INFECTION AND IMMUNITY, Feb. 1994, p. 333–340
0019-9567/94/$04.00+0

Vol. 62, No. 2

MINIREVIEW

Proteolytic Activation of Bacterial Toxins: Role of Bacterial and Host Cell Proteases

VALERY M. GORDON AND STEPHEN H. LEPPLA*

Laboratory of Microbial Ecology, National Institute of Dental Research, Bethesda, Maryland 20892

INTRODUCTION

Many bacterial and plant toxins achieve their high potency by delivering a catalytically active polypeptide fragment of the toxin to the cytosol of eukaryotic cells. Toxins acting in this manner include diphtheria toxin (DT), anthrax toxin, *Pseudomonas* exotoxin A (PE), Shiga toxin and the Shiga-like toxins (ST/SLTs), botulinum toxin type C_2, cholera toxin, and the tetanus and botulinum neurotoxins. When these toxins were first purified and characterized, they were found to be mixtures of intact toxin and toxin that was cleaved into several polypeptides, apparently because of the activity of endogenous proteases of the producing organisms. For certain of the toxins in which the limited proteolysis (nicking) by the endogenous proteases was incomplete, mild treatment with trypsin would complete the nicking, and this was accompanied by increases in potency. These observations led to the concept that many protein toxins require proteolytic cleavage to express full toxic activity. Further analysis showed that proteolysis was needed to release the catalytic domain from other domains having receptor-binding and translocation functions. Activation occurred by proteolytic cleavage at a defined site, often followed by reduction of a disulfide bond, thereby freeing the catalytic domain so that it could act in the cytosol. More recently, proteolysis was found to have a different role in several binary toxins, in which cleavage within a specific sequence exposes binding sites or surfaces by which several toxin proteins associate.

Many of these toxins were initially obtained in nicked forms produced by bacterial proteases, acting either during secretion or extracellularly. However, it seems unlikely that extracellular proteases would be able to activate the toxins during an infection or intoxication, because bacterial proteases would be greatly diluted in body fluids and would be inhibited by eukaryotic protease inhibitors. According to this argument, any proteolytic cleavage shown to be essential by in vitro experiments must be accomplished in vivo by proteases of the target cells and tissues. However, the fact that the toxins were usually obtained in an active (i.e., nicked) form diverted attention from the possible role of eukaryotic proteases in toxin activation. Only in the last few years have there been renewed interest and detailed experimental analyses of the role of eukaryotic proteases in activation of bacterial toxins. This was facilitated by improved purification procedures that yield pure, uncleaved forms of several of the toxins, and by the characterization of several toxins that are produced in an uncleaved form and therefore have an absolute requirement for activation by target cell proteases.

A number of eukaryotic proteases are active in extracellular spaces under certain circumstances (plasmin during blood clotting, neutrophil elastase during inflammation, etc.). However these proteases are normally inhibited by protease inhibitors such as α_1-antitrypsin and α_2-macroglobulin and are therefore unlikely to play a role in activation of toxins. Recent work has implicated furin, a newly recognized eukaryotic protease, in the activation of several bacterial toxins (38, 39, 54, 57). Furin is a ubiquitous, subtilisin-like eukaryotic protease that cleaves mammalian proproteins and prohormones (7, 31, 90). The discovery of furin ended a search extending over many years for the proteases that cleave prohormones at sites containing paired basic amino acids, such as KR and RR. Furin was found to specifically recognize the longer sequence, RX(K/R)R (where X is any amino acid), present at cleavage sites in many proproteins such as proinsulin, proalbumin, and the insulin receptor (31). Other members of the family of subtilisin-like eukaryotic proteases, including PC1, PC2, and PC4, appear to be responsible for cleavage of prohormones at KR and RR sequences to produce active peptide hormones. Consistent with these roles in biosynthesis, furin is produced by nearly all types of cells, whereas the other processing proteases are produced only in neuroendocrine cells. The subtilisin-like endoproteases have recently been reviewed (7, 83). In this review, we discuss the requirements for activation of selected bacterial toxins and propose a role for furin in the cleavage of several of the toxins.

ROLE OF FURIN IN ACTIVATION OF BACTERIAL TOXINS

Evidence implicating furin in activation of toxins emerged from a systematic study of activation of anthrax toxin protective antigen (PA). This protein is synthesized in an inactive form and must be cleaved to become toxic. Mutagenesis of the PA gene showed that in order to be activated, PA must at a minimum contain the sequence RXXR (39). This sequence was recognized to be very similar to the cleavage sites in the normal eukaryotic substrates of furin. Work with other probable furin substrates (8) and with PA mutants (39) confirmed that a basic residue at the −2 position relative to the bond cleaved is not absolutely required for cleavage by furin. In addition, Nakayama et al. (60) showed that a basic residue at the −6 position may substitute for the −4 or −2 Arg. Further analysis of the prorenin test system (94) showed that efficient cleavage by furin occurred when Arg occupied any two of the sites at −2, −4, and −6. Arg was absolutely required at −1, and the +1 position could not contain a hydrophobic aliphatic residue (e.g., Leu).

* Corresponding author. Mailing address: Laboratory of Microbial Ecology, NIDR, Bldg. 30, Room 309, NIH, Bethesda, MD 20892. Phone: (301) 402-0730. Fax: (301) 402-0396. Electronic mail address: Leppla@irp.nidr.nih.gov.

TABLE 1. Toxins known or suspected to be activated by furin

Toxin	Source	Cleavage site[a]	Reference(s)
Anthrax toxin PA	*Bacillus anthracis*	SNS**R**K**KR**ST	39, 57
PE	*Pseudomonas aeruginosa*	TRH**R**QP**R**GW	65
DT	*Corynebacterium diphtheriae*	AGN**R**VR**R**SV	18
ST, SLT-I	*Shigella dysenteriae* type I, *Escherichia coli*	HAS**R**VAR**M**A	41
SLT-II, SLT-IIv	*Escherichia coli*	QGAR**S**VR**A**V	73
Botulinum toxin C_2	*Clostridium botulinum* types C and D	Not known[b]	66

[a] Residues in boldface are those which constitute a site for furin recognition.
[b] Protein sequence is unknown.

The discovery that PA is activated by furin (39, 57) prompted us to consider whether other protein toxins might be activated by this protease. Table 1 shows the cleavage sites for the toxic proteins listed in the introduction, except that botulinum C_2 toxin is omitted because the sequence has not been reported. As shown, the toxins that are considered to be processed by eukaryotic proteases all contain sequences recognized by furin, whereas toxins that are nicked by bacterial enzymes have various other cleavage sites. In this review, we discuss the data available on processing of these toxins and provide evidence supporting the hypothesis that furin is the primary eukaryotic enzyme which activates protein toxins.

