Part I  Toxins from Microorganisms
1 Bacterial Toxins with Metalloprotease Activity

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1.1 INTRODUCTION

Metalloproteases are hydrolytic enzymes characterised by an active site containing a metal atom, usually zinc. They include amino-peptidases, carboxypeptidases and endopeptidases depending on whether they remove an N- or a C-terminal residue or cleave internal peptide bonds of the protein substrate. Zinc-dependent endopeptidases are characterised by the presence of a zinc-binding motif consisting of His-Glu-X-X-His [1]. Hundreds of different metalloproteases are produced by microorganisms and are involved in their metabolic activities or are released outside to cleave substrates not elaborated by the microorganism itself. The present chapter will only deal with bacterial metalloproteases which act on a specific cellular or tissue target of the mammalian bacterial host.

The recent determination of their primary sequence has led to the discovery of the metalloproteolytic activity of the bacterial toxins responsible for tetanus, botulism and anthrax. The protease domain of these toxins enters into the cytosol where it displays a zinc-dependent endopeptidase activity of remarkable specificity. Tetanus (TeNT) and botulinum toxins (BoNTs) cleave three protein components of the neuroexocytosis machinery leading to the blockade of neurotransmitter release and consequent paralysis. BoNTs are increasingly used in medicine for the treatment of human diseases characterised by hyperfunction of cholinergic terminals.

The lethal factor of Bacillus anthracis is specific for the MAP kinase-kinases that are cleaved within their amino-terminus. In this case, however, such a specific biochemical lesion has not yet been correlated with the pathogenesis of anthrax.

Fragilysin (BFT) is produced by toxigenic strains of the intestinal pathogen Bacteroides fragilis and attacks E-cadherin.

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1.2 TETANUS AND BOTULINUM NEUROTOXINS

1.2.1 INTRODUCTION

Tetanus neurotoxin (TeNT) and botulinum neurotoxin (BoNT) were identified as the sole cause of tetanus and botulism, respectively, a little over a century ago, after the discovery of the anaerobic and spore-forming bacteria of the genus Clostridium [2–4]. There are seven types of BoNT (indicated with letters from A to G) which differ in antigenicity and biochemical activity [5, 6]. BoNTs bind to and enter peripheral cholinergic terminals, causing a sustained block of acetylcholine (ACh) release, with ensuing flaccid paralysis and autonomic symptoms. Tetanus neurotoxin (TeNT) acts on the CNS and blocks neurotransmitter release at the inhibitory interneurons of the spinal cord, resulting in a frequently lethal spastic paralysis. Despite the opposite clinical symptoms of tetanus and botulism, the neurotoxins affect the same neuronal function: neuroexocytosis [7, 8].

1.2.2 GENETICS AND STRUCTURE

In C. tetani and in C. botulinum G, the neurotoxin genes are contained within large plasmids, whereas in C. botulinum A, B, E and F the neurotoxin genes have a chromosomal localisation and in C. botulinum C and D toxins are encoded by bacteriophages. Usually one bacterium harbours one toxin gene, but several cases of multiple toxin genes have been reported [9]. These genes do not contain a secretion signal sequence and the protein neurotoxins are released by bacterial autolysis as single polypeptide chains of 150 kDa, which are later activated by a specific proteolytic cleavage within a loop subtended by a highly conserved disulphide bridge [10]. The heavy chain (H, 100 kDa) and the light chain (L, 50 kDa) remain associated via noncovalent interactions and by the conserved interchain S–S bond, whose integrity is essential for neurotoxicity, but which has to be reduced to allow the display of the metalloproteolytic activity of the L chain in the cytosol [11] (Figure 1.1A).

