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ExoY from *Pseudomonas aeruginosa* is a nucleotidyl cyclase with preference for cGMP and cUMP formation

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ABSTRACT

In addition to the well known second messengers cAMP and cGMP, mammalian cells contain the cyclic pyrimidine nucleotides cCMP and cUMP. Soluble guanylyl cyclase and soluble adenylyl cyclase produce all four cNMPs. Several bacterial toxins exploit mammalian cyclic nucleotide signaling. The type III secretion protein ExoY from *Pseudomonas aeruginosa* induces severe lung damage and effectively produces cGMP. Here, we show that transfection of mammalian cells with ExoY or infection with ExoY-expressing *P. aeruginosa* not only massively increases cGMP but also cUMP levels. In contrast, the structurally related CyaA from *Bordetella pertussis* and edema factor from *Bacillus anthracis* exhibit a striking preference for cAMP increases. Thus, ExoY is a nucleotidyl cyclase with preference for cGMP and cUMP production. The differential effects of bacterial toxins on cNMP levels suggest that cUMP plays a distinct second messenger role.

1. Introduction

cAMP and cGMP are established second messengers [1,2]. In mammals, cAMP is produced by nine membranous G_s-protein-activated ACs and the soluble bicarbonate-stimulated soluble AC [3,4]. cGMP is produced by the nitric oxide-stimulated soluble GC and seven membranous GCs regulated by diverse mechanisms [5,6]. In addition to cAMP and cGMP, mammalian cells contain substantial concentrations of the cyclic pyrimidine cNMPs cCMP and cUMP [7,8]. Soluble GC and soluble AC, but not the corresponding membranous NCs, are rather promiscuous with respect to their substrate-specificity and produce all four cNMPs [7–10].

Bacteria have developed effective strategies to highjack mammalian cell cNMP signaling to corrupt host defence and to facilitate bacteria survival. Specifically, cholera toxin from *Vibrio cholerae* ADP-ribosylates G_s to induce massive cAMP production *via* membranous ACs [11]. By this mechanism *V. cholerae* causes life-threatening diarrhea. CyaA from *Bordetella pertussis* is an AC that inserts into the plasma membrane of host cells and is activated by calmodulin [12]. EF from *Bacillus anthracis* is an AC that is taken up into host cells *via* PA and is activated by calmodulin as well [13]. CyaA contributes to the pathogenesis of whooping cough [14], and EF contributes to the pathogenesis of anthrax disease [15]. ExoY is a type III secretion protein from *Pseudomonas aeruginosa* that possesses structural similarity with CyaA and EF in the catalytic domain and generates cAMP, too [16]. ExoY induces severe lung damage [17].

Not only soluble mammalian NCs possess a rather broad substrate-specificity but also bacterial AC toxins. Particularly, both purified CyaA and EF produce cCMP and cUMP [8], but it is unknown whether this activity is also present in intact cells. Surprisingly, ExoY turned out to be much more effective at producing cGMP than cAMP in intact cells [18]. In view of the increasing

Abbreviations: cAMP, adenosine 3′,5′-cyclic monophosphate; cGMP, guanosine 3′,5′-cyclic monophosphate; cCMP, cytidine 3′,5′-cyclic monophosphate; cNMP, nucleoside 3′,5′-cyclic monophosphate; cUMP, uridine 3′,5′-cyclic monophosphate; EF, edema factor; PA, protective antigen; AC, adenylyl cyclase; GC, guanylyl cyclase; NC, nucleotidyl cyclase; PI, propidium iodide; HPLC–MS/MS, high performance liquid chromatography coupled to quadrupole tandem mass spectrometry.

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interest in cCMP and cUMP as potential second messengers [19], these findings prompted us to examine the effects of ExoY on cNMP production in intact cells and to compare its effects with those of CyaA and EF. We took advantage of a highly sensitive and specific HPLC–MS/MS methodology [7–10].