Anthrax toxins. *Bacillus anthracis* secretes three proteins that form two bipartite toxins, edema toxin and lethal toxin (46). These toxins both utilize PA (83 kDa), the protein responsible for binding to eukaryotic cells. The catalytic polypeptides which enter the cytosol of target cells are edema factor (EF), which is a calmodulin-dependent adenylate cyclase (45), and lethal factor (LF), which recently has been shown to have properties characteristic of a metalloprotease (36, 37). The events leading to intoxication of cells begin when PA binds to a cellular receptor and becomes activated by proteolytic cleavage. The cell-bound, protease-nicked PA is able to form ion-conductive channels in lipid membranes (40) and in cells (52). Release of a 20-kDa amino-terminal fragment exposes a site to which either EF or LF binds (47). The complexes are then internalized by receptor-mediated endocytosis (22, 25), and the enzymatically active components, EF and LF, translocate to the cytosol. The cleavage of PA occurs after the sequence R-164-KKR-167. Singh et al. (82) showed that deletion of residues 163 to 168 produced a PA protein that could not be cleaved by trypsin or by target cell proteases and was nontoxic for cells and animals. Purified recombinant human furin was found to rapidly cleave PA between R-167 and S-168 (57). Klimpel et al. (39) prepared mutants of PA and demonstrated that Arg was required at both the -1 and -4 positions for maximal cytotoxic activity. Mutants having a single Arg or Lys within the four-amino-acid sequence became toxic only after cleavage with trypsin. Comparison of the potency and kinetics of action of some of the mutants suggested that PA may enter cells by endocytosis, be cleaved by proteases, and then recycle to the surface to bind LF or EF. Inhibitor studies showed that PA bound to receptors on the surface of fixed cells was cleaved by a surface protease having the catalytic properties of furin (39). The latter result suggests that at least a small amount of furin is on the plasma membrane of cells, in apparent contradiction to evidence that the protease is located exclusively in the Golgi compartment. There is also evidence that PA can be cleaved by a Ca^{2+}-dependent protease in serum or plasma (20). However, taken together, the data strongly suggest that furin is the major protease responsible for activation of the anthrax PA protein.

***Pseudomonas* exotoxin A.** The first of two well-studied ADP-ribosylating toxins to be discussed here is PE, a 66-kDa polypeptide secreted by *Pseudomonas aeruginosa*. The toxin binds to the α_2-macroglobulin receptor (42) and enters cells by receptor-mediated endocytosis. A requirement for the sequence REDLK at the carboxyl terminus of PE (9), together with protection of cells by the Golgi compartment-disrupting agent brefeldin A (79, 97), supports the view that PE must be trafficked through the endoplasmic reticulum and Golgi compartment. Biochemical studies have identified a 37-kDa fragment which is eventually translocated to the cytosol (64). This carboxyl-terminal region contains a catalytic domain that arrests protein synthesis by ADP-ribosylating elongation factor 2. A number of studies have helped to characterize the intracellular proteolytic step that produces the 37-kDa fragment. Amino acid sequencing showed that the fragment begins at G-280 (65). Thus, cleavage must occur after R-279, in the sequence R-274-HRQPRG-280, located within a disulfide loop formed by C-265 and C-287. The three-dimensional structure of PE (4) shows that this region is on the exterior surface of the protein, where it would be readily accessible to proteases. Extensive mutagenesis studies showed that nearly all replacements of R-276 eliminate toxicity and that substitutions of R-279 also greatly reduce potency (33). Replacement of R-276 with histidine did not affect activity. PE containing a glycine substitution at position 276 bound to and entered cells but was unable to translocate to the cytosol and was nontoxic (33). Fryling et al. (23) partially purified an activity from mouse L929 cells that cleaves PE to produce the 37-kDa fragment. In vitro cleavage of PE by the protease was optimal at pH 5.5 and required Ca^{2+}. The protease had properties like those of furin. It could be obtained only in very small amounts, consistent with evidence that furin is a low-abundance protein in all cell types so far studied.

Very strong evidence that furin is needed for PE activation comes from analysis of a Chinese hamster ovary (CHO) cell mutant, RPE.40, that was selected by exposure to PE and found to be cross-resistant to a number of RNA viruses (56, 95). At the time that RPE.40 was obtained, the authors explicitly and presciently suggested that the mutant cells were deficient in a protease required for cleavage of both PE and viral proteins. Recently, they have shown that RPE.40 cells transfected with a furin cDNA regained sensitivity to PE and to viruses (54). In trying to relate their results with the earlier view that furin recognizes RX(K/R)R sites, the authors pointed to the PE sequence R-182-REKR-186 as a possible furin cleavage site. Considering the more recent evidence that furin can cleave RXXR sites, and the other data discussed above, it is much more probable that intracellular activation of PE involves cleavage of the R-279–G-280 bond by furin. All the available data are consistent with the hypothesis that furin acts in an interior compartment to cleave PE distal to R-279 so as to release the 37-kDa fragment which then translocates to the cytosol. Because PE lacks a basic residue at the -2

position, it may be less rapidly cleaved by furin than proteins having the RX(K/R)R sequence. However, the presence of a basic residue at the −6 position (R-274) may partially compensate (60, 94) so as to increase the rate of cleavage and thereby increase the toxin's potency.

Diphtheria toxin. DT is a 58-kDa polypeptide that binds to the heparin-binding epidermal growth factor-like precursor (59), enters cells by receptor-mediated endocytosis, and translocates from acidified endosomes directly to the cytosol (71, 76). Toxicity results from inactivation of elongation factor 2 by a catalytic mechanism identical to that of PE (71). Expression of enzymatic activity in vitro, and presumably also in vivo, requires that the 21-kDa fragment having the catalytic domain be separated from the 37-kDa fragment that has receptor-binding and membrane translocation functions (13). Cleavage occurs at the sequence R-190-VRR-193, which is located within a disulfide loop shown in the three-dimensional structure to be highly exposed on the protein surface (11). DT may be activated in vitro by cleavage with trypsin (18) after any of the three Arg residues in this loop (12, 58) or by cleavage with urokinase distal to R-193 (12).

For many years, the DT preparations available to most researchers were predominantly nicked because of action of corynebacterial proteases. For this reason, the involvement of target cell proteases in activation was not addressed. However, Sandvig and Olsnes (76) purified unnicked DT and showed that it was up to 30-fold less toxic to Vero cells than nicked DT in short-term experiments in which nicking by serum proteases was prevented. These investigators also showed that the catalytic 21-kDa fragment of receptor-bound, nicked DT could enter cells that were exposed to low external pH, in a process considered to mimic the membrane insertion event that occurs when endosomes become acidified. Unnicked DT was at least 10-fold less potent than nicked DT in this protocol. More recently, these investigators have studied a fusion protein of DT with staphylococcal protein A which can be internalized by acid shock after binding to cells coated with antibody (50). The fusion protein was toxic in this protocol only if it was first nicked after R-193 with urokinase. These results suggest that DT is not efficiently cleaved by proteases on the surface of cells. Therefore, for unnicked DT to kill cells, it must be cleaved by cellular proteases subsequent to endocytosis.

Direct studies of the susceptibility of DT to cleavage by furin were done by Klimpel et al. (38, 39), who found that purified furin cleaved DT to produce fragments of 21 and 37 kDa, equal in size to those produced by trypsin. Whereas trypsin treatment gave a mixture of three or four fragments differing in isoelectric point, presumably reflecting cleavage at each of the three Arg residues (58), cleavage with furin gave only the species with the most positive net charge, as would be expected if cleavage occurred after R-193.