The crystallographic structures of BoNT/A and BoNT/B and of the C-terminal part of the heavy chain of TeNT revealed that the 50 kDa receptor binding domain, termed Hc, consists of two sub-domains [12–14]. The N-terminal part of Hc (HcN) consists of sixteen β-strands and four α-helices arranged in a jelly roll motif, closely similar to that of carbohydrate-binding proteins of the legume lectin family [14, 15]. The amino acid sequence of this sub-domain is highly conserved among BoNTs and TeNT, suggesting a closely similar three-dimensional structure. In contrast, the sequence of the C-terminal part of Hc (HcC) is poorly conserved, but folds similarly to proteins of the trypsin inhibitor family. On the basis of experiments performed with TeNT, it was suggested that HcC plays a major role in neurospecific binding [16].
Figure 1.1 The three-functional domain structure of CNTs. a) Schematic structure of activated di-chain CNTs. The neurotoxin is composed of two polypeptide chains held together by a single disulphide bridge. The C-terminal portion of the heavy chain (H, 100 kDa) is responsible for neurospecific binding (domain Hc) while the N-termis (HN) is implicated in the translocation of the light chain in cytosol and pore formation. Structurally Hc can be further subdivided into two portions of 25 kDa. HcN and HcC. The light chain (L, 50 kDa) is a zinc-endopeptidase responsible for the intracellular activity of CNTs. The segments presenting high homology between different serotypes are in black. A short α-helix (217–229 in BoNT/A and 227–239 in TeNT), in the central part of the L chain, shows the highest homology and contains the zinc-binding motif of metallo-endopeptidases. Amino acids involved in the coordination of zinc or in the hydrolysis of the substrate are indicated by an arrow. The glutamic acid coordinating a water molecule responsible for the target hydrolysis is indicated by an asterisk. b) The active site architecture shows a primary sphere of zinc coordinating residues (circle) and a secondary layer of residues, not as close to the zinc centre (broken circle).
The N-terminal part of the heavy chain (H N) features two ~ 100 Å-long antiparallel α-helices, similar to those of the membrane interacting proteins colicins and influenza hemagglutinin [14, 15]. The H Ns of the CNTs are highly homologous and their predicted secondary structures are also highly similar, in agreement with their proposed role in transmembrane translocation of the L chain [15, 17].

The L chain is a metalloprotease with little protein–protein interaction with the adjacent translocation domain (H N), which is in turn linked to the receptor-binding domain. At the centre of the long cleft-shaped active site there is a zinc atom coordinated via the two histidines and the glutamic residues of the zinc-binding motif, and by Glu262 in BoNT/A and Glu268 in BoNT/B, a residue conserved among clostridial neurotoxins which corresponds to Glu271 of TeNT (Figure 1.1A). The Glu residue of the motif is particularly important because it coordinates the water molecule which actually performs the hydrolytic reaction of proteolysis. Its mutation leads to complete inactivation of these neurotoxins [18]. The critical role of Glu271 of TeNT and Glu262 in BoNT/A has been shown to be that of providing a negatively charged carboxylate moiety ([19] and in preparation). This active site architecture is similar to that of thermolysin and identifies a primary sphere of residues essential to the catalytic function, which coincides with the zinc coordinating residues. In addition, it appears that a secondary layer of residues, less close to the zinc centre, is present at the active site of clostridial neurotoxins (Figure 1.1B). Among these residues, Arg363 and Tyr366 in BoNT/A could play a role in the catalytic activity of this family of metalloproteases. In particular, Tyr366 in BoNT/A (corresponding to Tyr373 in BoNT/B and to Tyr375 in TeNT) point its phenolic ring inside the cleft-shaped active site of the toxin [14, 15]. The mutation of Tyr375 with an alanine inactivates the TeNT L chain, clearly indicating that this residue plays a critical role in the hydrolysis of the substrate [19]. It has been proposed that Tyr373 of BoNT/B assists the hydrolysis reaction by donating a proton to the amide nitrogen of VAMP Phe77 which, together with bound water molecules, stabilises the leaving group [20].

The active site of the L chain faces the H chain in the unreacted form, accounting for its lack of proteolytic activity, and becomes accessible to the substrate upon reduction of the interchain disulphide bridge. Their proteolytic activity is zinc-dependent and heavy metal chelators such as ortho-phenantroline, which remove bound zinc, generate inactive apo-neurotoxins, but the active site metal atom can be reacquired upon incubation of apo-toxin in zinc-containing buffers [11]. The biochemical and structural properties of clostridial neurotoxins define them as a distinct group of metalloproteases, whose origin cannot at present be traced to any of the known families of metalloprotease [11].

