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Anthrax edema factor potency depends on mode of cell entry ☆

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Abstract

Anthrax edema factor (EF) is a highly active calmodulin-dependent adenylyl cyclase toxin that can potently raise intracellular cAMP levels causing a broad range of tissue damage. EF needs anthrax protective antigen (PA) to enter into the host cell and together they form edema toxin. Here, we examine factors that are critical for edema toxin cell entry and potency. In Y1, 293T and mouse embryonic fibroblast cells, EF causes cell rounding, aggregation, and sometimes detachment via protein kinase A but not Epac. The rate-limiting step for these EF-mediated effects is cellular entry via the anthrax toxin receptor. Finally, EF potency is also enhanced if the EF adenylyl cyclase domain is transfected into host cells, even in the absence of the anthrax PA-binding domain. These results indicate that the effects of EF in cells can differ dependent upon the mode of cellular entry of the adenylyl cyclase.

Keywords: Anthrax; Edema toxin; Edema factor; Adenylyl cyclase; Protective antigen; Protein kinase A; Cyclic AMP

Bacterial toxins have evolved to effectively modulate communication processes in host cells and have served as invaluable pharmacologic tools to probe eukaryotic cell signaling. *Bacillus anthracis* is a spore-forming, gram-positive bacterium that causes anthrax. The anthrax spore is a potent bioweapon based on its hardiness and the effective morbidity and mortality of inhalational anthrax. The combination of anthrax toxins and rapid bacterial growth causes a plethora of tissue damage and makes anthrax deadly. Anthrax bacteria secrete three exotoxin components: edema factor (EF), lethal factor (LF), and protective antigen (PA). EF is a calcium and calmodulin-dependent adenylyl cyclase (AC) that converts cellular ATP to cAMP [1]. LF is a Zn^{2+}-dependent metalloprotease [2] that cleaves and inactivates mitogen-activated protein kinase kinases (MAPKKs) [3,4]. PA binds to a cell surface anthrax toxin receptor (ATR/TEM-8 or CMG-2) [5–7], is activated by proteolytic cleavage [8], and forms a heptameric pore [9] that enables cellular entry of LF and EF.

The combination of PA and EF forms edema toxin which can block the phagocytic ability of human monocytes [10], impair the functions of dendritic cells [11], and impair antigen presentation of T-cells [12]. Edema toxin is lethal to mice and can cause multiple tissue damage as well as cardiovascular malfunction [13,14]. Lethal toxin, a combination of LF and PA, can induce apoptosis of macrophage and endothelial cells [15–18], and impairment of dendritic cells [19]. Edema and lethal toxin synergize their action against host innate immunity [11,12]. Consequently, the deletion of the EF or LF gene leads to the reduction of virulence of anthrax bacteria [14,20].

☆ *Abbreviations:* EF, edema factor; PA, protective antigen; ATR, anthrax toxin receptor; TEM-8, tumor endothelial marker-8; CMG-2, capillary morphogenesis protein-2; LF, lethal factor; GFP, green fluorescent protein; PKA, protein kinase A.

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Edema toxin can raise intracellular cAMP levels, activating not only protein kinase A (PKA) but also cyclic nucleotide-gated ion channels and Epac, a guanine nucleotide-exchange factor for Rap1 [21]. To understand the pathologic consequences of exposure to edema toxin of various tissues and to realize the potential of edema toxin as a pharmacologic tool, we need to examine the effect of edema toxin on given cell types. After receptor-mediated endocytosis and subsequent translocation across the endosomal membrane, EF remains membrane associated [22]. Recent structural analysis reveals that the PA-binding domain of EF is dislodged from the remaining catalytic domain upon binding with calmodulin [23]. Whether the PA-binding domain plays a further role in the cytoplasmic action of EF, either through differential cellular localization or direct PA-mediated interactions, has not been directly tested.

Elucidation of the exact biological action of EF has been hampered by lack of highly purified, bacterially expressed EF protein. We have recently optimized the expression and purification of EF [24]. Here, we report that EF causes cytoskeletal and cell shape changes in a number of mammalian cell systems via activation of PKA. The PA-binding domain is not required for EF potency inside the cells, but expression of the anthrax toxin receptor is a key limiting factor. Our results suggest that both the specific cell type and the efficiency of cellular EF uptake are determining factors in the physiological response to edema toxin exposure.

Materials and methods

Materials. Protective antigen (PA) and full-length edema factor purified from *B. anthracis* (*BA*-EF) were purchased from List Biological Laboratories (Campbell, CA). Alexa Fluor 594 phalloidin and Alexa Fluor 488 C_5-maleimide were from Molecular Probes (Eugene, Oregon). Dibutyryl-cAMP was purchased from Calbiochem (La Jolla, CA). Epac activator 8-(4-chlorophenylthio)-2′-*O*-methyl-cAMP was from BIOLOG Life Science Institute (Bremen, Germany). Rabbit polyclonal antibody against green fluorescent protein (GFP), and a mouse monoclonal antibody against α-tubulin were from Santa Cruz Biotechnology (San Diego, CA). EF was produced in *Escherichia coli* as previously described [24].