Restrictions against cloning of active, recombinant DT have delayed analysis of the DT cleavage site by mutagenesis. However, the effects of alterations to the DT cleavage sequence were examined in a chimeric fusion toxin (96) containing DT residues 1 to 486 and interleukin-2 (DAB_{486}-IL-2). Comparison of proteins having substitutions in the cleavage sequence R-190-VRR-193 (the residue numbering used here corresponds to that of native DT) showed that R-193 is absolutely required for intoxication of target cells, and that replacing either R-190 or R-192 with Gly decreased potency 20-fold. An inactive mutant containing the sequence RVRG-193 became toxic only after incubation with trypsin. Thus, like anthrax toxin PA, the DT fusion proteins are most toxic to cells when an optimum furin recognition site is present. These data strongly suggest that the DT fusion protein (and by inference, DT) is activated by furin during its binding and internalization by cells.

Shiga and Shiga-like toxins. ST, produced by *Shigella dysenteriae* type I, and the SLTs, produced by enterohemorrhagic *Escherichia coli*, are functionally identical and very similar in sequence (62). All consist of a single A subunit (~32 kDa) in noncovalent association with a pentamer of B subunits (~7.5 kDa) (61, 70). The B subunits bind to eukaryotic cell receptors which are glycolipids of the globoseries (32, 49). The toxins enter cells by endocytosis and appear to be routed in a retrograde manner through the Golgi compartment and endoplasmic reticulum before reaching the cytosol (75). The A subunit of ST/SLTs has specific *N*-glycosidase activity which inactivates 28S rRNA, resulting in inhibition of protein synthesis (19). The A subunit of SLT type I (SLT-I), which is virtually identical to ST (88), is usually obtained in an intact form if care is taken to inhibit bacterial proteases during purification from culture supernatants. The A chain of ST/SLTs can be separated into an enzymatically active A_1 polypeptide (~28 kDa) and an A_2 polypeptide (~4 kDa) by mild treatment with trypsin and a reducing agent. Trypsin cleavage (41) occurs at one or both of the two Arg residues within a disulfide loop containing the sequence R-270-VAR or RSVR, a potential furin site (residue numbering is that in ST [Table 1]). Kittell et al. (35) performed amino-terminal amino acid sequencing on a preparation of SLT-I A subunit that had been nicked by endogenous bacterial proteases and found that cleavage had occurred after R-273, as would be expected for trypsin-like enzymes.

In contrast to the situation with other toxins discussed above, the necessity for cleavage and separation of the ST/SLT A chains into A_1 and A_2 is in question. Kongmuang et al. (41) found no difference in cytotoxic activity between intact ST and ST that was nicked between A_1 and A_2 with trypsin. However, those workers exposed cells to toxin for 24 h, a time that might allow alternate enzymes to activate the toxin. Samuel and Gordon (73) replaced either one or both of the Arg residues of the putative furin site of SLT-IIv with His and found that enzymatic and cytotoxic activities were unaffected. In contrast, replacements of either Arg with Gln eliminated toxicity, suggesting that this site is important. Sandvig and colleagues deleted six residues at the cleavage site of ST, S-269-RVARM, and found that this decreased toxicity (74). Clearly, conservation of a furin recognition site across the whole family of SLTs suggests a potential role for furin in their activation. Perhaps cleavage at this site by furin or other eukaryotic proteases augments but is not essential to activity of the toxins in certain cells or tissues. This may become evident when analysis of mutant ST/SLTs is extended.

Clostridial binary toxins. In addition to producing the better-known and highly potent neurotoxins, *Clostridium botulinum* types C and D produce a toxin designated C_2 toxin. This toxin consists of two separate proteins, I and II (67). Subunit I ADP-ribosylates actin, leading to inhibition of cell division and eventual cell death (2, 68). Subunit II, like the anthrax PA, binds to the surface of target cells. Although most isolates of *C. botulinum* types C and D are proteolytic, subunit II does not appear to be efficiently nicked during production and purification. The potency of the purified toxin is greatly increased if subunit II is treated with trypsin (66). Trypsin cleavage causes subunit II to aggregate and gives it the ability to bind subunit I (66). By incubating fluorescently labelled subunits with Vero cells, Ohishi and Yanagimoto (69) showed that subunit I will bind to subunit II only if the latter has undergone proteolytic processing, a situation which mimics the pattern of interaction of the anthrax PA with LF and EF.

TABLE 2. Toxins having cleavage sites that are not recognized by furin

Toxin	Source	Cleavage site[a]	Reference or source
Cholera toxin	*Vibrio cholerae*	NAPRSSMS	51
Tetanus toxin	*Clostridium tetani*	IRENLYNRTASL	43
Botulinum neurotoxin type A	*Clostridium botulinum* type A	TKSLDKGYNKAL	15
Perfringens iota toxin	*Clostridium perfringens*	NNFFDVRFFSAA	Popoff[b]

[a] The carboxyl side of the boldface residue is the preferred site of cleavage by the bacterial proteases of the producing organism.
[b] Probable cleavage region, deduced from the unpublished sequence provided by M. R. Popoff, Pasteur Institute.

Toxins that share with the C_2 toxin a binary structure and an ADP-ribosylating activity are produced by *Clostridium spiroforme* and *Clostridium perfringens* (3, 81), and it is anticipated that these three toxins will be found to have substantial sequence similarity. The first member of this family for which the complete sequence has been determined is the *C. perfringens* iota toxin (72a). Surprisingly, the cell-binding subunit of iota toxin, designated Ib, has extensive sequence homology to residues 170 to 590 of anthrax toxin PA. Apparently, this is the region in both toxins that is involved in membrane translocation and in binding of the catalytic subunit. Only a single Arg is located in the sequence near the point in iota toxin that is cleaved to remove the amino-terminal proregion. This site does contain two closely spaced occurrences of a Phe-Phe sequence. Interestingly, a Phe-Phe sequence at residues 312 to 313 in anthrax toxin PA is highly susceptible to cleavage by chymotrypsin and by bacterial metalloproteases such as thermolysin. Therefore, the iota toxin may usually be activated by clostridial proteases rather than proteases of the target eukaryotic cells. The fact that the C_2 toxin requires trypsin treatment suggests that it may differ from the iota toxin at the protease cleavage site, and may more closely resemble the anthrax toxin PA in this respect. Whether C_2 toxin contains a furin-susceptible sequence will be known only after its sequence is determined. The fact that iota toxin and anthrax toxin PA have both incorporated a similar translocation domain, as well as sequences that constitute a latent site for subunit interaction, shows that both these features must be functionally efficient motifs for protein toxins.

ROLE FOR ENDOGENOUS PROTEASES IN ACTIVATION OF TOXINS

A number of other bacterial toxins exist which require proteolytic processing for activity, but which contain cleavage sites that are not expected to be susceptible to furin. These toxins include cholera toxin, tetanus toxin, and the botulinum neurotoxins. As shown in Table 2, the sequences at which these toxins are nicked differ widely. Evidence is not yet available to determine whether these toxins are normally activated by bacterial or eukaryotic proteases. All of these toxins are secreted. Bacterial proteases could cleave toxins during or after their secretion. In the case of gram-negative bacteria, cleavage could also occur in the periplasm. Cholera toxin, and the closely related *E. coli* heat-labile enterotoxin, are assembled in the periplasm and then secreted through the outer membrane (30). In *E. coli*, a periplasmic protease, DegP (85), which could nick toxins during their passage through this compartment has been identified. Outer membrane proteins such as the *E. coli* cell surface protease OmpT (26) could also play a role in activation of secreted toxins. Other bacterial proteases could be involved in nicking of toxins, but no direct evidence exists that cleavage by bacterial proteases is involved in the intoxication process in vivo.