Such structural organisation of the CNTs has been shaped by evolution to fulfil the requirements of their mode of neuron intoxication which consists of four steps [17, 21]: 1) binding, 2) internalisation, 3) membrane translocation, and 4) proteolytic cleavage of their substrates (Figure 1.2).
**Figure 1.2** Entry of BoNTs and TeNT inside nerve terminals. ① BoNTs and TeNT bind to the presynaptic membrane at as yet unidentified receptors of peripheral nerve terminals. ② The protein receptor of TeNT would be responsible for its inclusion in an endocytic vesicle that moves in a retrograde direction all along the axon to the inhibitory interneurons of the spinal cord (CNS), whereas BoNT protein receptors would guide them inside vesicles that acidify within the NMJ. ③ At low pH, BoNTs and TeNT change conformation, insert into the lipid bilayer of the vesicle membrane and translocate the L chain into the cytosol of peripheral and central neurons respectively. ④ Inside the cytosol the L chain catalyses the proteolysis of one of the three SNARE proteins.

### 1.2.3 NEURONAL INTOXICATION

From the site of production or adsorption (intestine or wounds), BoNTs and TeNT diffuse in the body fluids, up to the presynaptic membrane of cholinergic terminals where they bind very specifically. The Hc domain plays a major role in neurospecific binding [22], but additional regions may be involved in determining the remarkable specificity for cholinergic terminals of CNTs.

Identification of the presynaptic receptor(s) of CNTs has been attempted by several investigators. Polysialogangliosides are certainly involved [23–25] together with as yet unidentified proteins of the presynaptic membrane [26]. The presence of both lectin-like and protein binding sub-domains in the Hc domain supports the suggestion that CNTs bind strongly and specifically to the
presynaptic membrane because they display multiple interactions with sugar- and protein-binding sites [14, 15]. Recently, BoNT/B was shown to bind strongly to the synaptic vesicle protein synaptotagmin II only in the presence of polysialogangliosides [24], but its role in vivo remains to be established. Identification of the receptors for the various CNTs will constitute a major advance in the understanding of the mechanism of neuron intoxication and help to improve current therapeutic protocols employing BoNT to treat human syndromes of hyperfunction of cholinergic terminals and excessive muscle contraction.

As depicted in Figure 1.2, the L chains of CNTs block neuroexocytosis by acting in the cytosol and they reach this cell compartment following endocytosis and membrane translocation. They are internalised inside acidic cellular compartments via a temperature- and energy-dependent process [27]. Nerve stimulation facilitates intoxication by CNTs [28] and a close link exists between stimulus-contraction coupling and endocytosis at nerve terminals [29]. Hence endocytosis and other factors such as nerve stimulation-dependent proteolytic activity in the cytosol [30] may partly account for this effect, which is potentially very relevant for the development of novel protocols of therapy employing BoNT. The protein receptor of TeNT would be responsible for its inclusion in an endocytic vesicle that moves in a retrograde direction all along and inside the axon, whereas BoNTs’ protein receptors would guide them inside vesicles that acidify within the NMJ. The TeNT-carrying vesicles reach the cell body in the spinal cord and then move to dendritic terminals to release the toxin in the intersynaptic space. TeNT equilibrates between pre- and post-synaptic membranes and then binds and enters the inhibitory interneurons of the spinal cord via synaptic vesicle endocytosis.

To reach the cytosol the L chain has to cross the hydrophobic barrier of the vesicle membrane (Figure 1.2) and the acidity of the lumen is essential for such a movement. CNTs have to be exposed to a low pH step for nerve intoxication to occur [31]. Acidic pH does not induce a direct activation of the toxin via a structural change, but is required in the process of transmembrane translocation of the L chain itself. CNTs undergo a low pH-driven conformational change from a water-soluble ‘neutral’ structure to an ‘acid’ structure characterised by the surface exposure of hydrophobic patches, which lead the H and L chains in the hydrocarbon core of the lipid bilayer [32]. Following the low pH-induced membrane insertion, BoNTs and TeNT form transmembrane ion channels in planar lipid bilayers of low conductance [33, 34]. There is a general consensus that the toxin channels participate in the process of transmembrane translocation of the L domain, from the vesicle membrane to the nerve terminal cytosol, but there is no agreement on how this process may take place. The BoNT translocation domain is different from those of other pore-forming toxins since the long pair of α-helices, with their triple helix bundle at either end, resemble more some coiled coil viral proteins [14, 15] which do not translocate through pores but change structure at low pH and insert into membranes. It has been proposed that the L chain translocates across the
vesicle membrane within a channel opened laterally to lipids, rather than inside a wholly proteinaceous pore [21], accounting for the fact that the L chain does contact the fatty acid chains of lipids during translocation [33]. The H chain is suggested to form a transmembrane hydrophilic cleft that nests in the passage of the partially unfolded L chain with its hydrophobic segments facing the lipids. Facing the cytosolic neutral pH, the L chain refolds and regains its water-soluble neutral conformation. This model is also supported by the finding that the protein-translocating channel of the endoplasmic reticulum has been shown to be open laterally to lipids.

