The synthesis, segregation, intracellular transport, and exocytic export of secretory proteins in eukaryotic cells is now well understood. Synthesis begins on free cytosolic ribosomes that subsequently attach to the ER, resulting in the cotranslational discharge of the nascent proteins into the ER lumen. The targeting signals and the cytosolic and ER membrane components that mediate the translocation step have been extensively characterized and reviewed (for example, see 29). The ER lumen contains a range of resident proteins responsible for modifying newly synthesized polypeptides, and for ensuring correct folding into the biologically active conformation (9). Secretion entails transport of the proteins from the ER, via the Golgi stack and the TGN, to secretory vesicles that ultimately fuse with the plasma membrane to complete protein export. The secretory pathway was elucidated by Palade and his colleagues (24). Protein transport between the various compartments of the secretory pathway is mediated by carrier vesicles that bud from one compartment and fuse with the next. Once again, many of the cellular components that are required for, and that regulate, vesicular transport have been identified, and the mechanisms by which cytoplasmic protein coats drive the individual transport steps are now emerging (31). It has been recognized for some time that the secretory pathway is at least partially reversible. For example, retrograde vesicular transport from the Golgi to the ER is needed to retrieve resident ER proteins that have escaped from this compartment (25), and endocytosis can transport proteins from the cell surface to the TGN (2), which is the cellular location where the secretory and endocytic pathways converge (31). ER resident proteins contain a retrieval signal, the COOH-terminal tetrapeptide Lys-Asp-Glu-Leu (KDEL),1 which binds to a membrane receptor in the cis-Golgi region and returns them to the ER. Indeed the KDEL receptor is capable of retrieving escaped ER resident proteins from as far along the secretory pathway as the TGN; an exogenous synthetic peptide with a COOH-terminal KDEL sequence, which was introduced into the TGN from the cell surface was subsequently transported to the ER lumen (20). Recently, it has become apparent that certain protein toxins follow this same route from the surface to the ER lumen. To reach their targets in the cytosol of mammalian cells the toxins apparently go one step further and cross the ER membrane. The emerging experimental evidence in support of this is reviewed here.

Toxin Structure and Mode of Action

Certain bacteria and plants produce proteins that are able to enter and kill mammalian cells. These proteins all act by catalytically modifying essential cellular components so that their normal function is prevented. In this review, we focus on a subset of these toxins that act by inhibiting cellular protein synthesis. Other catalytic toxins enter cells by the same mechanisms, and reference will be made to them (where appropriate). Bacterial toxins that inhibit protein synthesis in eukaryotic cells include diphtheria toxin (DT), Pseudomonas exotoxin A (PE), and Shiga toxin (ST), whereas the best characterized of a group of related plant toxins is ricin. DT, PE, and ricin are all synthesized as single polypeptide chains but in their functional form they exist as heterodimers covalently joined by a single disulfide bond. DT and PE are cleaved into dimers after entering mammalian cells by the ubiquitous protease furin (7), whereas ricin is cleaved in the plant cells that produce it (17). In the heterodimeric toxins, one polypeptide (often designated the A chain or fragment) is catalytically active whereas the other (the B chain or fragment) is responsible for binding the toxin to receptors on the surface of susceptible cells. ST differs in structure from the other toxins described here in that the catalytic A chain is associated with a pentamer of B subunits, the so called AB5 structure possessed by other bacterial toxins such as cholera toxin and Escherichia coli heat labile enterotoxin.

DT and PE inhibit protein synthesis by ADP-ribosylating elongation factor–2 (45), whereas ST and ricin act by removing a specific adenine residue from a highly conserved loop present in 28S rRNA (5). Since the toxin substrates are in the cell cytosol, the A chain must enter the target cell cytosol and retain its biologically active conformation. To enter mammalian cells, the toxins must first bind to a surface component. Binding can be to a specific protein such as the α2-macroglobulin receptor (used by PE) (15) or a heparin-binding epidermal growth factor (EGF)-like growth factor precursor (used by DT) (21), to glycolipids such as globotriaosyl ceramide (used by ST)
(11), or ganglioside GM_{1} (used by cholera toxin) (44), or to any appropriate glycoprotein or glycolipid (as in the case of ricin whose B chain is a galactose-specific lectin) (17).

**Cell Entry**

Surface-bound toxin enters cells by endocytosis; the endocytic route depends on the nature of the receptor. DT, for example, appears to enter exclusively via clathrin-coated pits and vesicles. As such, overexpression of mutant dynamin, which prevents clathrin-dependent endocytosis, protects cells from intoxication by DT (40). Such cells remain completely sensitive to ricin, however. Normally ricin uses both clathrin-dependent and -independent endocytosis, so when the former route is effectively blocked the latter remains available. The clathrin-independent route used by ricin does not appear to be mediated by caveolae since it is unaffected by treatment with cholesterol-binding drugs such as nystatin (40). ST appears to enter cells exclusively via clathrin-dependent endocytosis (33), suggesting that its glycolipid receptor becomes fixed in coated pits, possibly by interacting with a protein already anchored there. Both the clathrin-dependent and the clathrin-independent uptake routes converge in endosomes. At this stage the endocytosed toxins fall into two different behavioral groups: those that translocate into the cytosol from an endosomal compartment, and those that do not. Toxins whose A fragment crosses the endosomal membrane include DT, which has been most thoroughly studied, and others such as anthrax, tetanus, and botulimum toxins. DT translocation depends crucially on the low pH of the endosomal compartment, which induces a conformational change in DT B causing it to insert into the endosomal membrane and form, or contribute to the formation of, a proteinaceous pore through which the A fragment is able to pass. Further discussion of the DT translocation mechanism can be found elsewhere (37).