Cholera toxin. Cholera toxin has a subunit composition like that of ST. The A subunit is cleaved into A1 and A2 peptides, with the former having ADP-ribosylation activity for certain GTP-binding proteins. No furin site is present. The cholera toxin A subunit is cleaved by *Vibrio cholerae* hemagglutinin/protease at a Ser-Met bond (51). Little is known about whether cleavage can take place within target cells.

Tetanus toxin. Culture supernatants of toxigenic strains of *Clostridium tetani* contain active tetanus toxin (1, 80), which contains two disulfide-linked polypeptides, a heavy chain (105 kDa) with cell-binding activity and a light chain (55 kDa) that is a zinc metalloprotease (77). The toxin is synthesized as a single polypeptide of ~160 kDa, and this unnicked, intracellular toxin can be isolated by special procedures from within the bacteria. Helting et al. (29) isolated a protease from *C. tetani* which was able to nick the intact toxin. Extensive studies with a purified clostridial protease and five other reagent-quality proteases (43) showed that all these proteases cleaved tetanus toxin within residues 445 to 461, which is part of a disulfide loop formed by C-438 and C-466. This region contains two Arg residues but does not contain a recognition site for furin. The purified clostridial protease cleaved a Glu-Asn and then an Ala-Ser bond, whereas the other proteases such as trypsin cleaved at positions expected for their established specificities. Studies with various animal cells and tissues showed that eukaryotic proteases can also cleave tetanus toxin into the heavy and light chains, thereby increasing its potency (27). The current data clearly show that the toxin requires activation and that this could occur in animal cells. Mutagenesis of residues within the disulfide loop will provide information as to the identity of the cellular enzymes that actually participate in activation.

Botulinum neurotoxins. The botulinum neurotoxins (1, 80), of which eight types are now recognized, are synthesized as single polypeptides of approximately 150 kDa. Most of the toxin types, including types A and B, are cleaved by clostridial proteases (14) into two polypeptide chains of ~100 and ~50 kDa. These are held together by a disulfide linkage (44, 87). Type E toxin is typically obtained in an intact form and is toxic only after being activated with trypsin (44). The heavy chains of the botulinum neurotoxins bind to ganglioside or glycolipid receptors on target cells (34), and the light chains are, like the light chain of tetanus toxin, zinc metalloproteases (77, 78). Dekleva and DasGupta (16) characterized a clostridial protease that activates botulinum type A neurotoxin. Cleavage of type A toxin by the clostridial protease occurred first at a Lys-Gly bond, and more slowly at a Lys-Ala bond located four residues carboxyl terminal from the first cleavage site (15). Activation of type E toxin by trypsin was shown to result from cleavage at an Arg-Lys bond (24). None of the botulinum neurotoxins has a furin site at the junction of the heavy and light chains. As in the case of tetanus toxin, the possibility that furin or other eukaryotic proteases are involved in activation may be addressed most efficiently by mutagenesis of the toxins.

PROTEOLYTIC ACTIVATION OF FUSION TOXINS USED AS THERAPEUTIC AGENTS

Many investigators are designing immunotoxins and fusion toxins to be used as therapeutic agents to treat cancer, immune system diseases, and human immunodeficiency virus type 1 (HIV-1) infection. This work has been reviewed (21, 72, 86, 91). Typically, the fusion proteins contain a polypeptide that binds to the target cell receptors, fused to the membrane translocation and catalytic domains of a toxin. In most cases, the proteolytic activation site derived from the toxin is present, and this must be cleaved for the toxin to be active. For fusion toxins containing disulfide-linked chains, the proteins can be nicked prior to administration, although this risks dissociation of the domains. For constructs lacking disulfide bridges, it is important to consider whether the trafficking of the fusion toxin will bring it to an intracellular site where proteolytic activation can occur efficiently.

Manipulation of the protease cleavage site can potentially enhance fusion toxin potency. O'Hare et al. (63) prepared fusion proteins containing ricin A chain linked to staphylococcal protein A. The chimeric protein was not toxic to antibody-coated cells, as was expected from prior work showing that ricin A chain chemical conjugates having uncleavable linkers are inactive. To make a cleavable fusion protein, a 30-residue linker containing the disulfide loop and cleavage site from DT was inserted between the two functional domains. The ricin A-protein A fusion with the linker killed antibody-coated Daudi cells with a 50% effective concentration of 100 pM. Trypsin treatment increased toxicity by a further fourfold. The high potency without trypsin treatment showed that cellular proteases, possibly furin, were cleaving the DT linker during internalization and intracellular trafficking.

A different approach to dealing with the requirement for proteolytic activation was taken in designing a fusion toxin of PE and transforming growth factor alpha (TGF-α). The 37-kDa fragment that is normally generated from PE by intracellular cleavage after R-279 in the sequence R-274-HRQPRG-280 was fused to TGF-α (89). The strategy of making Gly-280 the amino terminus of the fusion protein created a translocation-competent fragment, eliminating the requirement for proteolytic activation. The conjugate was highly toxic to cells having abundant epidermal growth factor receptors (to which TGF-α binds), and it was 20-fold more toxic than the analogous construct having the complete PE sequence, which requires proteolytic activation. The authors concluded that proteolytic cleavage of PE within the cell is normally rate limiting, and that the increased potency resulted from eliminating the need for this processing step.

In a conceptually similar approach, we have designed fusion toxins specific for HIV-1-infected cells by exploiting the absolute requirement that PA be cleaved to be active. The furin site in PA was replaced by a sequence of eight amino acids recognized by HIV-1 protease (48). This mutant PA was shown to be cleaved appropriately by purified HIV-1 protease. It is anticipated that when this altered PA is administered in vivo, it will be activated only after being internalized by HIV-1-infected cells. The activated toxin will then bind and internalize LF or fusions of LF to ADP-ribosylating domains of PE or DT (6), causing specific killing of the HIV-1-infected cells.

FUTURE PROSPECTS AND APPROACHES FOR STUDY OF TOXIN ACTIVATION

Although the evidence for involvement of furin in activation of toxins is compelling, recent evidence has shown that other proteases may contribute to toxin activation in certain circumstances. Two of the toxins discussed above, DT and anthrax PA, have sites containing more than the two Arg residues that constitute the minimal furin recognition sequence. These two toxins may have evolved to be targets for cleavage by several proteases. This could be a safeguard for the toxin to ensure activation by any cell that possesses the toxin receptor. Klimpel et al. (39) found that the PA mutant PA31 having the cleavage site sequence AKKR retained some residual toxicity for macrophages, and subsequent work has shown that PA mutants containing the sequences XXRR are also quite active, while those with XXXR are not (24a). The AKKR and XXRR mutants are evidently being activated by a protease other than furin, perhaps one specific for paired basic residues. The presence of alternate proteases able to activate toxins is also suggested by the fact that selection of somatic cell mutants resistant to DT by a number of different groups never yielded mutants having a phenotype expected for deficiency in furin or other proteases (17, 55). Furin-deficient mutants were obtained only when selection was done with intact PE having the normal cleavage site, RQPR, which can be viewed as a pure furin substrate, i.e., one not susceptible to cleavage by alternate proteases.