Once in the cytosol, CNTs exploit their catalytic activity. BoNTs and TeNT are remarkably specific proteases that recognise and cleave only three proteins, the so-called SNARE proteins, which form the core of the neuroexocytosis machinery [17, 26]. TeNT, BoNT/B, /D, /F and /G cleave VAMP, at different single peptide bonds [17, 35]; BoNT/A and /E cleave SNAP-25 at different sites within the COOH-terminus whereas BoNT/C cleaves both syntaxin and SNAP-25 [36–38]. Strikingly, TeNT and BoNT/B cleave VAMP at the same peptide bond (Gln76–Phe77) and yet, when injected into the animal, they cause the opposite symptoms of tetanus and botulism, respectively [35], conclusively demonstrating that the different symptoms of the two diseases derive from different sites of intoxication rather than from a different molecular mechanism of action.

VAMP, SNAP-25 and syntaxin form a heterotrimeric coil-coiled complex, termed the SNARE complex, which induces the juxtaposition of vesicle to the target membrane [39] and is involved in their fusion [40]. VAMP is a family of vesicular SNAREs with a short C-terminal tail facing the vesicle lumen, a single transmembrane domain and the remaining N-terminal part exposed to the cytosol. Different VAMP isoforms are located on different cell vesicles and contribute to address each vesicle to its appropriate target membrane with which it will fuse. VAMP-1 and -2 are the isoforms mainly involved in the binding and fusion of neurotransmitter-containing synaptic vesicles with the presynaptic membrane (neuroexocytosis). Syntaxin is anchored to target membranes via a C-terminal hydrophobic tail. Of the many syntaxin isoforms presently known, syntaxin 1A, 1B and 2 are the isoforms mainly involved in neuroexocytosis. SNAP-25 (few isoforms) are 25 kDa SNARE proteins bound to the target membrane via fatty acids covalently linked to cysteine residues present in the middle of the polypeptide chain.

The proteolysis of one SNARE protein prevents the formation of the complex and consequently the release of the neurotransmitter. The SNARE complex is insensitive to CNT proteolysis [41], as expected on the basis of the fact that proteases are known to attack predominantly unstructured exposed loops.

The molecular basis of the specificity of the metalloprotease activity of the clostridial neurotoxins for the three SNAREs is only partially known. The sequences flanking the cleavage sites of the three CNT substrates do not show a conserved pattern that could account for such specificity. Experimental
evidence indicates the involvement of a nine-residue-long motif, termed hereafter SNARE motif and characterised by three carboxylate residues alternated with hydrophobic and hydrophilic residues [42–44]. The motif is present in two copies (V1 and V2) in VAMP and syntaxin and four copies in SNAP-25. The various CNTs differ with respect to the specific interaction with the SNARE motif [26]. The findings that only protein segments including at least one SNARE motif are cleaved by the toxins and that the motif is exposed at the protein surface [42, 43, 45] clearly indicate the involvement of the SNARE motif in the specificity of action of botulinum neurotoxins. Moreover, different SNARE isoforms coexist within the same cell [46], but only some of them are susceptible to proteolysis by the CNTs and it has been shown that resistance is associated with mutations in SNARE motifs or at the cleavage site [17].

