The Adenylate Cyclase Toxin from *Bordetella pertussis*: Structure-function Studies and Biotechnological Applications

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Introduction

Bordetella pertussis, the causative agent of whooping cough, produces among various toxins, an adenylate cyclase (AC), that is one of the major virulence factors of this organism. It contributes to the early establishment of the infection most likely by targeting the innate immune responses. The toxin exhibits several striking features: (1) it is secreted by the virulent bacteria; (2) it is activated by the eukaryotic protein calmodulin (CaM), and (3) it has the ability to enter into eukaryotic cells where, upon activation by endogenous CaM, it catalyzes high-level synthesis of cAMP that in turn alters cellular physiology (for reviews, see Weiss and Hewlett, 1986; Mock and Ullmann, 1993). In our group we are attempting to decipher the molecular mechanisms underlying the biogenesis of the AC toxin, its entry into eukaryotic target cells and its interaction with cellular effectors. In parallel, we have exploited some of the original properties of this molecule in various applications in vaccinology and biotechnology.

Role of the Adenylate Cyclase Toxin in *Bordetella pertussis* Virulence

The adenylate cyclase toxin (designated either as AC toxin or CyaA, according to the name of the structural gene) plays an important role in the early stages of respiratory tract colonization by *B. pertussis* (Weiss and Goodwin, 1989). In an infant mouse model of infection, mutants deficient in CyaA were shown to be avirulent (Weiss et al., 1984). Furthermore, both passive and active immunization with CyaA significantly shortened the period of bacterial colonization of the mouse respiratory tract by *B. pertussis*. Early studies by Confer and Eaton have shown that partially purified adenylate cyclase inhibits phagocytic functions by impairing chemotaxis and oxidative response, through elevation of intracellular cAMP (Confer and Eaton, 1982). Later, it was shown that cell cytotoxicity caused by *B. pertussis* occured through apoptosis and that the induction of macrophage apoptosis was dependent on the expression of CyaA (Khelef et al., 1993). Internalization of bacteria was not necessary for cytotoxicity and purified CyaA appeared to be the only *B. pertussis* virulence factor having the ability to induce apoptosis. More recently, in a murine model of infection, *in vivo* studies identified neutrophils and macrophages as the primary targets of the AC toxin from *B. bronchiseptica*, a related animal pathogen (Harvill et al., 1999). The AC toxin may therefore represent an essential mechanism of defense against the early steps of innate immune responses.

Biogenesis of the Adenylate Cyclase Toxin

Adenylate cyclase is encoded by the *cyaA* gene (Glaser et al., 1988a) and its expression, like that of other virulence genes of *B. pertussis*, is coordinately regulated by environmental signals (Arico et al., 1989). The 1706 residue-long CyaA is a bifunctional protein endowed with both adenylate cyclase (AC) and haemolytic activities (Glaser et al., 1988b; Bellalou et al., 1990). Synthesized as an inactive precursor, CyaA is converted to the active toxin by palmitoylation of Lys 860 and Lys 983, a process that is dependent on the product of an accessory gene, *cyaC* (Hackett et al., 1994). The acylated (i.e. active) CyaA polypeptide is then secreted across the bacterial envelop by a dedicated type I secretion machinery encoded by the *cyaB*, *cyaD*, and *cyaE* genes, that form an operon with the *cyaA* gene (Glaser et al., 1988b). Recent studies indicate that the exported CyaA might remained attached to the outer membrane of the bacteria rather than being released in the outside medium and could be delivered directly to the eukaryotic cells upon contact (Zaretzky et al., 2002).

Structure/Function Relationships

The structural organization of CyaA is shown in Fig.1. The protein is constructed in a modular fashion: the ATP-cyclizing, CaM-activated, catalytic domain is located in the 400 amino-proximal residues while the carboxy-terminal 1306 residues are responsible for the haemolytic phenotype of *B. pertussis*. Both activities can function independently as adenylate cyclase and haemolysin, respectively. The AC domain exhibits a high catalytic activity (k_{cat} = 2000 s^{-1}) upon activation by CaM (Wolff et al., 1980; Ladant et al., 1988). Its structural organization will be discussed in detail below/later.

