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The International Journal of Biochemistry & Cell Biology 39 (2007) 1771–1775
IJBCB
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Pathogens in focus

Vibrio cholerae: Cholera toxin

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Received 27 February 2007; received in revised form 5 July 2007; accepted 9 July 2007
Available online 20 July 2007

Abstract

The bacterial protein toxin of *Vibrio cholerae*, cholera toxin, is a major agent involved in severe diarrhoeal disease. Cholera toxin is a member of the AB toxin family and is composed of a catalytically active heterodimeric A-subunit linked with a homopentameric B-subunit. Upon binding to its receptor, $GM0_1$, cholera toxin is internalized and transported in a retrograde manner through the Golgi to the ER, where it is retrotranslocated to the cytosol. Here, cholera toxin reaches its intracellular target, the basolaterally located adenylate cyclase which becomes constitutively activated after toxin-induced mono-ADP-ribosylation of the regulating G_S-protein. Elevated intracellular cAMP levels provoke loss of water and electrolytes which is manifested as the typical diarrhoea.

The cholera toxin B-subunit displays the capacity to fortify immune responses to certain antigens, to act as a carrier and to be competent in inducing immunological tolerance. These unique features make cholera toxin a promising tool for immunologists.

Keywords: Cholera toxin; *Vibrio cholerae*; Retrograde traffic; Immunological adjuvant

1. Introduction

Vibrio cholerae is a major pathogen responsible for the life-threatening acute diarrhoea, cholera, which mainly affects third world populations. *V. cholerae* are Gram-negative bacteria which belong to the *Vibrionaceae* family. Despite rehydration therapy with uncontaminated water and electrolytes, *V. cholerae* infection still results in high morbidity and mortality rates. In regions where sanitary provisions are poor, cholera is often endemic, and symptomless carriers usually induce epidemic outbreaks mostly among people with an impaired immune system such as young children, the elderly or travellers. Historically, seven pandemic outbreaks have been described, of which the seventh is still ongoing in South-Asia and Bangladesh with over 1 million cases and more than 10,000 deaths (WHO, 2006).

The epidemic spread of cholera was first recognized by John Snow in 1854 during the fourth pandemic and in the same year, the bacteria was described and termed *V. cholerae* by Filippo Pacini. The discovery of the causative protein factor, cholera toxin (CT), was first suggested by Robert Koch in 1884 and demonstrated 75 years later (Finkelstein, Mukerjee, & Rudra, 1963).

2. Overview of pathogenesis

The *V. cholerae* genome comprises two chromosomes, of which chromosome I harbours all virulence factors, including CT and the toxin-co-regulated pilus (TCP).

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doi:10.1016/j.biocel.2007.07.005

V. cholerae colonises the small bowel using TCP and interacts with receptors on the intestinal epithelium. Once attached, the bacterium secretes its toxin which is accompanied by the release of hemagglutinin/protease (HA/protease). This extracellular HA/protease is responsible for nicking the CT-A subunit at Arg192, yielding discrete CT-A_1 and CT-A_2 subunits which are solely connected by a single disulfide bond. This post-translational modification is critical for full activity of the toxin, leading to an increase in cAMP-production. This causes massive secretion of electrolytes and water into the intestinal lumen, paralleled by excretion of the bacteria. The patient's stool bears resemblance to rice water and can amount to over 10 l a day (Sanchez & Holmgren, 2005). Feaco-oral transmission of *V. cholerae* is the main transmission channel of the pathogen, often resulting in violent outbursts.

Treatment of cholera relies on forced rehydration of the patient, orally or intravenously, often in combination with electrolytes and antibiotics (Guerrant, Carneiro-Filho, & Dillingham, 2003). From a prophylactic point of view, administration of killed *V. cholerae* bacteria or attenuated strains could serve as a vaccine.

A major breakthrough was the observation that more powerful immune responses could be elicited using combinations of killed whole cell *V. cholerae* preparations with an excess of purified or recombinant CT-B subunit. Currently, the most efficient vaccine is based on these findings and protects adults up to 2 years after immunization. (Hill, Ford, & Lalloo, 2006).

