capable of binding galactose (12) was not cytotoxic to macrophages even though the toxin, which is itself N-glycosylated, was able to enter the cells via the macrophage mannose receptor (13). It is possible that ricin binds intracellularly to an endogenous, galactosylated KDEL-containing protein, such as the resident rat liver ER luminal protein calreticulin (14), which is retrieved by the KDEL receptor from the trans Golgi cisternae or the trans Golgi network (TGN).

In order to examine further the potential involvement of the KDEL receptor in the retrograde transport of toxins, we prepared mutant ricin in which the tetrapeptide KDEL (or a related sequence that does not function as an ER retention signal, KDEA (15)) was added to the carboxyl terminus of RTA. Holotoxin containing RTAKDEL was significantly (6- or 7-fold) more cytotoxic to Vero cells than holotoxin containing either RTAKDEA or wild type RTA (16). This type of analysis is inevitably complicated by the presence of RTB that also contributes significantly to the potent cytotoxicity of the holotoxin. In order to focus more directly on the contribution of the KDEL tag, we repeated the analysis using free RTAs that enter cells by fluid phase uptake. The results, which are presented here, clearly confirm that the addition of KDEL to RTA markedly enhances its cytotoxicity in comparison to either RTA or RTAKDEA.

Cytotoxic proteins such as diphtheria toxin (DT),1 Pseudomonas exotoxin A (PE), and Shiga toxin (ST) from bacteria and ricin from plant seeds are able to kill mammalian cells by catalytically inactivating cellular components essential for protein synthesis. DT and PE achieve this by the ADP-ribosylation of elongation factor-2 (1), whereas ST and ricin specifically depurinate 28 S ribosomal RNA, removing an adenine residue that is essential for binding elongation factors to the ribosome (2, 3). Because these target substrates are present in the host cell cytosol, a catalytically active fragment or subunit of the toxins has to enter this compartment. The toxins bind to cell surface receptors and enter cells by endocytosis via coated pits, coated vesicles, and endosomes (4). At some stage during uptake, the catalytic toxin fragment has to cross an intracellular membrane in order to enter the cytosol.

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1 The abbreviations used are: DT, diphtheria toxin; PE, Pseudomonas exotoxin A; ST, Shiga toxin; RTA, ricin A chain; RTB, ricin B chain; TGN, trans Golgi network; GMES, Glasgow's modification of Eagle's medium; MES, 2-(N-morpholino)ethanesulfonic acid; ER, endoplasmic reticulum.
Production of Mutant RTA—Plasmids encoding the mutant forms of RTA were created, and the recombinant proteins expressed in E. coli TO2 were purified to homogeneity by two rounds of CM-Sephrose chromatography and quantified exactly as described previously (16). Recombinant wild type RTA was kindly provided by Zeneca Pharmaceuticals (Alderley Edge, Cheshire, United Kingdom).

Cytotoxicity Assays—Vero and HeLa cells were grown in Glasgow's modification of Eagle's medium (GME) containing 10% fetal calf serum and 2 mM L-glutamine. The cells were plated out in 96 well microtiter plates at a density of 1.5 × 10⁵ cells per well. After cell adhesion, the medium was replaced with GME containing a range of toxin concentrations. Plates were incubated in 5% CO₂ at 37°C for 4 h unless stated otherwise. Protein biosynthetic capacity was then determined by replacing the medium with 50 μl of GME containing 1 μCi of [35S]methionine per well followed by further incubation in 5% CO₂ at 37°C for 1-2 h. Protein was precipitated in the wells by the addition of 10% trichloroacetic acid washed twice in 10% trichloroacetic acid and three times in phosphate-buffered saline and redissolved in 0.2 M NaOH. Incorporated radioactivity was determined by liquid scintillation counting. For the time course experiments, the labeling period with [35S]methionine was 30 min.