2. Materials and methods

2.1. Materials

FuGene HD transfection reagent was purchased from Promega (Mannheim, Germany). Annexin V-allophycocyanin was supplied by MabTag (Friesoythe, Germany). *B. anthracis* EF was kindly provided by Dr. Wei-Jen Tang (Department for Cancer Research, University of Chicago, Chicago, IL, USA) and PA was obtained from List Biological Laboratories (Campbell, CA, USA). All cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured under the recommended conditions. The *P. aeruginosa* strains PA103Δ*exoUexoT::Tc* pUCP*exoY* and the non-active PA103Δ*exoUexoT::Tc* pUCP*exoY*-K81M were generated as described [16]. *B. pertussis* CyaA holotoxin (CyaA-wt) and the enzymatically inactive CyaA mutant (lacking AC activity as a result from a Leu-Gln dipeptide insertion between Asp188 and Ile189 in the catalytic core of the enzyme) were expressed in *Escherichia coli* and purified to near homogeneity by a previously established procedure [20]. The specific activity of CyaA was >500 μmol/min/mg. For transfection studies, coding sequences of ExoY and ExoY-K81M were cloned into pcDNA3.1 using standard molecular biology techniques.

2.2. Transfection and infection

For transfection, cells (4×10^5 per well) were seeded in 6-well plates and transfected with FuGene HD transfection reagent, 2 μg of pcDNA3.1-FLAG, pcDNA3.1-FLAG-ExoY-K81M or pcDNA3.1-FLAG-ExoY plasmid, respectively, following the manufacturer's recommendations. After various time points, cells were processed for cNMP determination or annexin-V/PI analysis. For infection, cells (4×10^5 per well) were seeded in 6-well plates, so that cells achieved a confluency of 80–100%. For the bacterial infection, both *P. aeruginosa* strains PA103Δ*exoUexoT::Tc* pUCP*exoY* and the non-active PA103Δ*exoUexoT::Tc* pUCP*exoY*-K81M, maintained on Vogel–Bonner medium supplemented with antibiotics (400 μg/ml carbenicillin), were streaked out on plates and incubated at 37 °C overnight. Bacteria were suspended in serum-free MEM and the number of CFU/ml was determined by measuring optical density ($OD_{540} = 0.25 = 2 \times 10^8$ CFU/ml). Serial dilutions of the applied bacterial suspension were made and incubated overnight in order to control the number of CFU. The cell culture medium was removed from the well which then was rinsed twice with PBS. The bacteria suspension was added to mammalian cells with a MOI of 5. After different time periods of incubation at 37 °C and 5% CO_2 (v/v), the bacterial suspension was removed and a mixture of 100 μg/ml gentamicin and 200 μg/ml ciprofloxacin diluted in serum-free culture medium was added to the cells. After an additional incubation period of 2 h, cells were harvested and prepared for HPLC–MS/MS quantitation of cNMPs.

2.3. HPLC–MS/MS

cNMP quantitation in cells was performed *via* HPLC–MS/MS as described using a QTrap5500 triple quadrupole mass spectrometer (ABISCIEX, Foster City, CA, USA) [7–10].

2.4. Analysis of cell viability

In order to distinguish between apoptotic and necrotic cell death, an annexin V/PI staining was performed. Cells (4×10^5 per well) were seeded in a 6-well plate. Five hundred microliter of transfected or stimulated cells were transferred to a 1.5 ml Eppendorf tube and centrifuged at a speed of 300*g* for 10 min. The supernatant fluid was discarded, 100 μl of FACS binding buffer (10 mM HEPES, 140 mM NaCl, 2.5 mM $CaCl_2$ in water, pH 7.4 was adjusted by adding 1 M NaOH) was added and the mixture was immediately transferred into a FACS tube. Annexin V-allophycocyanin (MabTag, 5.8 μl) was added and the samples were incubated for 20 min in the dark. Directly before the flow-cytometric analysis a volume of 10 μl PI (50 μg/ml) was added to each sample. The flow-cytometric analysis was performed using a MACS Quant Analyzer with MAQS Quant running buffer (Miltenyi Biotec, Bergisch Gladbach, Germany). Annexin V-allophycocyanin was excited at a wavelength of 635 nm and PI at a wavelength of 487 nm. Emission was determined between 655 and 730 nm.