Further progress in identifying and characterizing the eukaryotic proteases that activate protein toxins will depend on methods that modulate protease activity in target cells. Methods available include use of sequence-specific protease inhibitors (84), intracellular expression of furin or specific peptide inhibitors of furin (8, 53, 93), and protease gene knockouts (10). One of the most powerful and accessible methods, and one that has already proven invaluable, is the selection of toxin-resistant somatic cell mutants, as discussed above for the PE-resistant CHO cell mutant RPE.40 (54). Methods for selection of CHO cell mutants are well established, and the functionally haploid behavior of many genes in CHO cells makes it possible to obtain mutations at certain sites at frequencies of 10^{-5}. We have recently selected six independent CHO cell mutants that are cross-resistant to PE and to the PA mutant, PA33, having the cleavage site sequence RAAR (39), but remain sensitive to the wild-type PA having the normal sequence RKKR (24a). These mutants are believed to be deficient in furin and are presumed to be similar to RPE.40. Subsequently, we have obtained other CHO cell mutants resistant to a PA protein having the cleavage site sequence XXRR. These mutant CHO cells are highly resistant to the XXRR toxin, but only slightly increased in resistance to native PA, suggesting that they are deficient in enzymes or trafficking mechanisms distinct from those involved in activation by furin.

It is of interest to consider why so many bacterial toxins require proteolytic activation. One answer may be inferred from the fact that this requirement is characteristic of toxins that act catalytically inside cells, but not of those that act in extracellular spaces (staphylococcal enterotoxins, etc.) or on cell surfaces (hemolysins, etc.). The toxins of the former type necessarily contain domains involved in receptor binding and internalization, and distinct domains having catalytic activity. These domains must initially be linked and then later separated. The catalytic domain can only enter target cells successfully if physically attached to the receptor-recognition and translocation domains, but it must then become detached to express its full enzymatic activity. The toxins must have evolved through recombination events that fused domains having the separate functions, and then selective pressures would have favored mutation to produce sites that allow their proteolytic separation. The role of proteolytic activation is less clear for

anthrax toxin and the clostridial binary toxins (C_2, iota, and spiroforme). The amino-terminal domain removed by proteolysis has no role in toxin binding or internalization. It may be needed for proper secretion and folding of the protein, as shown for many proteins secreted by gram-positive bacteria (92), or it could serve to shield the surface involved in interaction with the second toxin component (EF and LF in the case of anthrax) until binding to the receptor has occurred and capture of the second toxin component is needed.

Continued analysis of the pathways for activation of bacterial toxins may point to ways to block the action of the toxins, and thus to prevent bacterial infections and/or the resulting damage caused by the toxins. There is already evidence that furin inhibitors can slow the rate of HIV-1 infection of T cells by blocking the essential cleavage of the envelope gp160 (28). Although furin would seem to be an essential enzyme, the CHO cell mutants deficient in furin discussed above grow at nearly normal rates, suggesting that it may be feasible to use specific furin inhibitors in vivo without causing overt toxicity. A particularly promising approach to inhibition of furin in vivo is the recent construction of a mutant human α_1-antitrypsin inhibitor in which the recognition sequence AIPM-358 is changed to RIPR (5). This protein inhibits furin with a K_i of 30 ng/ml. Because it is only slightly altered from an endogenous human protein, this inhibitor will have a long half-life in plasma and be of low immunogenicity. In addition to helping to explain the virulence properties of pathogenic bacteria, the continuing analysis of proteolytic activation of toxins will undoubtedly aid in the design of improved protein fusion toxins for therapeutic purposes.

ACKNOWLEDGMENTS

We thank Kurt R. Klimpel, Naveen Arora, Peter J. Nicholls, David J. FitzGerald, and April R. Robbins for discussions, Michel R. Popoff for providing the sequence of *C. perfringens* iota toxin prior to publication, and Jerry M. Keith for the continuing encouragement and support that makes our work possible.

REFERENCES

1. **Ahnert-Hilger, G., M. E. Dauzenroth, E. Habermann, A. Henschen, K. Krieglstein, F. Mauler, and U. Weller.** 1990. Chains and fragments of tetanus toxin, and their contribution to toxicity. J. Physiol. (Paris) **84:**229–236.
2. **Aktories, K., M. Barmann, I. Ohishi, S. Tsuyama, K. H. Jakobs, and E. Habermann.** 1986. Botulinum C_2 toxin ADP-ribosylates actin. Nature (London) **322:**390–392.
3. **Aktories, K., and A. Wegner.** 1992. Mechanisms of the cytopathic action of actin-ADP-ribosylating toxins. Mol. Microbiol. **6:**2905–2908.
4. **Allured, V. S., R. J. Collier, S. F. Carroll, and D. B. McKay.** 1986. Structure of exotoxin A of *Pseudomonas aeruginosa* at 3.0-Angstrom resolution. Proc. Natl. Acad. Sci. USA **83:**1320–1324.
5. **Anderson, E. D., L. Thomas, J. S. Hayflick, and G. Thomas.** Inhibition of HIV-1 gp160-dependent membrane fusion by a furin-directed alpha-1-antitrypsin variant. J. Biol. Chem., in press.
6. **Arora, N., and S. H. Leppla.** 1993. Residues 1-254 of anthrax toxin lethal factor are sufficient to cause cellular uptake of fused polypeptides. J. Biol. Chem. **268:**3334–3341.
7. **Barr, P. J.** 1991. Mammalian subtilisins: the long-sought dibasic processing endoproteases. Cell **66:**1–3.
8. **Brennan, S. O., and R. J. Peach.** 1991. The processing of human proinsulin and chicken proalbumin by rat hepatic vesicles suggests a convertase specific for X-Y-Arg-Arg or Arg-X-Y-Arg sequences. J. Biol. Chem. **266:**21504–21508.
9. **Chaudhary, V. K., Y. Jinno, D. FitzGerald, and I. Pastan.** 1990. *Pseudomonas* exotoxin contains a specific sequence at the carboxyl terminus that is required for cytotoxicity. Proc. Natl. Acad. Sci. USA **87:**308–312.
10. **Chauhan, S. S., and M. M. Gottesman.** 1992. Construction of a new universal vector for insertional mutagenesis by homologous recombination. Gene **120:**281–285.
11. **Choe, S., M. J. Bennett, G. Fujii, P. M. Curmi, K. A. Kantardjieff, R. J. Collier, and D. Eisenberg.** 1992. The crystal structure of diphtheria toxin. Nature (London) **357:**216–222.
12. **Cieplak, W., C. Hasemann, and L. Eidels.** 1988. Specific cleavage of diphtheria toxin by human urokinase. Biochem. Biophys. Res. Commun. **157:**747–754.
13. **Collier, R. J., and J. Kandel.** 1971. Structure and activity of diphtheria toxin. I. Thiol-dependent dissociation of a fraction of toxin into enzymically active and inactive fragments. J. Biol. Chem. **246:**1496–1503.
14. **DasGupta, B. R.** 1971. Activation of *Clostridium botulinum* type B toxin by an endogenous enzyme. J. Bacteriol. **108:**1051–1057.
15. **DasGupta, B. R., and M. L. Dekleva.** 1990. Botulinum neurotoxin type A: sequence of amino acids at the N-terminus and around the nicking site. Biochimie **72:**661–664.
16. **Dekleva, M. L., and B. R. DasGupta.** 1990. Purification and characterization of a protease from *Clostridium botulinum* type A that nicks single-chain type A botulinum neurotoxin into the di-chain form. J. Bacteriol. **172:**2498–2503.
17. **Draper, R. K., D. Chin, D. Eurey-Owens, I. E. Scheffler, and M. I. Simon.** 1979. Biochemical and genetic characterization of three hamster cell mutants resistant to diphtheria toxin. J. Cell Biol. **83:**116–125.
18. **Drazin, R., J. Kandel, and R. J. Collier.** 1971. Structure and activity of diphtheria toxin. II. Attack by trypsin at a specific site within the intact toxin molecule. J. Biol. Chem. **246:**1504–1510.
19. **Endo, Y., K. Tsurugi, T. Yutsudo, Y. Takeda, T. Ogasawara, and K. Igarashi.** 1988. Site of action of a Vero toxin (VT2) from *Escherichia coli* O157: H7 and of Shiga toxin on eukaryotic ribosomes. RNA N-glycosidase activity of the toxins. Eur. J. Biochem. **171:**45–50.
20. **Ezzell, J. W., Jr., and T. G. Abshire.** 1992. Serum protease cleavage of *Bacillus anthracis* protective antigen. J. Gen. Microbiol. **138:**543–549.
21. **Frankel, A. E.** 1992. Genetically engineered toxins. Marcel Dekker, Inc., New York.
22. **Friedlander, A. M.** 1986. Macrophages are sensitive to anthrax lethal toxin through an acid-dependent process. J. Biol. Chem. **261:**7123–7126.
23. **Fryling, C., M. Ogata, and D. FitzGerald.** 1992. Characterization of a cellular protease that cleaves *Pseudomonas* exotoxin. Infect. Immun. **60:**497–502.
24. **Gimenez, J. A., and B. R. DasGupta.** 1990. Botulinum neurotoxin type E fragmented with endoproteinase Lys-C reveals the site trypsin nicks and homology with tetanus neurotoxin. Biochimie **72:**213–217.