1.3 THE ANTHRAX LETHAL FACTOR

1.3.1 INTRODUCTION

Anthrax is a disease of animals and humans, caused by toxigenic strains of Bacillus anthracis, a Gram positive spore-forming bacterium, which secretes three distinct proteins, acting in binary combinations [47]. They are: the protective antigen (PA) which elicits a protective immune response against anthrax [48], the lethal factor (LF) and the oedema factor (EF). The three proteins are encoded by genes included in a large plasmid (pXO1) and are synthesised and secreted independently. The injection of PA+LF (LeTx, lethal toxin) causes a rapid death of laboratory animals [49]. LeTx lyses some cell lines and primary cultures of murine macrophages [50, 51] whereas intradermal injection of PA+EF (EdTx, oedema toxin) produces oedema in the skin [47]. Separately, none of these proteins is toxic. It is now well established that they represent a unique variation in the A-B toxin pattern. PA is the common cell-binding domain (B) which mediates the entry into the cytosol of two different enzyme domains (A): EF and LF, which elicit cell damage. EF is a calmodulin-dependent adenylate cyclase [52] whereas LF is a zinc-binding protein which includes the HEXXH motif and acts in the cytosol via a metalloproteolytic activity [53, 54].

1.3.2 THE PROTECTIVE ANTIGEN PA AND TOXIN INTERNALISATION AND TRANSLOCATION

The mature protective antigen (PA83) is a 735-amino-acid protein (82 kDa) and the crystal structure shows that it is a long, flat protein, rich in β-sheet structure [55]. The role of PA is to cause binding of LF and EF to the cell
surface, so that they will be internalised by endocytosis, and to provide a membrane channel for their translocation from the endosome to the cytosol (Figure 1.3). The first step corresponds to the highly specific binding of PA to the cell surface receptor. The receptor of PA, partially proteinaceous, is present on many cell types [56] and is still unidentified. Upon cell binding, PA is cleaved by furin or furin-like proteases and the N-terminal 20 kDa fragment (PA20) is released [57]. The proteolytic activation of PA83 is essential since it exposes the binding site for EF and LF and allows the oligomerisation of the remaining 63 kDa fragment (PA63) in a ring-shaped heptamer. The heptamer then binds LF and EF competitively. Some data support the hypothesis that each PA63 monomer binds one EF or LF, suggesting the binding of seven molecules of LF and/or EF per heptamer. Formation of the heptamer and fixation of EF and/or LF is then followed by the internalisation of this hetero-oligomeric complex. Oligomerisation triggers receptor-mediated endocytosis of receptor-bound PA63 [58] and both EdTx and LeTx require passage through an acidic vesicle to enter the cytosol, because inhibitors of endosomal acidification or of endocytosis prevent toxicity [57, 59]. At acidic pH the PA63-heptamer pre-pore converts to an active pore and it is assumed that LF/EF translocate through the lumen of the pore, but little is known of how this process occurs. Several data suggest that EF and LF participate actively in their translocation. The two

Figure 1.3 Schematic representation of anthrax LF toxin action. Upon cell binding, PA is cleaved by furin and the N-terminal 20 kDa fragment (PA20) is released. The proteolytic activation of PA83 is essential since it exposes the binding site for LF (or EF) and allows the oligomerization of the remaining 63 kDa fragment (PA63) in a ring-shaped heptamer. Formation of the heptamer and fixation of LF is then followed by the internalization of this hetero-oligomeric complex through an acidic vesicle. At acidic pH the PA63-heptamer pre-pore converts to an active pore and LF translocates through the lumen of the pore to the cytosol where it catalyses the proteolysis of the MAP kinase kinases (MAPKK) (modified from [57])
components interact with lipid bilayers in a pH-dependent manner [54] and this interaction, optimal at acidic pH, is irreversible for EF and reversible for LF. Moreover, recent experiments on CHO-K1 cells, show that LF is completely translocated into the cytoplasm whereas EF remains membrane-associated [60]. The two components may be translocated in a partially unfolded state [61, 62].