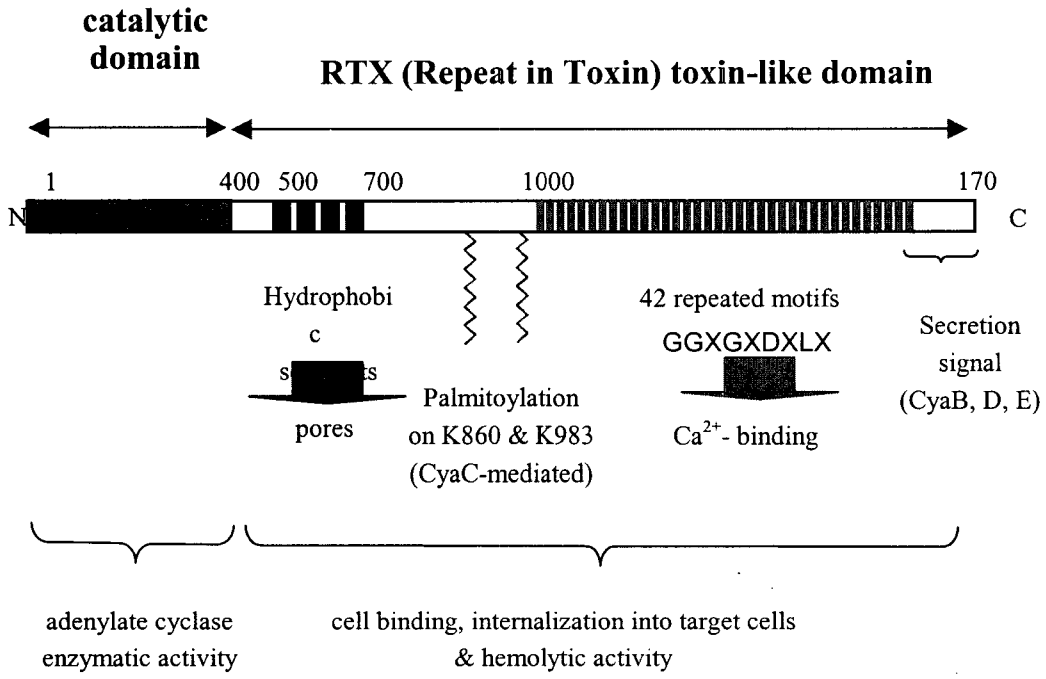

Fig. 1. Structural organization of *B. pertussis* adenylate cyclase. Regions involved in the different functions are indicated along the primary structure of CyaA. Numbers represent amino acid residues.

The haemolysin domain (residues 400 to 1706) displays structural characteristics that link CyaA to a family of bacterial toxins known as RTX (Repeat in ToXin), the prototype of which is the *E. coli* alpha-haemolysin (Coote, 1992). This part of the molecule (like several other RTX toxins) is endowed with an intrinsic, albeit low, haemolytic activity which results from its ability to form cation-selective channels in membranes (Benz et al., 1994). More importantly, this domain mediates the binding and internalization of the toxin into eukaryotic cells. Several sub-regions can be identified in this haemolysin domain (for a review, see Ladant and Ullmann, 1999): (i), a pore-forming domain with four hydrophobic segments (residues 500 to 700); (ii), a region where the CyaC-dependent palmitoylation occurs; (iii), a region with a series of 38-42 copies of a characteristic glycine and aspartate-rich nonapeptide repeats (residues 913 to 1613), representing the main Ca^{2+} binding site of the protein and (iv), a nonprocessed C-terminal secretion signal that is recognized by the CyaB/CyaD/CyaE secretion machinery (Glaser et al., 1988b).

Mechanisms of CyaA Entry into Eukaryotic Cells

CyaA can interact with a variety of cell types and deliver its catalytic domain into the host cell cytoplasm where, upon binding CaM, it produces supraphysiologic levels of cAMP. The main originality of the CyaA toxin, with respect to other bacterial or plant toxins, stems from its unique mechanism of penetration into eukaryotic cells. The catalytic domain appears to be translocated directly across the plasma membrane of the target cells into the cytosol in a process referred to as internalization or intoxication (Rogel et al., 1989). Several lines of evidences support this direct translocation mechanism: (i) the onset of intoxication is rapid: an increase of cAMP levels in target cells can be detected within seconds after addition of the CyaA toxin (entry of toxins through endocytosis and vesicular trafficking requires a much longer exposure time); (ii) its entry is independent of the acidification of endocytic vesicles; (iii) more importantly, CyaA can invade cells that are lacking membrane traffic, like mammalian erythrocytes.