3. Structure of the virulence factor

CT belongs to the superfamily of AB toxins and is an oligomeric protein composed of a heterodimeric A-subunit (CT-A, $M_r \sim 27{,}400$) and a homopentameric B-subunit (CT-B, $M_r \sim 58{,}000$) (Fig. 1). The five identical B monomers ($M_r \sim 11{,}600$) are arranged in a ring-like configuration with each a single binding site for the plasma membrane receptor of the jejunal intestinal epithelial cells, the monosialoganglioside GM_1 (Chinnapen, Chinnapen, Saslowsky, & Lencer, 2007). The CT-A subunit, extending well above the plane of the pentameric ring, consists of two distinct polypeptide chains (CT-A_1, $M_r \sim 22{,}000$ and CT-A_2 $M_r \sim 5{,}400$) linked by a single disulfide bridge. The CT-A_2 polypeptide has a linker function and occupies the central channel and goes through the doughnut-like structure of the CT-B pentamer, tethering CT-A_1 and CT-B subunits. Additionally, based on the primary sequence of the CT-gene, the CT-A_2 subunit possesses a C-terminal KDEL (Lys/Asp/Glu/Leu) retrieval signal, inferred to play a role in retrograde trafficking from Golgi to the endoplasmic reticulum (ER) (Dams, De Wolf, & Dierick, 1991). CT-A_1 is a catalytic polypeptide displaying mono-ADP-ribosyltransferase activity, involved in ADP-ribosylation of the $G_{s\alpha}$-subunit of a stimulatory GTP-binding regulatory protein, followed by stimulation of basolateral adenylate cyclase (AC) (De Haan & Hirst, 2004).

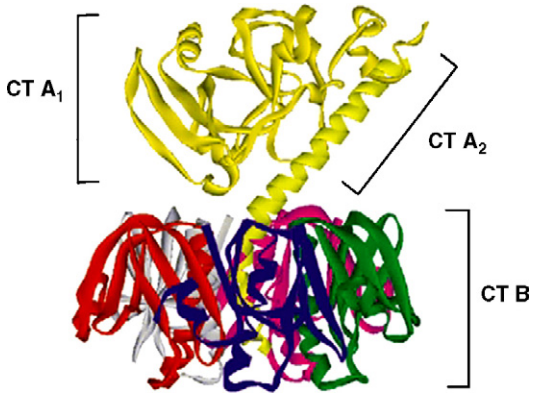

Fig. 1. CT structure. Crystal structure of cholera toxin. The hetero dimeric CT-A subunit (depicted in yellow) consists of two polypeptide chains, CT-A_1 and CT-A_2, linked by a single disulfide bond. CT-A_1 is enzymatically active and displays mono-ADP-ribosyltransferase activity while CT-A_2 functions as a linker between CT-A_1 and CT-B. The B subunit is comprised of five identical polypeptide chains (illustrated in green, purple, red, grey and blue), each with GM_1 binding capacity, arranged in a torus like conformation. (Reprinted from Lencer and Tsai (2003), reprinted with permission of Elsevier). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

4. Biological function

After colonization of the small intestine, the bacteria start to produce and release CT at the luminal side. The toxin initiates it's action by binding with high affinity and specificity to it's apical cell membrane receptor, GM_1, of the host enterocytes. The assertion that multivalent binding is a prerequisite for CT uptake and cell intoxication has been refuted using hybrid CT possessing only one or two functional binding pockets (De Wolf, Dams, & Dierick, 1994). Morphologic studies show that CT preferentially clusters into non-coated membrane invaginations, the caveolae, and enters several cell types via smooth, non-clathrin coated vesicles (Chinnapen et al., 2007). Furthermore, studies using cholesterol perturbing agents and chimeric toxins indicate involvement of detergent-resistant membrane fractions (DMRS)/lipid rafts in toxic CT entry (Fig. 2). Although CT partitions in DMRS/lipid rafts is currently used as a marker for non-clathrin-mediated endocytosis, it appears to be internalized simultaneously through both clathrin-dependent