Cell culture. Y1 mouse adrenal cortical cells and Chinese hamster ovary (CHO) cells were grown as previously described [25]. Other cell lines used in the paper, including HEK293, 293T, and T24E (a mouse fibroblast line immortalized with large-T antigen, a gift from Dr. Akira Imamoto at the University of Chicago), were cultured in DMEM containing 10% FBS and 1% penicillin and streptomycin at 37 °C in a 5% CO_2 incubator. For treatment with EF, cells were pre-treated with PA for 15 min.

DNA constructs. To construct a mammalian expression vector for the EF catalytic domain (GFP-ΔEF), pEGFP-C1 (BD Biosciences Clontech, Palo Alto, CA) and pProEx-EF3 [26] were digested with *Bgl*II and *Not*I, respectively. The resulting DNA was filled in with DNA polymerase Klenow, digested with *Kpn*I, ligated together, and digested with *Sal*I. The mammalian expression vector for GFP-ΔmutEF (D491N) was made by introducing a mutation in the appropriate codon of GFP-ΔEF using the Stratagene QuickChange mutagenesis kit. For the GFP–PKA construct, the GFP sequence was amplified by PCR using the forward primer A 5′-GCGGATCCACCATGGATTACAAG-3′ and the reverse primer A 5′-GGCGGCGGCGTTGCCATTGGGCCCTCCTTGAA TTC-3′ from pGL1-FlagGFP(D)FAT [27]. The catalytic domain of PKA was amplified by PCR from pCα EV [28] (a gift from Dr. G. Stanley McKnight, University of Washington) using the forward primer B 5′-AATTCAAGGAGGGCCCAATGGGCAACGCCGCCGCC-3′ and the reverse primer B 5′-CCCCTAAAACTCAGTAAACTCC-3′. The two PCR fragments carrying overlapping sequences were then annealed together and amplified by another PCR using the forward primer A and the reverse primer B. The resulting product was then subcloned into pGEM-T-Easy from Promega (Madison, WI), cut with *Bam*HI and *Spe*I, and then subcloned into the *Bam*HI and *Spe*I sites of pGL1-FlagGFP(D)FAT. The ATR expression vector pKB311 [5] was a gift from Dr. John A.T. Young (Salk Institute, San Diego).

Transfection. Lipofectamine reagents (Invitrogen, Carlsbad, CA) were used in 4–5:1 lipofectamine to DNA ratio for transfection of T24E cells. All other transfections were performed using TransIT-LT1 with a 3:1 LT1 to DNA ratio (Madison, WI).

Actin stress fiber staining. T24E cells were seeded on a coverslip (1.2×10^5 cells per plate in 60 mm plates). Actin was stained with Alexa Fluor 594 phalloidin (3 U/ml) as previously described [27].

Assay of cellular cAMP. For T24E cells treated with ET, cells were seeded at 0.48×10^5 cells per well in 12-well plates. For T24E cells transfected with GFP-ΔEF, cells were either seeded at 0.48×10^5 cells per well in 12-well plates or 1.15×10^5 cells per plate in 35 mm plates and transfected with 0.4 μg DNA or 1 μg DNA, respectively. Cells were lysed with 250 and 480 μl of 0.1 N HCl for 12-well plates and 35 mm plates, respectively. Fifty microliters of lysate for 12-well plates and 40 μl lysate for 35 mm plates were used for measurement of cAMP using Assay Designs's Direct cAMP assay kit (Ann Arbor, MI). Data were plotted according to manufacturer's procedure using the non-acetylated version. For CHO cells, cells were seeded in a 24-well plate at a density of 7.5×10^4 cells/well. After 24 h incubation, the cells were treated with either *B. anthracis* EF (*BA*-EF) or EF from *E. coli* at different concentrations with 100 ng/ml PA and were incubated for two hours. cAMP levels were measured using the Amersham Biosciences EIA kit.

In vitro enzymatic assay. Adenylyl cyclase activities were measured after 10 min at 30 °C in the presence of 30 mM Hepes, pH 7.2, 10 mM ATP with a trace amount of [^{32}P]ATP, 1 mM EDTA, 10 mM $MgCl_2$, and 1.2 mM free $CaCl_2$ as calculated by WEBMAXC STANDARD (http://www.stanford.edu/-cpatton/webmaxcS.htm). ATP and cAMP were separated by Dowex and alumina columns as described [26].

Cell lysate extraction and Western blot analysis. Cell lysates were collected and Western blots using anti-GFP antibody or anti-tubulin antibody at a dilution of 1:1000 were generated as previously described [29].

Fluorescence labeling of PA and surface-binding assay. The PA-cysteine mutant (PA_{83}E733C) plasmid was a gift from Dr. Ken Bradley (UCLA, LA) [30]. The purification of PA protein was performed as previously described [31]. The purified PA was labeled with Alexa Fluor 488 C_5 maleimide according to methods described [32]. For the surface-binding assay, cells were washed from plates, bound to labeled PA for 2 h on ice, then washed three times, and fixed with formaldehyde. All the binding and washing steps were performed in ice-cold growth medium containing 10% FBS. Cells were then sorted and assessed by FACS analysis. Fluorescence was monitored within a region defined using a plot of Forward scatter (FSC) versus Side scatter (SSC) to exclude cell debris.

Results and discussion

EF causes cell shape changes in a variety of cell types

In order to study the function of EF, an *E. coli* bacterial expression vector containing a histidine-tagged,