24a. **Gordon, V. M., et al.** Unpublished data.

25. **Gordon, V. M., S. H. Leppla, and E. L. Hewlett.** 1988. Inhibitors of receptor-mediated endocytosis block the entry of *Bacillus anthracis* adenylate cyclase toxin but not that of *Bordetella pertussis* adenylate cyclase toxin. Infect. Immun. **56:**1066–1069.
26. **Grodberg, J., and J. J. Dunn.** 1988. *ompT* encodes the *Escherichia coli* outer membrane protease that cleaves T7 RNA polymerase during purification. J. Bacteriol. **170:**1245–1253.
27. **Habermann, E., U. Weller, and M. Hudel.** 1991. Limited proteolysis of single-chain tetanus toxin by tissue enzymes, in cultured brain tissue and during retrograde axonal to the spinal cord. Naunyn-Schmiedeberg's Arch. Pharmacol. **343:**323–329.
28. **Hallenberger, S., V. Bosch, H. Angliker, E. Shaw, H. D. Klenk, and W. Garten.** 1992. Inhibition of furin-mediated cleavage activation of HIV-1 glycoprotein gp160. Nature (London) **360:**358–361.
29. **Helting, T. B., S. Parschat, and H. Engelhardt.** 1979. Structure of tetanus toxin. Demonstration and separation of a specific enzyme converting intracellular tetanus toxin to the extracellular form. J. Biol. Chem. **254:**10728–10733.
30. **Hirst, T. R., and J. Holmgren.** 1987. Conformation of protein secreted across bacterial outer membranes: a study of enterotoxin translocation from *Vibrio cholerae*. Proc. Natl. Acad. Sci. USA **84:**7418–7422.
31. **Hosaka, M., M. Nagahama, W. S. Kim, T. Watanabe, K. Hatsu-**

zawa, J. Ikemizu, K. Murakami, and K. Nakayama. 1991. Arg-X-Lys/Arg-Arg motif as a signal for precursor cleavage catalyzed by furin within the constitutive secretory pathway. J. Biol. Chem. **266:**12127–12130.