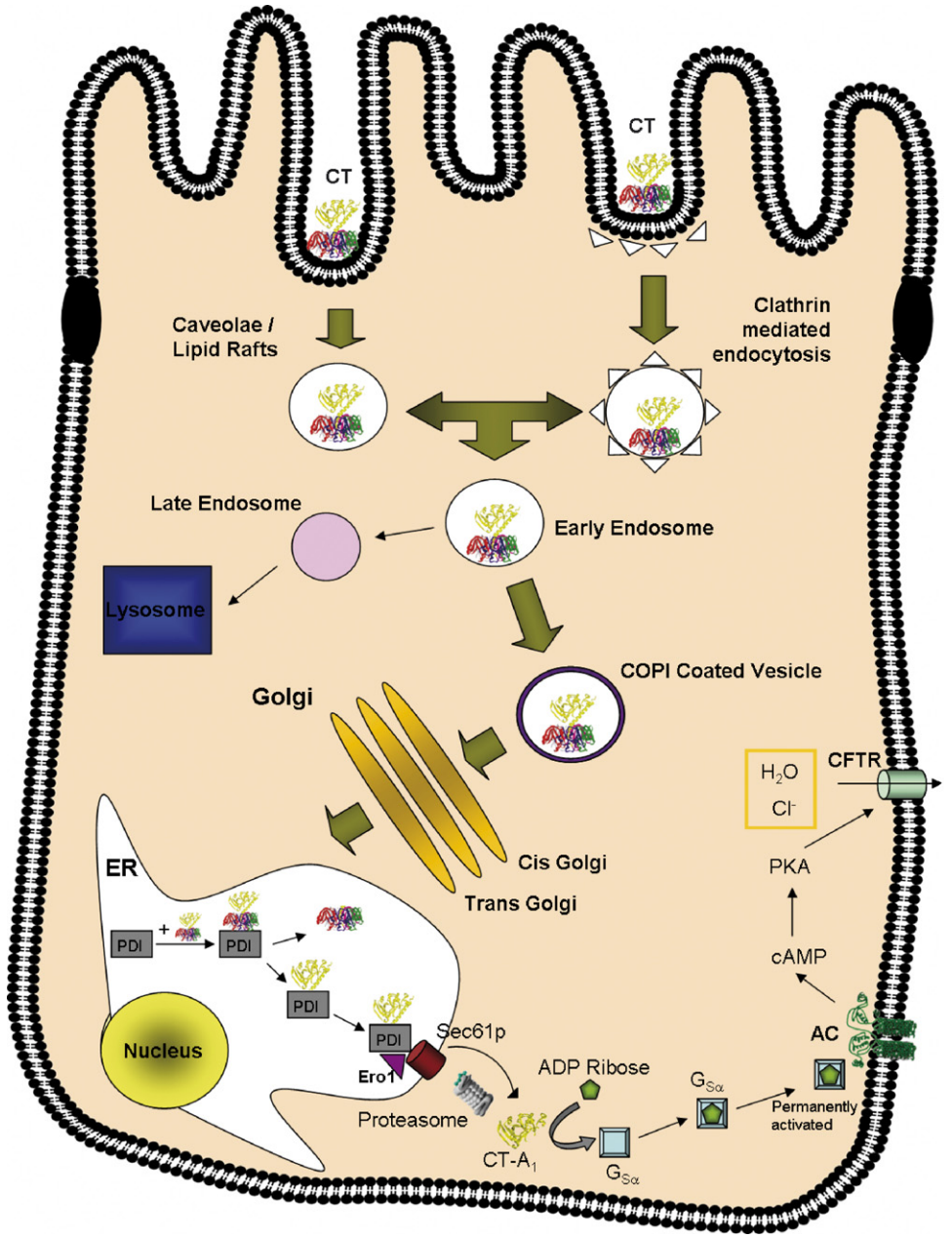

Fig. 2. Proposed model for the intoxication of the enterocyt by CT. CT binds with high affinity its receptor GM_1, after which it is actively internalized by the host cell machinery into the early and recycling endosomes, regardless of the mechanism of internalization. Subsequently, CT is transported as a holotoxin in a retrograde manner to the Golgi and further to the ER. Here, CT dissociates into a CT-A_1 and a CT-A_2/CT-B complex driven by PDI, which is also responsible for the unfolding of CT-A_1. CT-A_1 is further retrotranslocated via the Sec61p complex to the cytosol, avoiding proteasomal degradation, where it associates with basolateral AC. Transfer of mono-ADP-ribose from NAD^+ to $G_{S\alpha}$ constitutively triggers the adenylate cyclase (AC), resulting in a substantial increase in intracellular concentrations of cAMP, followed by a protein kinase (PKA)-mediated phosphorylation of the major chloride channel of intestinal epithelial cells, the cystic fibrosis transmembrane conductance regulator (CFTR). The net increase in Cl^--secretion is accompanied by osmotic movement of a large quantity of water into the intestinal lumen, resulting in severe diarrhoea.

and -independent routes (Hansen et al., 2005). In addition, fluorescence microscopy has shown that CT enters cells via an Arf6-regulated pathway or even by a fourth route that is dynamin- and Arf6-independent. However, after blocking all three known endocytotic pathways, CT-induced toxicity was hardly affected (Massol, Larsen, Fujinaga, Lencer, & Kirchhausen, 2004). These findings illustrate the difficulty in correlating morphologic

data with the functional entry of CT. Regardless of the internalization route, CT ends up in the early and recycling endosomes and in caveolin-containing endocytotic intermediates. Experiments using Brefeldin A, a fungal metabolite that disrupts the structural and functional integrity of the Golgi, show that cells become resistant