Since DT translocation requires low pH, treating cells with reagents that increase endosomal pH protects them from intoxication. Conversely, lowering the extracellular pH to that typically found in endosomes causes surface-bound DT to directly cross the plasma membrane. Increasing the intracellular pH does not protect cells against members of the second group of toxins; toxins that do not translocate from endosomes including PE, ST, and ricin. Members of this second toxin group must proceed beyond endosomes to reach the compartment for A chain translocation.

**Intracellular Transport Beyond Endosomes**

The trafficking of endocytosed ricin beyond endosomes was initially followed by electron microscopy (42). In addition to entering lysosomes or being recycled back to the cell surface, a significant amount of toxin accumulated in the TGN. Initially this was interpreted as suggesting that ricin A chain translocated to the cytosol from the TGN (43). With hindsight, a critical limitation of this experimental approach was its sensitivity. Whereas it clearly defined the fate of the bulk of the endocytosed toxin, the extreme potency of such proteins (entry of just a few molecules into the cytosol is believed sufficient to kill a cell) meant that the productive but inefficient trafficking to the translocation compartment went undetected. One of the first experimental indications that toxins such as PE, ST, and ricin might not translocate from the TGN was the demonstration that treating the cells with brefeldin A (BFA) protected against these toxins but had no effect on their susceptibility to DT (50). A careful morphological study of ricin trafficking in BFA-treated cells showed that in all lines where protection was seen, the typical BFA-induced disassembly of the Golgi stack had occurred (32). Transport of ricin to the TGN was unaffected by BFA treatment. In contrast, treating MDCK cells with BFA did not confer protection against ricin nor did it structurally perturb the Golgi apparatus.

Experiments with PE mutants suggested that PE might also undergo retrograde transport from the TGN to the ER, and provided a likely explanation of how this occurred. The last five COOH-terminal residues of the translocated PE fragment are Arg-Glu-Asp-Leu-Lys (REDLK). The effect of mutations in this pentapeptide on the catalytic activity and cytotoxicity of PE were determined. Deletions or substitutions predicted to prevent interaction with the KDEL receptor dramatically reduced cytotoxicity (3). Such changes had no effect on catalytic activity and apparently cause reduced cytotoxicity by preventing the mutant forms from reaching the cytosol. Replacing the wild-type REDLK sequence with KDEL produced a mutant toxin even more cytotoxic than wild-type PE. (36). The COOH-terminus of the catalytic polypeptide of cholera toxin is KDEL, and that of *E. coli* heat labile enteroxin is RDEL. Subsequent work has established that cholera and *E. coli* heat-labile toxin action are also blocked by BFA treatment (4). The cytotoxic activity of these G protein–modifying proteins is not absolutely dependent on the KDEL/RDEL tetrapeptide, although it apparently enhances delivery into the cytosol (16). EM studies have visualized endocytosed ST (34) and cholera toxin (35) in the ER lumen, and cholera toxin transport from the plasma membrane to the ER has been described (18). For toxins that contain KDEL or a KDEL analogue at the COOH-terminus of the translocated polypeptide, retrograde transport from the TGN to the ER is most probably achieved by interaction with the KDEL receptor which is now known to recycle between these compartments (8, 20). Presumably, after endocytosis, recycling cell surface receptor delivers a proportion of the toxin to the TGN where receptor–toxin dissociation occurs leaving the free toxin able to opportunistically interact with the KDEL receptor.

How do toxins such as ST and ricin, which do not possess KDEL or a related peptide, travel from the TGN to the ER? That ST can reach the ER lumen is beyond dispute since, as noted above, it has been visualized there. The effect of BFA treatment suggests that ricin must also traverse the Golgi stack to reach the ER. This contention was supported by the finding that addition of the KDEL retrieval signal to the COOH-terminus of ricin A chain significantly enhances cytotoxicity (46). It seems likely that ricin undergoes retrograde transport from the TGN to the ER by binding to a recycling galactosylated component. Ricin B chain contains two functional galactose-binding sites, and is itself glycosylated. Because it has a high mannose oligosaccharide sidechain, ricin is able to intoxicate cells with mannose receptors, such as macrophages, even when
binding to galactosylated surface components is prevented (38). The effect of abrogating one or other or both B chain galactose-binding sites on the cytotoxicity of ricin entering macrophages via the mannose receptor showed that toxin retaining either of the B chain sugar-binding sites remained cytotoxic, but when both sites were destroyed, toxicity was lost (22). The putative recycling galactosylated component used by ricin for retrograde transport might itself carry a KDEL retrieval signal, be a transmembrane glycoprotein with a retrieval signal such as KKKX (12), or may interact with another molecule that has a retrieval signal.