2.5. Statistics

Data are presented as means ± SD and are based on 3 independent experiments performed in triplicates. GraphPad Prism 5.01 (San Diego, CA, USA) was used for calculation of mean and SD. *p*-Values were calculated by means of one-way ANOVA with Bonferroni's multiple comparison test.

3. Results

As a major model system for our present studies we used B103 neuroblastoma cells because these cells endogenously express high levels of cUMP and cCMP [9]. Transfection of mammalian cells *per se* results in a decrease in cellular cNMP levels [7], and this was also true for B103 cells (data not shown). When ExoY was transfected into B103 cells we observed long-lasting increases in all four cNMPs in the order cUMP ~ cCMP ~ cGMP > cAMP (Fig. 1A–D). Note that these experiments were performed in the absence of phosphodiesterase inhibitors. As a control, we transfected cells with ExoY harboring a mutation in the catalytic center (K81M) that greatly reduces NC activity [16]. As expected [16–18], with ExoY-K81M we observed only very small if any cNMP increases. Treatment of B103 cells with EF + PA resulted in a large but more transient cAMP increase and a small cCMP increase; no cGMP and cUMP increases were observed (Fig. 1E–H). As expected [13,14], EF and PA alone had no effect on cNMP levels. A similar pattern as for EF was observed for CyaA; i.e. a large and transient cAMP increase, and a small and transient cCMP increase (Fig. 1I–L). A catalytically inactive CyaA mutant was ineffective. Similar to the data obtained with B103 cells, EF increased predominantly cAMP in J774 macrophages; a small cCMP increase was noted, too. cGMP and cUMP did not increase (Fig. S1A–D). In J774 macrophages and HL-60 leukemia cells, CyaA induced large cAMP increases and small cCMP increases; in J744 cells, an additional small cUMP increase was observed. cGMP remained unaffected (Fig. S1E–L).

In order to assess the functional consequence of toxin treatment, we studied the viability of B103 cells by FACS analysis. Compared to empty plasmid and ExoY-K81M control, ExoY increased the percentage of dying and necrotic cells (Fig. 2A). For CyaA, an increase in the percentage of necrotic cells was noted (Fig. 2B), and for EF, we observed an increase in the percentage of apoptotic and necrotic cells (Fig. 2C).

The studies described above were performed with cells into which ExoY was transfected *via* pcDNA3.1. However, under *in vivo*-conditions, ExoY is injected into host cells *via* the type III