to CT cytotoxicity and that the intracellular formation of CT-A_1 is blocked (Orlandi, Curran, & Fishman, 1993). Toxin trafficking to the *trans*-Golgi network possibly requires Rab7 and Rab9, since Golgi targeting of endogenous GM_1 has been found to be dependent on these small GTPase Rab proteins. Several lines of evidence have indicated that further retrograde transport to the ER involves cytosolic coat protein I (COPI) coated vesicles, consistent with the presence of a KDEL retrieval sequence at the C-terminus of CT-A_2. Other studies challenge this hypothesis suggesting an alternative Rab6-dependent pathway (Chem, Hu, & Mikoryak, 2002).

Whether CT enters the ER as holotoxin or dissociates into its subunits during passage through the Golgi is still under discussion. There is experimental evidence that only the A-subunit enters the ER, as dissociation of the holotoxin is a prerequisite to assure accessibility of the CT-A_2 C-terminal KDEL sequence for its receptor. However, other lines of evidence indicate that this is potentially incorrect. A major counterplea is the non-imperative role of the KDEL sequence in the functional intoxication of the cell, as proven by genetic mutants. Moreover, studies using a mutant CT-B subunit, containing sulfatation and *N*-glycosylation motifs, directly show passage of CT-B through both the Golgi and the ER (De Luca & Lencer, 2006).

Recently, the inhibitory effect of Cbz-Gly-Phe-NH_2 on the ability of CT to raise intracellular cAMP levels has been demonstrated (De Wolf, 2000). Cbz-Gly-Phe-NH_2 is a dipeptide metalloprotease inhibitor known to interfere with endosomal transport via a yet unidentified mechanism.

Inside the ER lumen, CT disassembles into its CT A_1-subunit and the CT-A_2/CT-B complex. In this process, a role for protein disulfide isomerase (PDI) has been suggested. PDI catalyzes the reduction of the disulfide bond that tethers CT-A_1 and CT-A_2 together, and is assisted by Ero1 (De Haan & Hirst, 2004).