**The ER as the Site of Toxin Translocation**

Recently the first direct biochemical evidence that endocytosed ricin reaches the ER before translocating to the cytosol has been presented (28). Ricin A chain was modified to contain a tyrosine sulfation site and overlapping N-glycosylation sites. Mutant A chain was reassociated with B chain and incubated with cells in the presence of Na<sub>2</sub>SO<sub>4</sub> and the A chain became radiolabeled. This labeling identified that proportion of the endocytosed toxin that had reached the Golgi, and labeling was prevented by treatment with BFA or ilimaquinone. The labeled A chain underwent retrograde transport to the ER where it became core glycosylated, and then translocated to the cytosol. Only free glycosylated A chain entered the cytosol, indicating that transport to the ER was a prerequisite for translocation. At this stage it is unclear whether the A and B chains dissociate in the ER before translocation, or in the cytosol after translocation of the holotoxin. A similar experimental approach has confirmed that ST B fragment also reaches the ER via the Golgi apparatus (13).

**Do Toxins Use the ER-associated Protein Degradation Pathway to Enter the Cytosol?**

It has been known for some time that small glycopeptides can enter the cytosol directly from the ER (30), and more recently it has become apparent that proteins and glycoproteins also do so as part of the ER quality control function (14). Nascent secretory proteins enter the ER through a proteinaceous channel whose central component is Sec61p, the Sec61p translocon. Only if secretory proteins are folded correctly, and if appropriate, correctly assembled into oligomers, are they packaged into ER–Golgi transport vesicles. Proteins that fail to fold or assemble properly are instead exported across the ER membrane to the cytosol, where they are degraded by the proteasomes (10, 47). Human cytomegalovirus (CMV) takes advantage of this export machinery to downregulate antigen presentation at the cell surface by the class I major histocompatibility complex (MHC). CMV proteins in the ER cause dislocation of the transmembrane MHC class 1 heavy chain from the ER membrane to the cytosol, and its subsequent degradation by the proteasomes (48, 49). Genetic and biochemical experiments in *Saccharomyces cerevisiae* demonstrated that soluble, misfolded proteins are also exported across the ER membrane to the cytosol for degradation (10, 19). Coimmunoprecipitation studies indicated that the MHC class 1 heavy chain in CMV-infected cells might be exported through the Sec61p translocon (49). Direct evidence for the involvement of Sec61p in the export of misfolded proteins from the ER to the cytosol has recently been demonstrated in yeast (26, 27). It is likely that when toxins such as PE, ST, or ricin reach the ER lumen, thiol exchange catalyzed by protein disulfide isomerase causes dissociation of the disulfide-linked A and B subunits. Reductive dissociation in the oxidizing environment of the ER lumen has been demonstrated for disulfide-linked cholera toxin A fragments (23). Dissociation of the toxin subunits exposes a hydrophobic region present in the A polypeptides that may promote unfolding or membrane interaction. In the case of ricin A chain, point mutations in this region do not affect catalytic activity but dramatically reduce ricin cytotoxicity, which may indicate that the hydrophobic stretch plays a role in the membrane translocation step (39). The free A subunits may be perceived as unassembled polypeptides or—if ER resident chaperones cause partial unfolding—as misfolded polypeptides. Evidence that both DT A and ricin A chain partially unfold during cell entry has been presented (1, 6). This may result in export to the cytosol by the ER quality control system. If this is the case, some of the exported toxin must avoid proteasomal degradation and assume its biologically active conformation in order to kill the cell. An in vitro study has reported that the NH<sub>2</sub>-terminal region of the translocated PE fragment promotes its own export after microsomal membrane insertion (41).

**Summary and Future Prospects**

Bacterial and plant toxins that kill mammalian cells by modifying intracellular targets are unusual in that they both fold and traverse a membrane twice. They cross a membrane and fold for the first time as they are secreted from the producing bacteria or enter the ER lumen of the eukaryotic plant cells. During the intoxication process, the toxin A subunits partially unfold and again cross a membrane before refolding in the cytosol. Whereas some toxins, including DT, take advantage of the low pH to cross the endosomal membrane, others such as PE, ST, and ricin are apparently unable to form or become part of a translocation channel. Therefore, these toxins undergo retrograde transport through the secretory pathway to reach a cellular compartment where protein-conducting channels are already present—the ER. This is achieved by parasitizing normal cellular components that recycle between the plasma membrane and the TGN, and between the TGN and the ER. From the ER, the toxins enter the cytosol, possibly via the Sec61p translocon, and perhaps by masquerading as substrates for the ER export machinery. It may be possible to take advantage of the ability of these toxins to reach the ER. For example, toxins may be able to directly deliver antigenic peptides to MHC class 1 molecules. Finally, toxin entry demonstrates that the eukaryotic secretory pathway is completely reversible. This is physiologically significant in that it provides a route by which these exogenous proteins reach their cytosolic targets.

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