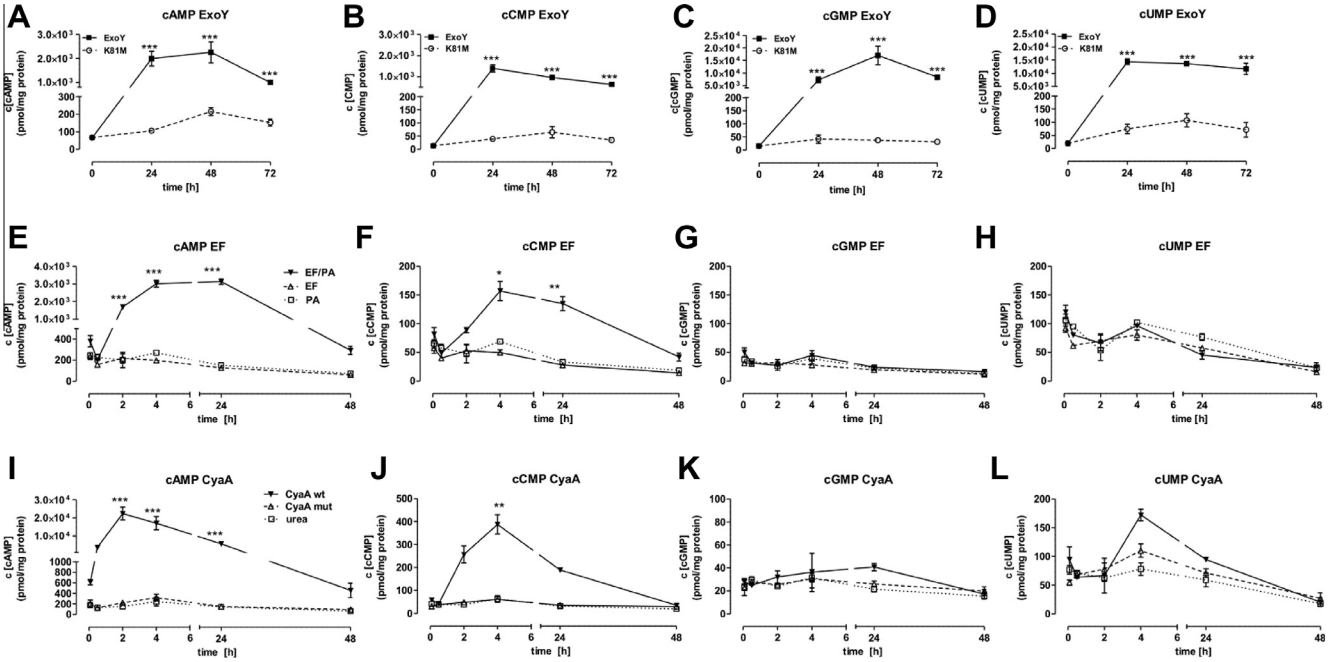

Fig. 1. cNMP accumulation in B103 cells following transfection with ExoY and treatment with EF or CyaA. A–D, B103 cells were transfected with pcDNA3.1-ExoY or pcDNA3.1-ExoY-K81M and incubated for various periods of time. Thereafter, cNMPs were extracted and determined by HPLC–MS/MS. E–H, B103 cells were treated with 1 μg/ml EF, 2.3 μg/ml PA or a combination of 1 μg/ml EF +2.3 μg/ml PA for various periods of time. Thereafter, cNMPs were extracted and determined by HPLC–MS/MS. I–L, B103 cells were treated with 10 μg/ml CyaA holotoxin, 10 μg/ml CyaA mutant or urea carrier for various periods of time. Thereafter, cNMPs were extracted and determined by HPLC–MS/MS. ∗, $p < 0.05$; ∗∗, $p < 0.01$; ∗∗∗, $p < 0.001$. Data shown are the means ± SD of three independent experiments performed in triplicates.

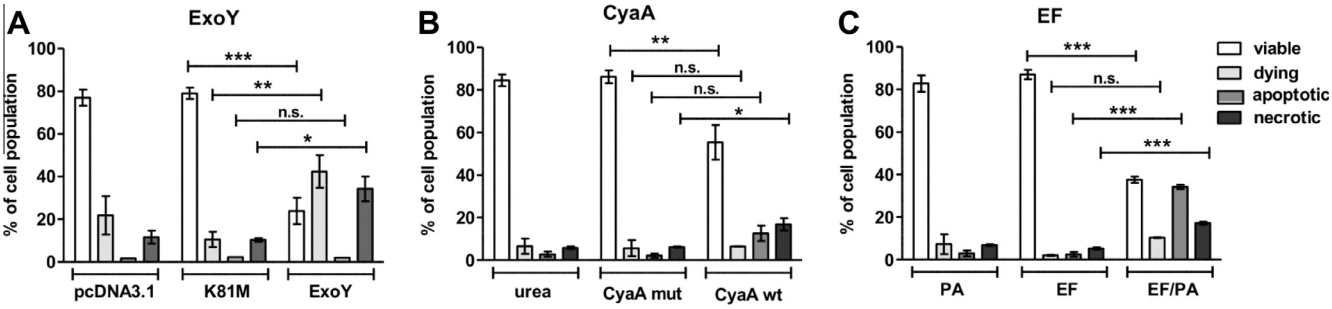

Fig. 2. Flow cytometric viability analysis of B103 cells following transfection with ExoY and treatment with EF or CyaA. A, B103 cells were transfected with pcDNA3.1-ExoY or pcDNA 3.1-ExoY-K81M and incubated for 72 h and cell viability was analyzed by annexin V/PI staining. B, B103 cells were treated with 10 μg/ml CyaA holotoxin, 10 μg/ml CyaA mutant or urea carrier for 72 h and cell viability was analyzed by annexin V/PI staining. C, B103 cells were treated with 1 μg/ml EF, 2.3 μg/ml PA or a combination of 1 μg/ml EF +2.3 μg/ml PA for 72 h and cell viability was analyzed by annexin V/PI staining. N.s., not significant; ∗, $p < 0.05$; ∗∗, $p < 0.005$; ∗∗∗, $p < 0.001$. Data shown are the means ± SD of three independent experiments performed in triplicates.