28 S rRNA Depurination—HeLa cells were plated out in 6 well plates at a density of 1.1 × 10⁵ cells/well and, after incubation for 7 h in 5% CO₂ at 37°C, the media were replaced by dilutions of toxin in GME. Incubation was continued for a further 18 h after which the media were removed and retained on ice. The cells were washed with 1 ml of ice-cold phosphate-buffered saline that was then removed and added to the retained media. The cells were released from the wells by trypsin treatment and added to the pooled media/wash as was the 3 ml of GME subsequently used to wash the cells. The cells were lysed by centrifugation at 250 g for 5 min at 4°C, and the supernatant was discarded. The cells were lysed by the addition of 150 μl of buffered phenol followed by 300 μl of 0.5% SDS, 0.15 M sodium acetate, pH 6.0, and 150 μl of chloroform. After vortexing, samples were transferred to 1.5 ml Eppendorf tubes and centrifuged for 10 min to separate the phases. Nucleic acid was precipitated from the aqueous phase with ethanol, followed by resuspension in 100 μl of 50 mM MES-NaOH, pH 7.0, 2 mM magnesium acetate, and incubated with 20 units of RNase-free DNase (Bohringer Mannhein) on ice for 30 min. The reaction was then extracted with phenol/chloroform, and, after precipitation of the RNA, determination was determined by treatment with aniline reagent followed by agarose gel electrophoresis as described previously (17). Cytotoxicity assays, as described above, were run concurrently using the same toxin concentrations and incubation time to determine the extent of protein synthesis inhibition.

Temperature Dependence of Toxin Uptake—Vero cells were plated out in 6 well plates as described above and incubated for 30 min on ice prior to adding various toxin concentrations in ice-cold GME. The plates were then either placed at 37°C or left on ice for a further 30 min before the toxin solution was removed and the cells washed with ice-cold phosphate-buffered saline. GME was then added to the cells that were incubated in 5% CO₂ at 37°C for 4 h after which time protein biosynthetic capacity was determined.

RESULTS

Plasmids encoding RTA, RTAKDEL, and RTAKDEA were expressed in E. coli, and the recombinant proteins were purified to homogeneity by two rounds of CM-Sepharose chromatography and quantified as described previously (16). The 28 S rRNA N-glycosidase activity of the three recombinant proteins was identical (16). This was confirmed in the present study since RTAKDEL and RTAKDEA had identical inhibitory effects on in vitro protein synthesis in rabbit reticulocyte lysates and were indistinguishable in this respect from RTA (data not shown). The purified proteins were tested for cytotoxic activity on Vero cells and HeLa cells (Fig. 1). For Vero cells RTAKDEL was 250-fold more cytotoxic than either RTAKDEA or RTA. The IC₅₀ for RTAKDEL was 0.2 μg/ml in comparison to values of 50 μg/ml or greater for RTAKDEA or RTA (Fig. 1A). The cytotoxic enhancement resulting from the addition of KDEL to RTA was dependent on the cell line assayed. As shown in Fig. 1B, the IC₅₀ for RTAKDEL on HeLa cells was 3.6 μg/ml, approximately 10-fold lower than that for either RTAKDEA or RTA. In the case of COS cells, RTAKDEL was approximately 50-fold more cytotoxic than RTAKDEA.²

The cytotoxicity of the RTA mutants resulted from the depurination of 28 S rRNA at a specific site close to the 3' end of the molecule. Depurination renders isolated rRNA susceptible to amine-catalyzed hydrolysis of the phosphodiester bonds on either side of the modification site. This cleavage generates a small RNA fragment of approximately 400 nucleotides that is diagnostic for RTA inactivation of ribosomes (2, 17). As shown in Fig. 2, the cytotoxicity of RTAKDEL or RTAKDEA to HeLa cells measured as the inhibition of cellular protein synthesis correlated with the appearance of the diagnostic rRNA fragment when the ribosomes were isolated and the 28 S rRNA was hydrolyzed with aniline. This result clearly indicates that the enhanced cytotoxicity in the case of RTAKDEL results from enhanced entry of the toxin into the cell cytosol. A time course for the cytotoxic effects of 4 μg/ml RTAKDEL or RTAKDEA on Vero cells is shown in Fig. 3. Once again, the increased cytotoxicity of RTAKDEL is apparent.

