Bacterial toxins deliver the goods

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Some toxins produced by bacteria are so potent that scientists have long considered putting them to good use. This became possible when their structures were determined through the combined use of molecular biology and structural biochemistry. The current status of targeted toxin technology is perhaps best illustrated by exotoxin A produced by Pseudomonas aeruginosa, a Gram-negative bacterium that frequently infects superficial wounds and especially burns, and causes serious lung infections in cystic fibrosis patients. Exotoxin A, one of the huge battery of virulence factors produced by P. aeruginosa, is a 66-kDa protein composed of three important domains, one required for binding to the cell surface, one for intoxication of the target cell (through ADP ribosylation of elongation factor 2 with consequent arrest of protein synthesis), and one for translocation of the intoxication domain into the cell. Toxin adsorbed to its receptor is endocytosed by means of coated pits into endosomes, reduced, and then processed (by furin proteases (1, 2)) to release the translocation and intoxication domains that are translocated into the cytosol. Only the second and third domains are crucial for translocation and toxicity; the first domain can be artificially replaced by others that allow the chimeric toxin to bind to receptors different from those to which the toxin normally binds. This seminal observation by Ira Pastan and his colleagues at the National Institutes of Health (Bethesda, MD) (3) led to the creation of chimeric toxins that kill very specific cell types (4). Some of the most impressive examples are hybrid toxins in which the receptor binding domain is the Fv region of monoclonal antibodies raised against proteins that are only present on the surface of specific cell types (the so-called single-chain immunotoxins) (5). In other chimeras, the receptor-recognition domain is a ligand that binds to a cell-type-specific receptor (6). Chimeras produced by recombinant bacteria form cytoplasmic inclusion bodies that can be separated relatively easily from other proteins, denatured, and then renatured to produce fully active toxons.

These “magic bullet” toxins will revolutionize some aspects of cancer chemotherapy. The basic technology is also likely to have a major impact on the analysis of cell and tissue development, in studies of degenerative disorders (7) and as a method for modulating gene expression, or for the treatment of genetic disorders leading to the loss of an essential protein. Most importantly, as pointed out in the article in this issue of the Proceedings from John Collier’s laboratory at Harvard Medical School (8), this and related methods of introducing proteins directly into the cytosol do not have any of the deleterious effects on cell viability that are associated with other methods, such as partial permeabilization of the plasma membrane, which are difficult to control and that induce leakage of cell contents.

Many toxins produced by Gram-negative bacteria are composed of multiple subunits that assemble, prior to their release, in the periplasm between the two membranes of the cell envelope. Indeed, the high viscosity, high protein concentration, and plethora of folding catalysts in the periplasm provide an ideal environment for the assembly of multimeric protein complexes such as toxins (9). Gram-positive bacteria do not produce preassembled multisubunit toxins but some produce so-called binary toxins, the intoxication component of which interacts with its cognate receptor recognition and uptake component only after the latter has bound to its receptor on the target cell surface. The binary anthrax toxin studied by Collier’s group is produced by the spore-forming Gram-positive bacterium Bacillus anthracis, the highly infectious causative agent of anthrax in domestic livestock, wild animals, and, occasionally, man. The bacterium usually infects minor cutaneous wounds, leading to the formation of pustules with a characteristic black eschar of dead or disorganized tissue at its center. Most of the clinical symptoms are caused by the toxin, a mixture of three proteins that act in synergy (10): edema factor (EF), a calcium and calmodulin-dependent adenylate cyclase that dramatically increases cellular cAMP levels; lethal factor (LF), which appears to be a zinc-dependent, presumably target-specific protease; and the receptor component, the so-called protective antigen (PA).

PA (83 kDa) must be cleaved (again by furin proteases) to give the carboxyl terminal, active 63-kDa form (PA63), which probably remains bound to the receptor, before EF or PA can bind to it (1). The complex is then endocytosed, whereupon the acidic environment of the endosome triggers the insertion of PA into the membrane and permits the translocation of EF or LF into the cytosol. PA63 can form channels in artificial membranes at low pH, conditions that also lead to the formation of stable heptameric rings with a 12Å-channel through which LF or EF are probably transported (11, 12). In support of this idea, an N-terminal fragment of EF (see below) interacts with PA63 in a voltage-dependent manner (11), and binding of LF to PA63 at low pH prevents solute and ion flux through the channel (13). Some studies indicate that both LF and EF might also penetrate into the lipid bilayer (14), but the results reported by Blanke et al. (8) indicate that any contribution they make to channel formation is negligible.

How then do LF and EF recognize the same receptor (PA) in order to be internalized? The considerable sequence similarity between their N-terminal regions (15) led Stephen Leppla and his colleagues at the National Institutes of Health to propose that this could be a common receptor (PA)-recognition domain. In support of this idea, they found that the ADP ribosylation domain of exotoxin A could be converted into a potent PA-dependent toxin by fusing it to the first 254 amino acids of LF (16). This phenomenon is not specific to the exotoxin A fragment and is independent of the end of the LF fragment to which the heterologous fragment is fused (17, 18).