1.3.3 THE METALLOPROTEOLYTIC ACTIVITY OF THE LETHAL FACTOR LF

The mature protein contains 776 residues with an apparent molecular mass of 85 kDa. The molecule can be divided into three parts. The aminoterminal part of LF (LF254) is involved in the binding to PA and has a substantial similarity with the aminoterminal part of EF [63]. The central part of the molecule (307–383) contains a series of four imperfect repeats rich in glutamate, and deletions in this region render the protein unstable and inactive [64]. The catalytic domain resides in the C-terminal part and the recognition that LF contains the consensus sequence of zinc metalloprotease [53] started a process that eventually led to the identification of its catalytic activity. Substitution of Ala for H686, E687 or H690 in the sequence $\text{HEFGH}_{690}$ abolishes the binding of zinc to LF and its toxicity on macrophage cell lines and on Fisher rat 344 [53, 65]. From these results it became quite obvious that, like the clostridial botulinum and tetanus toxins, LF is a zinc protease, and this hypothesis was supported by the discovery that inhibitors of zinc-dependent aminopeptidases (e.g. bestatin, aromatic amino acid amides and hydroxamates) protect macrophages from LF [53, 66]. However, searches for a cellular substrate continued until recently. Independently, and by two totally different experimental approaches, LF was shown to cleave the aminoterminus of mitogen-activated protein kinases (MAPKs) Mek1 and Mek2 [67, 68]. One group identified Mek1 and Mek2 as metalloproteolytic substrate of LF following the finding that the MAPKK inhibitor PD98059 and LF gave similar profiles of toxicity with respect to a series of tumoral cell lines [67]. The other group identified the same cytosolic substrates of LF metalloproteolytic activity by a yeast two-hybrid technique, using as bait an LF mutated at the glutamic acid 687 (LF$^{\text{E687A}}$) of the zinc-binding motif to screen a Hela c-DNA library. The MAPK pathway relays environmental signals to the transcriptional machinery in the nucleus and thus modulates gene expression via a burst of protein phosphorylation. Seven different MAPKs are known, composing three distinct MAPK cascades [69]. Recently, it has been reported that LF cleaves all the MAPKs except MAPKK5 and a consensus motif for the cleavage site was identified [70, 71]. Cleavage invariably occurs within the N-terminal proline-rich region preceding the kinase domain, and the release of the N-terminal part of MAPKs is accompanied by phosphorylation of the MAP kinases (MAPK) ERK in cultured macrophages [68]. Phosphorylated ERK is the nuclear active
form of the protein and it is possible that in macrophages phosphorylated ERK starts a pathway leading to their death. Although the signaling pathways involving MAPKKs play a crucial role in the activation of macrophages and are directly involved in the production of cytokines such as TNF, IL-1 and IL-6, the link between the cleavage of MAPKKs by LF, macrophage lysis and pathogenesis remains unclear. Release of proinflammatory cytokines potentially elaborated by macrophages upon treatment with LeTx could account for shock, and mice depleted of macrophages have been reported to become insensitive to LeTx challenge [72]. However, there are conflicting data concerning the modulation of TNF and IL-1 by LeTx. Whereas it was reported that sublytic doses of LeTx induced production of these cytokines [72], a lack of effect and an LeTx inhibition of the production of NO and TNF induced by LPS were reported [70, 73]. Moreover, macrophage cell lines resistant to the lytic effect of LeTx, and peritoneal macrophages isolated from mouse strains insensitive to LeTx, are still sensitive to the protease activity of LF on their MAPKKs. The lack of correlation seems to indicate that there are other cytosolic targets of LF involved in cytotoxicity.

1.4 FRAGILYSINS FROM BACTEROIDES FRAGILIS

1.4.1 INTRODUCTION

*Bacteroides fragilis* spp. are included in the normal commensal intestinal flora of the majority of adults (< 0.5% of the total), but have recently been identified as anaerobic bacteria in isolates from clinical specimens, bloodstream infections and abdominal abscesses [74, 75]. Toxigenic *B. fragilis* strains, termed entero-toxigenic *B. fragilis* (ETBF), were first identified during studies of an epidemic of diarrheal disease in lambs, and it was later found in many other animal species [76, 77]. A 20 kDa fraction of culture filtrates of ETBF stimulated a striking secretory response in lamb ligated intestinal segments [78], suggesting the presence of a secreted toxin, termed fragilysin and abbreviated BFT [79]. In different countries, the association of *B. fragilis* with human diarrheal diseases is well established [80, 81, 82], though we have yet to assess the link between severity of the disease and expression and release of BFT. Recently, an association between clinically active inflammatory bowel disease and infection with toxigenic *B. fragilis* was suggested [83].