The IC₅₀ for RTAKDEL on Vero cells (200 ng/ml) is considerably higher than that for whole ricin (1-2 ng/ml). In the former case the higher IC₅₀ reflects the absence of the cell

² M. J. Lewis and H. R. B. Pelham, personal communication.
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**FIG. 2.** Correlation of cytotoxicity with 28 S rRNA depurination. The cytotoxicity of various concentrations of RTAKDEL and RTAKDEA to HeLa cells was expressed as the percent inhibition of \[^{35}S\]methionine incorporation into protein. RNA was extracted from other HeLa cell samples treated in an identical fashion. After treatment with aniline, an RNA band of approximately 400 ribonucleotides diagnostic of RTA-catalyzed depurination of 28 S rRNA was visualized following agarose gel electrophoresis of 4.5 μg of total RNA. Only the portion of the RNA gel containing the diagnostic RNA fragment is shown.

**FIG. 3.** Time course for the intoxication of Vero cells. Vero cells were incubated with 4 μg/ml RTAKDEL (●), RTAKDEA (▲), or wild type RTA (■) for various time periods before their ability to incorporate \[^{35}S\]methionine into cellular protein was determined.

binding RTB subunit and means that a higher concentration of toxin is required to achieve the uptake of a cytotoxic dose by fluid phase endocytosis. The failure of RTAKDEL to bind to the cell surface was confirmed by incubation with Vero cells at either 37 °C or at 4 °C to prevent endocytosis. After 30 min any unbound RTAKDEL was washed away, and, after a further incubation at 37 °C for 4 h, cytotoxicity was measured. As shown in Fig. 4, preincubation at 37 °C with increasing concentrations of RTAKDEL produced a clear cytotoxic effect, whereas cells preincubated with RTAKDEL at 4 °C remained completely viable. Furthermore, the cytotoxicity of 4 μg/ml RTAKDEL to Vero cells at 37 °C was completely unaffected by the addition of the synthetic peptide YTSEK-DEL, even when the peptide/toxin ratio was as high as 1500 (data not shown). This peptide effectively competes with KDEL-terminating proteins for binding to the KDEL receptor in vitro and, as such, clearly eliminates the unlikely possibility that KDEL receptor was present on the Vero cell surface.

**DISCUSSION**

The enzymatic moieties of cytotoxic proteins such as DT, PE, ST, and ricin must reach their cytosolic target substrates in order to kill cells. With the exception of DT, known to translocate from acidified endosomes (5), the route taken by these toxins in moving from the cell surface to the cytosol is poorly understood. Recent findings in several laboratories are beginning to suggest that the toxins may travel the entire secretory pathway in reverse before entering the cytosol from the ER lumen. First, PE requires the carboxyl-terminal tetrapeptide REDL for cytotoxicity. This peptide resembles the ER retrieval sequence KDEL. Indeed PE is potently cytotoxic when the native REDLK sequence is replaced by KDEL or by other tetrapeptide variants known to cause ER retention, but not when replaced by related sequences that do not result in ER retrieval (8). The carboxyl-terminal lysine residue in native PE might be removed during cellular uptake by an enzyme that cleaves carboxyl-terminal basic residues (18). KDELK acts as a function ER retention signal when lysozyme KDELK is expressed in COS cells. Second, pretreating cells with brefeldin A protects against intoxication by PE, ST, and ricin, but not DT (19). Brefeldin A is capable of affecting eukaryotic cells at a variety of sites and may alter the structure of endosomes, the TGN, or the Golgi stack (20-23). Treating Madin-Darby canine kidney cells with brefeldin A affects endosome structure but not that of the Golgi complex, and treated cells remain susceptible to ricin poisoning (24). One possible interpretation of this and other considerations is that toxins must enter or pass through the Golgi stack en route to the cytosol. Third, ST endocytosed by butyric acid-treated A431 cells has been visualized in the TGN, all Golgi cisternae, and the ER lumen (25).

How might retrograde transport from the cell surface to the ER lumen be achieved? Electron microscopy has shown that a proportion of endocytosed PE, ST, or ricin reaches the TGN (26-28). With the exception of ST, it has not yet been possible