Collier’s group previously found that the 255 N-terminal amino acids of LF were capable of inducing PA-dependent internalization of the ADP ribosylation domain of another bacterial toxin, diptheria toxin (DT) (18). The DT fragment alone was not toxic, but the addition of a polyhistidine tag (to allow its facile purification by nickel chelate affinity chromatography) caused the reappearance of low but measurable levels of toxicity. This activity was dramatically increased when the histidines were replaced by more strongly-charged lysines, indicating that the tagged DT fragment probably binds to the surface of the target cells by electrostatic interactions and is then internalized. The observation is made even more astounding by the fact that the action of the polylysine-tagged DT
fragment is entirely dependent on the presence of PA and yet the PA recognition domain of LF does not contain a stretch of cationic amino acids. Furthermore, the PA recognition domain of LF does not block the action of the polylysine-tagged DT fragment, whereas it does block the action of the same DT fragment fused to the PA recognition domain of LF. One explanation for this observation could be that the tagged DT fragment binds productively to a different site on PA from that to which LF binds. Alternatively, because strongly basic regions of polypeptides bind avidly to negatively-charged surfaces, the polylysine tag on the DT intoxication fragment might allow it to attach nonspecifically to the cell surface whence it could be internalized in the same endocytic vesicles as PA (the polylysine-DT fragment and PA could even be internalized independently and interact in the endosome). Is PA interaction mediated solely by the polylysine tag or does the DT fragment play an active role? The only way to answer this question is to look for PA-dependent uptake of other polylysine-tagged polypeptides such as other intoxication fragments like the carboxyl-terminal domain of exotoxin A.

For the sake of argument, let's assume that uptake is exclusively dependent on the polylysine tag and consider its possible repercussions. Most exotoxin A-based chimeras retain their intoxication domain and are designed to kill specific cell types. On the other hand, proteins other than intoxication domains could be polylysine-tagged to permit their PA-dependent, nonspecific internalization. Blanke et al. (8) mention, for example, "protein complementation" as one potential application in the field of genetic disease therapy. This procedure is an attractive alternative to gene therapy, whose application is limited by the absence of efficient systems for DNA delivery to certain tissues, such as muscle cells in muscular dystrophy patients producing truncated forms of the protein dystrophin. Such patients could be treated with a mixture of a polylysine-tagged dystrophin and PA. Although attractive, the general applicability of such technology might be limited by at least three factors: (i) the need to repeatedly inject large amounts of the protein into the body to compensate for protein turn-over, (ii) possible side effects caused by introducing the protein into cells where it is not normally present, and (iii) nonapplicability to certain types of proteins, such as those that cannot withstand the low endosomal pH and cotranslationally modified proteins. The technique would also appear inappropriate for compartmentalized proteins such as endoplasmic reticulum (ER), Golgi, lysosomal, and plasma membrane proteins, which are almost invariably and obligatorily translocated across the ER membrane in a well-established cotranslational manner requiring specific targeting information absent from the polylysine-tagged variant. In addition, although the channel formed by PA oligomers is quite wide, proteins that are translocated through it might still tend to be partially unfolded and then refolded after translocation. Thus, it might not be possible to translocate and refold proteins with transmembrane segments of high overall hydrophobicity because of their inherent tendency to aggregate or to insert spontaneously into membranes. Nevertheless, there are many other potential applications both in disease therapy and in areas of fundamental research, such as protein-mediated gene regulation and modification of macromolecules.

It should be possible to produce comparable chimeras based on exotoxin A by replacing the intoxication domain by a heterologous protein. In fact, exotoxin A would appear to be readily amenable to such manipulation because its intoxication domain comprises the carboxyl-terminal portion of the protein. This raises the possible importance of the second difference between PA and exotoxin A-based delivery systems, which is that in the former, the transporter and the lysine-tagged heterologous protein are produced separately, whereas the latter are single-chain polypeptides that might be less amenable to genetic manipulation because of folding constraints and protein instability. Hybrid proteins are frequently unstable, but problems of this kind do not appear to have been encountered by Pastan and his colleagues in their studies with exotoxin A constructs. Indeed, the protein yields reported by this group are often very high, and renaturation does not appear to have posed any particular problems, which augurs well for future industrial-level production of such chimeras. However, there is no guarantee that chimeras in which the intoxication domain of the toxin is replaced by other polypeptide sequences would behave in the same way. On the other hand, the polylysine tag should enable proteins to be purified relatively easily by cation-exchange chromatography.

In conclusion, the latest paper from Colliers' group represents an important step toward the application of nondestructive methods for targeting heterologous proteins to living cells both in culture and in living organisms, including man. The toxin chimeras are entirely nontoxic (and cannot regain toxicity), can be produced in large amounts by "domesticated" bacteria, and can be purified by procedures currently used at industrial levels for the production of hormones or other therapeutic proteins. Besides their undoubtedly potential in many areas of fundamental research, these chimeras offer real and challenging possibilities for the treatment of diseases including cancers and inherited disorders.