1.4.2 GENETICS AND STRUCTURE OF FRAGILYSIN (BFT)

Three highly homologous genes encoding for BFTs have been identified so far [84–88] and were termed *bft-1* (from strain VPI 13784), *bft-2* (from strain 86-5443-2-2) and *bft-3* (from strain Korea 419) [79, 86, 89]. These genes are
included in a 6-kb region, present only in enteropathogenic strains of *B. fragilis* [84], which has several characteristics of a pathogenicity island [90]. This chromosomal region also includes two opening frames (ORF-A and ORF-B) in addition to *bfr-2* [91]. ORF-A is small (375 bp) and has no significant similarity to any protein sequence in the database. ORF-B encodes a predicted 44.4 kDa protein including a HExxH metalloprotease motif similar to those contained in the BFTs, a lipoprotein signal peptide and a nucleotidetriphosphate-binding site motif [92]. This protein has been termed metalloprotease II (MP-II) and neither its biologic activity nor its role in pathogenesis are known [91].

The BFT genes consist of one open reading frame encoding for a protein of nearly four hundred residues [84, 85]. The analysis of their sequences suggests that BFT is synthesised as a pre-pro-protein peptide and that it belongs to the intramolecular chaperone protease family [79, 93]. As depicted in Figure 1.4, this protein is composed of three consecutive parts: an 18 residues long signal sequence precedes a 193 residues long pro-portion which is essential for the correct folding of the 186 residues long enzymatic domain [79, 94]. Differences must exist in the intracellular synthesis, processing and secretion of BFTs among the various enteropathogenic *B. fragilis* strains, to account for a large variability in the net amount of secreted toxin [95–99], but this issue remains to be investigated in detail.

Unlike clostridial neurotoxins, whose structural and biochemical properties define a unique novel family of metalloproteases [19, 100], the analysis of the BFT genes indicates that they belong to the metzincins group of metalloproteases, characterised by the common presence of the motif HexxHxxGxxH and by the presence of a conserved methionine near the C-terminus [101, 102]. The catalytic zinc atom is coordinated by three conserved histidines and the water-binding glutamate of the motif. BFTs have both autoproteolytic activity and *in vitro* proteolytic activity for substrates such as actin, gelatin, casein and azocoll.

![Figure 1.4](image_url)  
**Figure 1.4** Schematic structure of BFT. The predicted amino acid sequences of the *bfr-1*, *bfr-2* and *bfr-3* genes suggest that the encoded proteins are pre-proprotein toxins. Cleavage between Arg and Ala residues appears to release the mature BFT protein. Each protein contains an HEXXXHXXGXXH motif (and a conserved methionine near the C-terminus) suggesting that they belong to the metzincins group of metalloproteases.
[88]. The bft-2 gene additionally includes a 20-residue-long COOH-terminal amphipathic segment [84], which has been suggested to mediate the oligomerisation and the membrane insertion of the protein toxin with creation of an ion channel [79]. This hypothesis was put forward to account for the fact that BFT-2 exhibits greater biologic activity than BFT-1 or BFT-3 when tested on HT29/C1 cells [79]. An alternative explanation is that the COOH-terminal segment of BFT-2 mediates the association of BFT-2 with the cell surface leading to a much more efficient cleavage of cell surface proteins, due to the presence of both toxin and substrate on the two-dimensional solvent constituted by the plasma membrane. The virulence of ETBF strains containing two copies of the bft and mpII genes in animal models of ETBF disease is not known at present.

Though BFT causes a morphological change in HT29/C1 cells, a human colonic carcinoma cell line [96], the most appropriate in vitro system to test for the activity of BFT consists of polarised epithelial cell monolayers grown on filters, which form tight junctions and develop a trans-epithelial resistance which is a sensitive measure of the tight junctional sealing [103, 104]. The three BFT isoforms have been purified and their in vitro activity was assayed on HT29/C1 cells. Their order of potency is BFT-2 > BFT-1 > BFT-3 [79]. The half-maximal concentration of BFT-2 altering HT29/C1 cell morphology is approximately 12.5 pM (measured at three hours, 0.5 pM at eighteen hours), whereas it is approximately 1 nM in polarised T84 monolayers [105, 106]. Such a difference could be due to a different degree of membrane association of BFT-2 with the two cell lines.