32. **Jacewicz, M., H. Clausen, E. Nudelman, A. Donohue-Rolfe, and G. T. Keusch.** 1986. Pathogenesis of shigella diarrhea. XI. Isolation of a shigella toxin-binding glycolipid from rabbit jejunum and HeLa cells and its identification as globotriaosylceramide. J. Exp. Med. **163:**1391–1404.
33. **Jinno, Y., M. Ogata, V. K. Chaudhary, M. C. Willingham, S. Adhya, D. FitzGerald, and I. Pastan.** 1989. Domain II mutants of *Pseudomonas* exotoxin deficient in translocation. J. Biol. Chem. **264:**15953–15959.
34. **Kitamura, M., M. Iwamori, and Y. Nagai.** 1980. Interaction between *Clostridium botulinum* neurotoxin and gangliosides. Biochim. Biophys. Acta **628:**328–335.
35. **Kittell, F. B., V. V. Padhye, and M. P. Doyle.** 1991. Characterization and inactivation of verotoxin 1 produced by *Escherichia coli* O157:H7. J. Agric. Food Chem.
36. **Klimpel, K. R., N. Arora, and S. H. Leppla.** 1993. Anthrax toxin lethal factor has homology to the thermolysin-like proteases and displays proteolytic activity, abstr. B-111, p. 45. Abstr. 93rd Gen. Meet. Am. Soc. Microbiol. 1993.
37. **Klimpel, K. R., N. Arora, and S. H. Leppla.** Unpublished data.
38. **Klimpel, K. R., S. S. Molloy, P. A. Bresnahan, G. Thomas, and S. H. Leppla.** 1992. Cleavage of diphtheria toxin and the protective antigen of *Bacillus anthracis* by the eukaryotic protease furin, abstr. B-32, p. 31. Abstr. 92nd. Gen. Meet. Am. Soc. Microbiol. 1992.
39. **Klimpel, K. R., S. S. Molloy, G. Thomas, and S. H. Leppla.** 1992. Anthrax toxin protective antigen is activated by a cell-surface protease with the sequence specificity and catalytic properties of furin. Proc. Natl. Acad. Sci. USA **89:**10277–10281.
40. **Koehler, T. M., and R. J. Collier.** 1991. Anthrax toxin protective antigen: low-pH-induced hydrophobicity and channel formation in liposomes. Mol. Microbiol. **5:**1501–1506.
41. **Kongmuang, U., T. Honda, and T. Miwatani.** 1988. Effect of nicking on Shiga-like toxin I of enterohaemorrhagic *Escherichia coli*. FEMS Microbiol. Lett. **56:**105–108.
42. **Kounnas, M. Z., R. E. Morris, M. R. Thompson, D. J. FitzGerald, D. K. Strickland, and C. B. Saelinger.** 1992. The alpha 2-macroglobulin receptor/low density lipoprotein receptor-related protein binds and internalizes *Pseudomonas* exotoxin A. J. Biol. Chem. **267:**12420–12423.
43. **Krieglstein, K. G., A. H. Henschen, U. Weller, and E. Habermann.** 1991. Limited proteolysis of tetanus toxin. Relation to activity and identification of cleavage sites. Eur. J. Biochem. **202:**41–51.
44. **Krysinski, S., Y. Oga, Y. Kamata, and G. Sakaguchi.** 1985. Activation of *Clostridium botulinum* type B and E derivative toxins with lysine-specific proteases. FEMS Microbiol. Lett. **27:**149–154.
45. **Leppla, S. H.** 1982. Anthrax toxin edema factor: a bacterial adenylate cyclase that increases cyclic AMP concentrations of eukaryotic cells. Proc. Natl. Acad. Sci. USA **79:**3162–3166.
46. **Leppla, S. H.** 1991. The anthrax toxin complex, p. 277–302. *In* J. E. Alouf and J. H. Freer (ed.), Sourcebook of bacterial protein toxins. Academic Press, London.
47. **Leppla, S. H., A. M. Friedlander, and E. Cora.** 1988. Proteolytic activation of anthrax toxin bound to cellular receptors, p. 111–112. *In* F. Fehrenbach, J. E. Alouf, P. Falmagne, W. Goebel, J. Jeljaszewicz, D. Jurgen, and R. Rappouli (ed.), Bacterial protein toxins. Gustav Fischer, New York.
48. **Leppla, S. H., K. R. Klimpel, and N. Arora.** Development of anthrax toxin-based fusion proteins for targeting of HIV-1-infected cells. *In* F. Fehrenbach (ed.), Bacterial protein toxins, in press. Gustav Fischer Verlag, Stuttgart, Germany.
49. **Lindberg, A. A., J. E. Brown, N. Stromberg, M. Westling-Ryd, J. E. Schultz, and K. A. Karlsson.** 1987. Identification of the carbohydrate receptor for Shiga toxin produced by *Shigella dysenteriae* type 1. J. Biol. Chem. **262:**1779–1785.
50. **Madshus, I. H., H. Stenmark, K. Sandvig, and S. Olsnes.** 1991. Entry of diphtheria toxin-protein A chimeras into cells. J. Biol. Chem. **266:**17446–17453.
51. **Mekalanos, J. J., D. J. Swartz, G. D. Pearson, N. Harford, F. Groyne, and M. de Wilde.** 1983. Cholera toxin genes: nucleotide sequence, deletion analysis and vaccine development. Nature (London) **306:**551–557.
52. **Milne, J. C., and R. J. Collier.** 1993. pH-dependent permeabilization of the plasma-membrane of mammalian-cells by anthrax protective antigen. Mol. Microbiol. **10:**647–653.
53. **Misumi, Y., K. Oda, T. Fujiwara, N. Takami, K. Tashiro, and Y. Ikehara.** 1991. Functional expression of furin demonstrating its intracellular localization and endoprotease activity for processing of proalbumin and complement pro-C3. J. Biol. Chem. **266:**16954–16959.
54. **Moehring, J. M., N. M. Inocencio, B. J. Robertson, and T. J. Moehring.** 1993. Expression of mouse furin in a Chinese hamster cell resistant to *Pseudomonas* exotoxin A and viruses complements the genetic lesion. J. Biol. Chem. **268:**2590–2594.
55. **Moehring, J. M., and T. J. Moehring.** 1979. Characterization of the diphtheria toxin-resistance system in Chinese hamster ovary cells. Somatic Cell Genet. **5:**453–468.
56. **Moehring, J. M., and T. J. Moehring.** 1983. Strains of CHO-K1 cells resistant to *Pseudomonas* exotoxin A and cross-resistant to diphtheria toxin and viruses. Infect. Immun. **41:**998–1009.
57. **Molloy, S. S., P. A. Bresnahan, S. H. Leppla, K. R. Klimpel, and G. Thomas.** 1992. Human furin is a calcium-dependent serine endoprotease that recognizes the sequence Arg-X-X-Arg and efficiently cleaves anthrax toxin protective antigen. J. Biol. Chem. **267:**16396–16402.
58. **Moskaug, J. O., K. Sletten, K. Sandvig, and S. Olsnes.** 1989. Translocation of diphtheria toxin A-fragment to the cytosol. Role of the site of interfragment cleavage. J. Biol. Chem. **264:**15709–15713.
59. **Naglich, J. G., J. E. Metherall, D. W. Russell, and L. Eidels.** 1992. Expression cloning of a diphtheria toxin receptor: identity with a heparin-binding EGF-like growth factor precursor. Cell **69:**1051–1061.
60. **Nakayama, K., T. Watanabe, T. Nakagawa, W. S. Kim, M. Nagahama, M. Hosaka, K. Hatsuzawa, K. Kondoh-Hashiba, and K. Murakami.** 1992. Consensus sequence for precursor processing at mono-arginyl sites. Evidence for the involvement of a Kex2-like endoprotease in precursor cleavages at both dibasic and mono-arginyl sites. J. Biol. Chem. **267:**16335–16340.
61. **O'Brien, A. D., and G. D. LaVeck.** 1983. Purification and characterization of a *Shigella dysenteriae* 1-like toxin produced by *Escherichia coli*. Infect. Immun. **40:**675–683.
62. **O'Brien, A. D., V. L. Tesh, A. Donohue-Rolfe, M. P. Jackson, S. Olsnes, K. Sandvig, A. A. Lindberg, and G. T. Keusch.** 1992. Shiga toxin: biochemistry, genetics, mode of action, and role in pathogenesis. Curr. Top. Microbiol. Immunol. **180:**65–94.
63. **O'Hare, M., A. N. Brown, K. Hussain, A. Gebhardt, G. Watson, L. M. Roberts, E. S. Vitetta, P. E. Thorpe, and J. M. Lord.** 1990. Cytotoxicity of a recombinant ricin-A-chain fusion protein containing a proteolytically-cleavable spacer sequence. FEBS Lett. **273:**200–204.
64. **Ogata, M., V. K. Chaudhary, I. Pastan, and D. J. FitzGerald.** 1990. Processing of *Pseudomonas* exotoxin by a cellular protease results in the generation of a 37,000-Da toxin fragment that is translocated to the cytosol. J. Biol. Chem. **265:**20678–20685.
65. **Ogata, M., C. M. Fryling, I. Pastan, and D. J. FitzGerald.** 1992. Cell-mediated cleavage of *Pseudomonas* exotoxin between Arg279 and Gly280 generates the enzymatically active fragment which translocates to the cytosol. J. Biol. Chem. **267:**25396–25401.
66. **Ohishi, I.** 1987. Activation of botulinum C_2 toxin by trypsin. Infect. Immun. **55:**1461–1465.
67. **Ohishi, I., M. Iwasaki, and G. Sakaguchi.** 1980. Purification and characterization of two components of botulinum C_2 toxin. Infect. Immun. **30:**668–673.
68. **Ohishi, I., and S. Tsuyama.** 1986. ADP-ribosylation of nonmuscle actin with component I of C_2 toxin. Biochem. Biophys. Res. Commun. **136:**802–806.
69. **Ohishi, I., and A. Yanagimoto.** 1992. Visualizations of binding and internalization of two nonlinked protein components of botulinum C_2 toxin in tissue culture cells. Infect. Immun. **60:**4648–4655.
70. **Olsnes, S., R. Reisbig, and K. Eiklid.** 1981. Subunit structure of Shigella cytotoxin. J. Biol. Chem. **256:**8732–8738.