secretion system [16,21]. Therefore, we performed experiments in which *P. aeruginosa* harboring ExoY as only pathogenic factor (PA103Δ*exoUexoT::Tc* pUCP*exoY*) [16–18] were co-cultured with adherent mammalian cells. After defined incubation times, *P. aeruginosa* bacteria were removed, and cNMP levels in the adherent mammalian cells were determined following an additional 2 h incubation period. In control experiments, we found that *P. aeruginosa* are devoid of endogenous cGMP, cCMP and cUMP, and cAMP levels were just above detection level (data not shown). In A549 lung epithelial cells, ExoY, delivered *via* the type III secretion system, increased cNMP levels in the order cUMP > cGMP > cCMP > cAMP (Fig. 3A–D). In B103 cells, ExoY increased cNMPs in the order cUMP ∼ cGMP > cAMP > cCMP (Fig. 3E–H). cUMP and cGMP increases in B103 cells were more rapid in onset than cAMP and cCMP increases. Both in A549 cells and B103 cells, ExoY-K81M had only little effect on cNMP levels. In HeLa cells, ExoY delivered *via* the type III secretion system increased cNMPs in the order cUMP > cGMP > cCMP > cAMP (Fig. S2A–D), and when delivered *via* transfection, the order was cUMP > cGMP ∼ cAMP > cCMP (Fig. S2E–H). In HEK293 cells transfected with ExoY, cNMPs increased in the order cUMP > cGMP ∼ cAMP > cCMP as well (Fig. S2I–L). Again, ExoY-K81M was ineffective at increasing cNMPs, regardless of the cell type or delivery method studied.

4. Discussion

The most important finding of this study is that ExoY is highly effective at producing cUMP in mammalian cells. The high efficiency of ExoY at producing cUMP was observed in all cell types studied and was independent of the delivery method, i.e. transfection or injection *via* the type III secretion system. Moreover, the catalytically inactive ExoY-K81M mutant failed to induce massive cUMP increases. ExoY has been recently shown to be effective at producing cGMP [17], and this efficacy is similar to the efficacy for cUMP formation. All these data indicate that ExoY constitutes