The molecular mechanism of toxin internalization into target cells is largely unknown but it can be described as a two-step process (Rogel and Hanski, 1992). In a first step, CyaA binds to the cell surface: this requires both the integrity of the C-terminal part of CyaA (residues 400 to 1706) and the CyaC-dependent palmitoylation of Lys860/Lys983 of CyaA. Binding of toxin to erythrocytes will eventually lead to cell lysis due to the pore forming capacity of CyaA (Bellalou et al., 1990; Benz et al., 1994). Indirect evidence suggests that haemolysis (which appears only after a lag of 30-100 min) requires oligomerization of the CyaA monomers embedded in the cell membrane. The second step consists in the actual translocation of the catalytic domain of the bound CyaA through the plasma membrane of the cells (Rogel and Hanski, 1992). This process is dependent upon the temperature (it occurs only above 15 °C), and the presence of calcium ions in the mM range. The Asp-Gly-rich repeat domain of CyaA (amino acid 1006 to 1638) might be directly involved in this Ca^{2+}-dependent translocation process, as this region harbors the main low affinity Ca^{2+}-binding sites and undergoes conformational changes upon calcium binding (Rose et al., 1995). Beside, Otero et al. (1995) showed that the internalization of CyaA is dependent upon the membrane potential of the target cells and later, we demonstrated that the electrostatic charge of the polypeptide chain around amino acids 224-242 of CyaA is critical for its translocation into eukaryotic cells (Karimova et al., 1998a). All together, these results suggest that AC might use the electrical field across the plasma membrane as a driving force to enter target cells.

Although CyaA, as stated above, is able to enter into a wide variety of target cells, recent results indicated that CyaA associates with high affinity and specificity with the $\alpha_M\beta_2$ integrin (Guermonprez et al., 2001). As the $\alpha_M\beta_2$ integrin is expressed on a restricted subset of leukocytes including neutrophils, macrophages and

dendritic cells, these results provide a molecular basis to the earlier observations that phagocytic cells are a primary target of the CyaA toxin. The precise mechanisms of entry of CyaA into cells that express the integrin receptor remain to be elucidated. In particular, because the $\alpha_M\beta_2$ integrin can be internalized by endocytosis, CyaA, after binding to its receptor, could reach endocytic vesicles from where it could predominantly escape to cytosol. Indeed, Khelef et al. (2001) recently reported that CyaA is internalized by macrophages into endocytic vesicles at least partly through macropinocytosis.

Engineering of Recombinant CyaA Toxins to Deliver Antigens into Antigen-Presenting Cells

The unique capacity of CyaA to deliver its N-terminal catalytic domain directly across the plasma membrane of eukaryotic cells, have been exploited to design novel vaccinal approaches. In collaboration with Claude Leclerc and her team (Biologie des Régulations Immunitaires, Institut Pasteur), we showed that antigenic peptides can be genetically grafted into defined permissive sites within the catalytic domain of CyaA without altering the biological activities of the molecule (Ladant et al., 1992) and we suggested that such recombinant toxins could deliver these antigens into antigen-presenting cells (APCs).

We produced different recombinant CyaA toxins carrying viral and/or tumoral $CD8^+$ T-cell epitopes (i.e. associated with the major histocompatibility complex (MHC) class I molecules) and showed that these purified recombinant AC toxins, when injected to mice, were able to stimulate strong cytotoxic T-cell (CTL) responses, mediated by MHC class I-restricted $CD8^+$ T-cells, against the grafted epitopes (Fayolle et al., 1996; Fayolle et al., 1999). More importantly, mice immunized with a recombinant toxin carrying a single epitope from the lymphocytic choriomeningitis virus (LCMV) were protected against infection with lethal doses of the LCMV virus (Saron et al., 1997). Protection was shown to be mediated by LCMV-specific $CD8^+$ T cells and independent of $CD4^+$ T-cell help. We also showed that CyaA-mediated delivery of the inserted T-cell epitope into the cytosol of APCs is essential for the induction of CTL responses *in vivo* (Karimova et al., 1998a), whereas the enzymatic activity of CyaA is not required; that is, genetically detoxified CyaA proteins were equally efficient in inducing protective immunity (Saron et al., 1997; Fayolle et al., 1999). Other experiments (Guermonprez et al., 1999) demonstrated that the epitopes inserted into CyaA were presented to CD8+ T cells through the classical cytosolic pathway for antigen presentation by the MHC class I complexes (i.e. dependent upon proteasome processing and "TAP" transporters).