71. **Pappenheimer, A. M., Jr.** 1977. Diphtheria toxin. Annu. Rev. Biochem. **46:**69–94.
72. **Pastan, I., V. Chaudhary, and D. J. FitzGerald.** 1992. Recombinant toxins as novel therapeutic agents. Annu. Rev. Biochem. **61:**331–354.
72a. **Popoff, M.** Personal communication.
73. **Samuel, J. E., and V. M. Gordon.** Evidence that proteolytic separation of Shiga-like toxin type IIv (SLT-IIv) A subunit into A1 and A2 subunits is not required for toxin activity. J. Biol. Chem., in press.
74. **Sandvig, K., E. Dubinina, O. Garred, K. Prydz, J. V. Kozlov, S. H. Hansen, and B. Vandeurs.** 1993. Entry of Shiga toxin into cells. Zentralbl. Bakteriol.-Int. J. Med. Microbiol. Virol. Parasitol. Infect. Dis. **278:**296–305.
75. **Sandvig, K., O. Garred, K. Prydz, J. V. Kozlov, S. H. Hansen, and B. van Deurs.** 1992. Retrograde transport of endocytosed Shiga toxin to the endoplasmic reticulum. Nature (London) **358:**510–512.
76. **Sandvig, K., and S. Olsnes.** 1981. Rapid entry of nicked diphtheria toxin into cells at low pH. Characterization of the entry process and effects of low pH on the toxin molecule. J. Biol. Chem. **256:**9068–9076.
77. **Schiavo, G., F. Benfenati, B. Poulain, O. Rossetto, P. Polverino de Laureto, B. R. Dasgupta, and C. Montecucco.** 1992. Tetanus and botulinum-B neurotoxins block neurotransmitter release by proteolytic cleavage of synaptobrevin. Nature (London) **359:**832–835.
78. **Schiavo, G., O. Rossetto, A. Santucci, B. R. Dasgupta, and C. Montecucco.** 1992. Botulinum neurotoxins are zinc proteins. J. Biol. Chem. **267:**23479–23483.
79. **Seetharam, S., V. K. Chaudhary, D. FitzGerald, and I. Pastan.** 1991. Increased cytotoxic activity of *Pseudomonas* exotoxin and two chimeric toxins ending in KDEL. J. Biol. Chem. **266:**17376–17381.
80. **Simpson, L. L.** 1986. Molecular pharmacology of botulinum toxin and tetanus toxin. Annu. Rev. Pharmacol. Toxicol. **26:**427–453.
81. **Simpson, L. L., B. G. Stiles, H. Zepeda, and T. D. Wilkins.** 1989. Production by *Clostridium spiroforme* of an iotalike toxin that possesses mono(ADP-ribosyl)transferase activity: identification of a novel class of ADP-ribosyltransferases. Infect. Immun. **57:**255–261.
82. **Singh, Y., V. K. Chaudhary, and S. H. Leppla.** 1989. A deleted variant of *Bacillus anthracis* protective antigen is non-toxic and blocks anthrax toxin action *in vivo*. J. Biol. Chem. **264:**19103–19107.
83. **Steiner, D. F., S. P. Smeekens, S. Ohagi, and S. J. Chan.** 1992. The new enzymology of precursor processing endoproteases. J. Biol. Chem. **267:**23435–23438.
84. **Stieneke-Grober, A., M. Vey, H. Angliker, E. Shaw, G. Thomas, C. Roberts, H. D. Klenk, and W. Garten.** 1992. Influenza virus hemagglutinin with multibasic cleavage site is activated by furin, a subtilisin-like endoprotease. EMBO J. **11:**2407–2414.
85. **Strauch, K. L., K. Johnson, and J. Beckwith.** 1989. Characterization of *degP*, a gene required for proteolysis in the cell envelope and essential for growth of *Escherichia coli* at high temperature. J. Bacteriol. **171:**2689–2696.
86. **Strom, T. B., P. L. Anderson, V. E. Rubin-Kelley, D. P. Williams, T. Kiyokawa, and J. R. Murphy.** 1991. Immunotoxins and cytokine toxin fusion proteins. Ann. N.Y. Acad. Sci. **636:**233–250.
87. **Syuto, B., and S. Kubo.** 1981. Separation and characterization of heavy and light chains from *Clostridium botulinum* type C toxin and their reconstitution. J. Biol. Chem. **256:**3712–3717.
88. **Takao, T., T. Tanabe, Y. M. Hong, Y. Shimonishi, H. Kurazono, T. Yutsudo, C. Sasakawa, M. Yoshikawa, and Y. Takeda.** 1988. Identity of molecular structure of Shiga-like toxin I (VT1) from *Escherichia coli* O157:H7 with that of Shiga toxin. Microb. Pathog. **5:**57–69.
89. **Theuer, C. P., D. FitzGerald, and I. Pastan.** 1992. A recombinant form of *Pseudomonas* exotoxin directed at the epidermal growth factor receptor that is cytotoxic without requiring proteolytic processing. J. Biol. Chem. **267:**16872–16877.
90. **van de Ven, W. J., J. Voorberg, R. Fontijn, H. Pannekoek, A. M. van den Ouweland, H. L. van Duijnhoven, A. J. Roebroek, and R. J. Siezen.** 1990. Furin is a subtilisin-like proprotein processing enzyme in higher eukaryotes. Mol. Biol. Rep. **14:**265–275.
91. **Vitetta, E. S., and P. E. Thorpe.** 1991. Immunotoxins containing ricin or its A chain. Semin. Cell Biol. **2:**47–58.
92. **Wandersman, C.** 1989. Secretion, processing and activation of bacterial extracellular proteases. Mol. Microb. **3:**1825–1831.
93. **Wasley, L. C., A. Rehemtulla, J. A. Bristol, and R. J. Kaufman.** 1993. PACE/furin can process the vitamin K-dependent pro-factor IX precursor within the secretory pathway. J. Biol. Chem. **268:**8458–8465.
94. **Watanabe, T., K. Murakami, and K. Nakayama.** 1993. Positional and additive effects of basic amino acids on processing of precursor proteins within the constitutive secretory pathway. FEBS Lett. **320:**215–218.
95. **Watson, D. G., J. M. Moehring, and T. J. Moehring.** 1991. A mutant CHO-K1 strain with resistance to *Pseudomonas* exotoxin A and alphaviruses fails to cleave Sindbis virus glycoprotein PE2. J. Virol. **65:**2332–2339.
96. **Williams, D. P., Z. Wen, R. S. Watson, J. Boyd, T. B. Strom, and J. R. Murphy.** 1990. Cellular processing of the interleukin-2 fusion toxin DAB_{486}-IL-2 and efficient delivery of diphtheria fragment A to the cytosol of target cells requires Arg194. J. Biol. Chem. **265:**20673–20677.
97. **Yoshida, T., C. C. Chen, M. S. Zhang, and H. C. Wu.** 1991. Disruption of the Golgi apparatus by brefeldin A inhibits the cytotoxicity of ricin, modeccin, and *Pseudomonas* toxin. Exp. Cell Res. **192:**389–395.