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I. Pastan, personal communication.
to locate toxin in other regions of the Golgi or the ER, perhaps because of the small amount of toxin that has to reach the translocationally competent compartment in order to kill the cell. The TGN is an intracellular site where the endocytic and secretory pathways meet (29, 30). Incoming toxin molecules might therefore switch receptors in the TGN, dissociating from their surface receptor and binding to another cellular component that is itself capable of retrograde transport beyond the TGN. PE is taken into cells by endocytosis after binding to the α2-macroglobulin receptor (31) but may have to interact intracellularly with the KDEL receptor in order to reach the cytosol (6). Toxins such as ricin and ST that do not normally possess a KDEL sequence might well interact with other cellular proteins that do. The rat liver ER lumen resident protein calreticulin is a KDEL-containing glycoprotein that possesses an oligosaccharide of the complex hybrid type with terminal galactose residues (14). The KDEL retrieval system therefore clearly operates from the trans Golgi cisternae where galactosyltransferase and newly galactosylated proteins are located (32), and it may even encompass the TGN. In keeping with this idea we have shown that ricin must be capable of binding galactose residues intracellularly for cytotoxicity (11), and we have proposed that galactose binding facilitates the retrograde transport of ricin beyond the trans Golgi region (33).

Since PE and ricin are believed to follow the same intracellular transport route, we argued that adding a KDEL tag to ricin might make its delivery to the cytosol even more efficient, precluding and/or supplementing the requirement for a galactosylated carrier protein. Alternatively, the “KDEL route” might represent a second uptake pathway, completely distinct from that normally used by ricin. Ricin holotoxin containing RTAKDEL was significantly more cytotoxic to Vero cells than holotoxin containing either RTAKDEA or wild type RTA (16). All such holotoxins are potently cytotoxic, however, since they are all capable of exploiting the normal RTB-mediated “galactose binding route.” In the present study we have examined the effect of adding KDEL to the isolated RTA subunit, in order to eliminate the contribution of RTB galactose binding to cytotoxicity. At appropriate concentrations, free RTA is cytotoxic, entering cells by fluid phase endocytosis. In this situation, addition of a KDEL sequence to the RTA carboxyl terminus markedly enhances cytotoxicity in comparison with RTA that is incapable of interacting with the KDEL receptor. While the recycling KDEL receptor may not be directly essential for the native cytotoxicity of ricin, it seems likely that RTAKDEL is capable of exploiting this route and may therefore utilize the retrograde pathway that is also responsible for the cytotoxicity of PE. It is worth noting that the carboxyl termini of cholera toxin and E. coli heat labile toxin are KDEL and RDEL, respectively (34, 35). It will be interesting to determine the effect of deleting or changing these carboxyl-terminal tetrapeptides on the ability of these bacterial toxins to ADP-ribosylate G-protein.

We have suggested that the ER lumen might be an appropriate environment for endocytosed toxins to accomplish the necessary step of membrane traversal (7). Reaching the ER would expose the toxins to the effects of protein disulfide-isomerase that could considerably increase the rate of thiol exchange in a folded or partially unfolding toxin molecule (36). Breaking the inter chain disulfide bond linking the cell binding and catalytically active toxin polypeptides is essential for cytotoxicity (37). Other ER luminal proteins that might facilitate membrane translocation include molecular chaperons such as immunoglobulin heavy chain binding protein, which may help maintain the toxin in an unfolded state prior to the translocation step (38). In addition, the ER membrane contains translocation machinery capable of transporting nascent secretory proteins (39) or peptide fragments resulting from cytosolic proteolysis (40) into the ER lumen. Toxins may exploit such machinery in the reverse direction. While the toxins might be transported from the TGN to the site of translocation by direct vesicular transport, it seems most likely that transport would be achieved by stepwise retrograde transport through the Golgi stack. If this is the case, then the uptake of such cytotoxic proteins by mammalian cells highlights two important new properties of eukaryotic cells. In addition to the efflux of protein from the ER into the cytosol discussed above, cellular intoxication would require that the secretory pathway of eukaryotes is completely reversible. Retrograde transport from the cell surface to the TGN (41, 42) and from trans Golgi cisternae to the ER (14) is now well documented. The crucial retrograde step from the TGN to other Golgi elements that would link the two established retrograde routes and allow transport from the cell surface to the ER lumen remains controversial. The preliminary data reported here and elsewhere (4, 10, 18) coupled to the electron microscopic evidence (19, 20) that exploited ST reaches not only the TGN but all other Golgi elements and the ER lumen (25) increasingly indicate that the secretory pathway is indeed reversible and, as such, represents a novel route capable of transporting extracellular proteins to the cell cytosol.

REFERENCES