Altogether, our results have shown that the genetically detoxified recombinant CyaA toxin might constitute an attractive non-replicative vector to induce cellular immunity toward pathogens or tumor cells. The remarkable efficacy of the recombinant CyaA toxins in CTL priming can likely be explained by the presence of the specific CyaA receptor, the $\alpha_M\beta_2$ integrin, at the surface of dendritic cells. Dendritic cells play a major role in the immune system by acting as professional antigen presenting cells. Indeed, recent experiments have shown that, *in vivo*, recombinant CyaA harboring foreign $CD8^+$ T cell epitopes are presented essentially by the $CD11b^+$ "myeloid" subset of dendritic cells (Guermonprez et al., 2002).

B. pertussis Adenylate Cyclase as a Signal Transducer in Screening Technologies for Protein-Protein Interactions and Proteolytic Activities

B. pertussis AC represents an original model of a bacterial enzyme activated by an eukaryotic protein, calmodulin. The AC catalytic domain,that encompasses the first 400 residues of CyaA, exhibits a high catalytic activity ($k_{cat} = 2000\ s^{-1}$) upon activation by CaM, which binds with a high affinity ($K_D < 0.1$ nM) to the enzyme and stimulates its activity by >1000-fold. The catalytic domain itself consists of two subdomains,

T25 and T18, that can be obtained *in vitro* by limited proteolysis (Ladant, 1988). The T25 and T18 fragments can associate with CaM in a fully active ternary complex. T25 (residues 1-224) carries the catalytic site, whereas T18 (residues 225-399) contains the main CaM-binding domain (Ladant et al., 1989).

As AC produces a regulatory molecule, cAMP, that is a pleiotropic regulator of gene transcription in *E. coli* (Ullmann and Danchin, 1983), we attempted to exploit this enzyme as a signal transducer to elaborate new screening technologies in *E. coli* with general biotechnological applications. We took advantage of the modular structure of the AC catalytic domain to design a genetic assay to detect protein/protein interactions *in vivo*, and more recently, to develop a sensitive screening procedure to detect proteolytic activities. Both assays are based on the reconstitution of an artificial cAMP signal transduction pathway in an *E. coli* adenylate cyclase deficient strain (*cya*).

A Bacterial Two-Hybrid System to Study Protein Protein Interaction in *E. coli*

The bacterial two-hybrid system we have set up (designated as BACTH standing for Bacterial Adenylate-Cyclase based Two-Hybrid) is based on the interaction-mediated reconstitution of AC enzymatic activity from hybrid proteins made of the two AC subdomains, T25 and T18, fused to polypeptides of interest (Fig. 2). This genetic assay allows an easy *in vivo* screening and selection of functional interactions between two proteins (Karimova et al, 1998b, 2001).

When the two AC subdomains, T25 and T18, are co-expressed as separate entities in an *E. coli* adenylate cyclase deficient strain (*cya*), they are unable to recognize each other and cannot reconstitute a functional enzyme. Yet, when T25 and T18 are fused to peptides or proteins that are able to interact, heterodimerization of these chimeric polypeptides results in functional complementation between the two AC fragments and leads to cAMP synthesis. Cyclic AMP, upon binding to the catabolite activator protein (CAP), activates the transcription of catabolic operons, allowing the bacteria to ferment carbohydrates such as maltose or lactose. This can be scored either on indicator plates or on selective media. We have shown that the BACTH system can reveal interactions between small peptides, bacterial, eukaryotic, or viral proteins (Karimova et al, 1998b, 2001). The system has also been used successfully by different researchers to reveal a variety of protein-protein interactions.

Altogether, these studies indicate that BACTH is a versatile system that permits the analysis of protein interactions occurring either at the DNA level within the cytosol or at the inner membrane level. Furthermore, as it is carried out in *E. coli*, the *in vivo* screening, as well as the *in vitro* characterization of interacting proteins are facilitated. Indeed, in a recent systematic study of interactions between proteins constituting the human peroxisome (peroxins), a comparative analysis of the BACTH and the yeast two-hybrid systems revealed excellent complementarities between these two techniques (Fransen et al., 2002).