CT-A_1 is unfolded before its transport to the cytosol and takes advantage of the ER-associated degradation (ERAD) pathway to accomplish its retrotranslocation, which seems to be contradictory since the ERAD system degrades misfolded proteins and ensures newly synthesized proteins fold into their native conformations. In order to take advantage of this proteolytic pathway, CT-A_1 peptides must masquerade as ERAD substrates, raising the critical question of why CT-A_1 remains intact while being translocated through a catabolic pathway. A plausible explanation resides in the unusually low lysine content of CT-A_1, an amino acid generally abundant in all proteins. These lysine residues are poly-ubiquitinated, hereby triggering the ERAD-mediated degradation, and by containing only a low number of accessible lysine residues CT-A_1 is protected from degradation by the host cell (Rodighiero, Tsai, Rapoport, & Lencer, 2002).

Once translocated to the cytosol, presumably via the Sec61p channel, the CT-A_1 subunit associates with AC, it's intracellular target enzyme which is located at the basolateral membrane. Mono-ADP-ribosylation of $G_{s\alpha}$, a component of the heterotrimeric GTP-binding protein that regulates AC-activity, leads to constitutive activation of this G-protein with subsequent continuous stimulation of AC and a marked increase in the intracellular cAMP concentration. This results in protein kinase (PKA)-mediated phosphorylation of the major chloride channel of intestinal epithelial cells, the cystic fibrosis transmembrane conductance regulator (CFTR). The net increase in Cl^--secretion is accompanied by the osmotic movement of a large quantity of water into the intestinal lumen, resulting in severe diarrhoea (Sanchez & Holmgren, 2005).

5. Clinical applications

Besides its established role as the causal mediator of diarrhoea, CT is now recognized as having remarkable immunological properties (Williams, Hirst, & Nashar, 1999). CT generates extremely potent anti-toxin antibodies following systemic immunization, even in the absence of classical adjuvants, potentially providing a strategy to enhance immunogenicity of orally delivered antigens. The obvious problem of using CT as an adjuvant is the potential for inducing pathological diarrhoea. However, CT acts as an adjuvant at doses lower than that required for the induction of toxic side effects, and importantly the B subunit has been identified as the immune adjuvant agent, at least when administered intranasally or parenterally. In orally delivered vaccines, CT-B alone displayed no immunological capacities, which might be explained by lack of stability during passage of the stomach, which requires the presence of the A-subunit (Hirst et al., 2002).

Important data from the research group of Czerkinsky and Holmgren (Sun, Holmgren, & Czerkinsky, 1994) have demonstrated that coupling of the antigen to CT-B, prior to oral administration, can induce immunologi-

cal tolerance. In this respect, promising results have been obtained in the area of autoimmune disorders, where coupling of an auto-antigen to CT-B induced tolerance e.g. the oral use of an insulin/CT-B complex as an effective therapy against diabetes in mice. Although no precise mechanism has been put forward to explain these findings, the need to directly couple antigen and CT-B stresses its role as a carrier, shuttling antigen into tolerance-inducing pathways via GM_1-receptor-mediated uptake across the intestinal epithelium. Moreover, the observation that CT-B, even in the absence of the auto-antigen, can induce tolerance suggests that CT-B has distinct immunological capacities way beyond that of a carrier function (Faria & Weiner, 2006).

6. Conclusion

CT is a remarkable protein in many aspects. Initially it emerged as a toxic protein with unusual characteristics such as its unique structure, retrograde transport, retrotranslocation and mono-ADP-ribosyltransferase activity. For these reasons, CT has revealed itself to be a useful vehicle for intracellular drug targeting as well as an anti-inflammatory agent. Its unique capacity to evoke immune responses or to induce immunological tolerance positions CT as a prominent player in novel therapeutic strategies for modulating immune disorders.

Acknowledgements

We are grateful to Prof. Dr. emeritus Albert Lagrou for his helpful discussions and his careful reading of the manuscript. The authors wish to express their apologies to many colleagues whose important contributions have not been referenced or whose work has been cited indirectly through references in review articles. Caroline Horvath is supported by the Institute for Promotion of Innovation through Science and Technology in Flanders.

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