1.4.3 THE METALLOPROTEOLYTIC ACTIVITY OF FRAGILYSIN (BFT)

In analogy with tetanus, botulism and anthrax, one is tempted to suggest that BFT is an essential virulence factor. However, wild-type ETBF strains and isogenic mutants of the same strain differing only in the in-frame deletion of the bft gene have not yet been compared for their virulence. Moreover, current knowledge of the pathogenicity of other gastrointestinal pathogens suggests a word of caution in attributing a predominant importance to single virulence factors. On the other hand, the activity of purified BFT is well documented at both the physiological and biochemical levels [79] and BFT promises to be a valuable addition for cell biologists and physiologists studying epithelia. In ligated intestinal loops isolated from different animal species, BFT stimulates dose-dependent secretion of fluids containing sodium, chloride and proteins [95]. At higher doses, BFT causes a hemorrhagic inflammation. Accordingly, BFT stimulates T84 cells to secrete the pro-inflammatory cytokine IL-8 and TGF-β, which promote the repair of ‘wounded’ epithelium in a dose-dependent manner [107]. At the single cell level, BFT induces time- and concentration-dependent changes in the structure of filamentous actin, without altering the
total F-actin content of the cells. In parallel, BFT stimulates a rapid and sustained increase in the volume of HT29/C1 cells [108]. However, the main activity of BFT is the decrease in the trans-epithelial resistance of polarised monolayers of epithelial cells in a dose- and time-dependent manner [106, 109, 110]. Electron microscopic analysis of BFT treated monolayers of T84 cells shows a decrease in microvilli and an effacement of some tight junctions and of the zonula adherens [105]. BFT is more active on the basolateral than on the apical domains of polarised (i.e. tight junction-forming) epithelial cells. T84 cells treated for 48 hours or more even with low concentrations of BFT undergo cell death with features of apoptosis. Unlike clostridial neurotoxins and LF, BFT does not appear to enter cells, but acts on the cell surface on selected targets, which include the tight junctions. Of the tight junctional proteins tested, only E-cadherin was cleaved by BFT, and cleavage could be detected within one minute from toxin addition in close agreement with the rapidity of BFT action. BFT cleavage of E-cadherin takes place in two steps: the extracellular domain of E-cadherin is degraded in an ATP-independent manner, followed by the degradation of its intracellular domain in an ATP-dependent manner, most likely mediated by intracellular proteases.

BFT is the first bacterial toxin known to remodel the intestinal epithelial cytoskeleton and F-actin architecture via cleavage of a cell surface molecule and represents the prototype of a novel class of bacterial toxins, while known actinomycotic bacterial toxins require cell internalisation and determine a covalent modification of cellular substrates [111].

These data suggested the following model for the pathogenesis of B. fragilis-induced intestinal secretion [79]. The toxigenic bacterial strains are presumed to attach to the apical membrane of intestinal epithelial cells and secrete BFT, which may diffuse through the zonula occludens to reach and cleave E-cadherin. Cleavage of the extracellular domain of E-cadherin is followed by loss of the intracellular domain of this molecule. Because the intracellular domain of E-cadherin is tethered to the apical network of F-actin in the epithelial cell, loss of these protein–protein interactions may precipitate focal morphological changes in the apical cellular cytoskeleton with loss of microvilli and decreased barrier function which allows the delivery of BFT to the basolateral membranes of the intestinal epithelial cells. Here, further cleavage of E-cadherin may increase the apical morphological changes initially stimulated by BFT. These dramatic changes in the apical membrane of epithelial cells caused by BFT are hypothesised to alter the function of one or more ion transporters, resulting in net intestinal secretion. Concomitantly, protein synthesis is stimulated in the intestinal epithelial cells resulting in secretion of the proinflammatory cytokine IL-8. This molecule initiates the recruitment of polymorphonuclear leukocytes to the intestinal submucosa. This resulting inflammatory response is predicted to contribute to the intestinal secretory response. However, it is not yet known if ETBF stimulate intestinal inflammation in humans, and several aspects of this model still require direct experimental testing.
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