More recent work has been focused on the improvement of the BACTH system in order to apply it to the screening of interactions between proteins on a genome-wide scale. Pilot experiments have indeed validated the screening potential of this technique. One obvious advantage of this technology concerns the study of membrane proteins. In particular, we have used the BACTH system to unravel the molecular basis of the association of the *E. coli* membrane proteins involved in cell division (Fts proteins). Although, no direct evidence for interactions between the Fts transmembrane proteins could been obtained up to now, we showed that the Fts proteins are connected to each other through multiple interactions that involve distinct polypeptide regions of each partners (Karimova *et al.*, in preparation). Our data suggest that the assembly of the cell division machinery is driven by the cooperative

association of the different Fts proteins to form a dynamic multiprotein structure at the septum site. This model contrasts the traditional view of a sequential recruitment of Fts proteins to the division site. This study also indicates that the cAMP-based two-hybrid system is particularly appropriate to analyze molecular interactions underlying the assembly of transmembrane protein complexes.

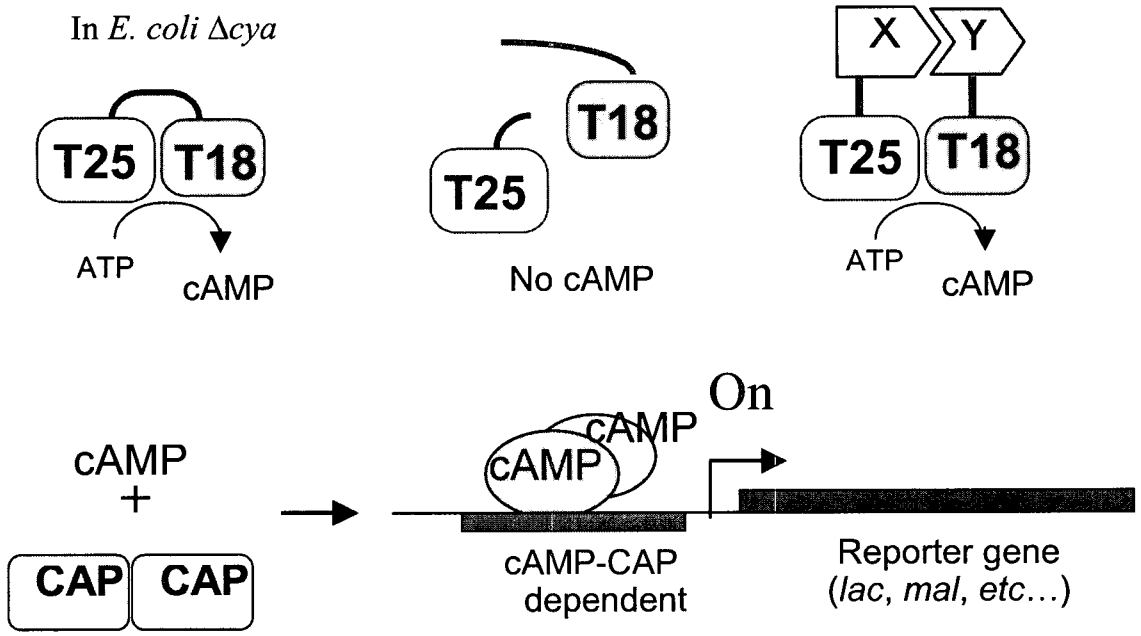

Fig. 2. Principle of the bacterial two-hybrid system based on AC fragment complementation (BACTH)

AC Based Genetic Screen for Protease Activity in *Escherichia coli*: Application to the Detection of Antiprotease-Resistant HIV Proteases

We also set up a genetic system that allows *in vivo* screening or selection of site-specific proteases and of their cognate specific inhibitors in *E. coli*. This genetic test is based on the specific proteolysis of the AC catalytic domain. As a model system, we tested the HIV protease that is responsible for the proteolytic processing of the HIV polyprotein precursor gag/pol into mature viral proteins (Dautin *et al.*, 2000). We showed that AC is an exquisitely sensitive reporter system for monitoring the proteolytic activity of the HIV protease and its inhibition by known inhibitors. In particular, we showed that this genetic test is able to distinguish between wild type HIV protease and protease-inhibitor-resistant variants that were isolated from patients under highly active antiretroviral therapy. This genetic test could represent a powerful approach to detect in patients, undergoing highly active antiretroviral therapy (HAART), the emergence of HIV variants harboring drug-resistant proteases (Perrin and Telenti, 1998). The AC-based genetic test could also be used to screen for new HIV protease inhibitors and, in a more general perspective, to search for novel proteolytic activities.

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