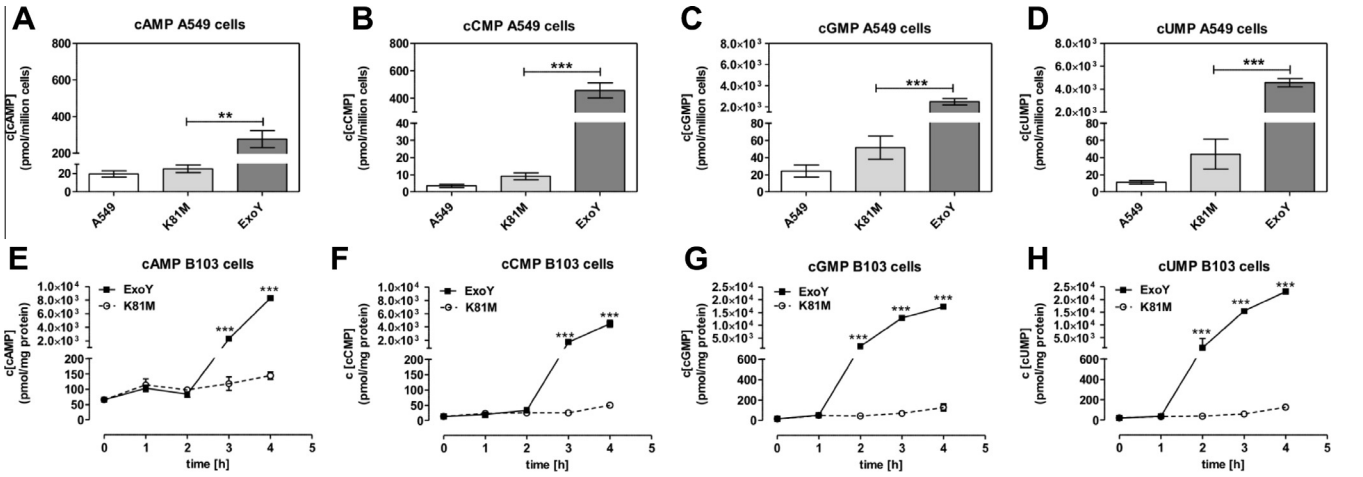

Fig. 3. cNMP accumulation in A549 cells and B103 cells following infection with ExoY-expressing *P. aeruginosa*. A–D, A549 cells were infected with *P. aeruginosa* strains PA103ΔexoUexoTpUCPexoY or PA103ΔexoUexoTpUCPexoY-K81M at a multiplicity of infection of 5 for 4 h with 5% CO_2 (v/v) at 37 °C. Bacteria were removed from A549 cells, and cells were incubated for additional 2 h. Thereafter, cells were harvested and cNMPs were extracted and determined by HPLC–MS/MS. E–H, B103 cells were infected with *P. aeruginosa* strains PA103ΔexoUexoTpUCPexoY or PA103ΔexoUexoTpUCPexoY-K81M at a multiplicity of infection of 5 for 0–4 h with 5% CO_2 (v/v) at 37 °C. Bacteria were removed from B103 cells, and cells were further incubated at 37 °C for additional 2 h. Thereafter, cells were harvested and cNMPs were extracted and determined by HPLC–MS/MS. ***, $p < 0.001$. Data shown are the means ± SD of three independent experiments performed in triplicates.

a NC with preference for cGMP and cUMP formation. ExoY also produced cAMP and cCMP to different extents. These differences may be due to different cellular and subcellular pools of the corresponding nucleoside 5′-triphosphates required for cNMP synthesis.

It has been suggested that ExoY, being injected into the cytosol of target cells, mimics and/or exaggerates the function of the soluble AC [22,23]. While on first glance, our data do not seem to support this notion because of the preference of ExoY for cGMP and cUMP, on second glance, our data actually support the original hypothesis regarding the soluble AC. Particularly, we have recently provided evidence, using removal and addition of bicarbonate and pharmacological inhibitors as experimental tools, that soluble AC is not only important for the maintenance of the high basal cAMP levels in HEK293 and B103 cells but also for maintenance of high cGMP, cCMP and cUMP levels [9]. In accord with a mimicry role of soluble AC, ExoY is highly effective at increasing all four cNMPs in HEK293 and B103 cells. As a functional result of the cNMP increases, ExoY induces necrosis, fitting to the deleterious effects of ExoY on *in vivo* tissue integrity [17].

The high efficacy of ExoY at producing cGMP and cUMP clearly distinguishes this NC from the structurally related CyaA and EF. These two toxins have a striking preference for cAMP formation relative to formation of the other cNMPs in intact cells. While ExoY, CyaA and EF exhibit similar functional effects in B103 cells, i.e. apoptosis and/or necrosis, it is well possible that in other cells, ExoY on one hand and CyaA and EF on the other hand, show different effects. Systematic comparative studies of the biological effects of the three NCs in various cell types are subject of future studies in our laboratory.

The large stimulatory effect of ExoY on cUMP levels in various cell types indicates that this cNMP plays a distinct role as second messenger. This notion is further supported by the fact that cUMP is specifically hydrolyzed by phosphodiesterases 3A and 9, but not by several other phosphodiesterases [24]. cUMP is a low-potency activator of cAMP- and cGMP-dependent protein kinases and of HCN channels 2 and 4 [25–27]. Given the very large cUMP increases following ExoY treatment, it is conceivable that cUMP reaches intracellular levels that are sufficient for activation of these cAMP- and cGMP effector proteins by this non-cognate cNMP. However, the much more logical hypothesis is that hitherto unrecognized specific cUMP effector proteins exist. Such new cUMP effector proteins could be identified with cUMP agarose binding techniques coupled with mass spectrometry detection [28]. We are currently performing such studies but cannot yet report a candidate cUMP-binding protein at this point of time. However, considering the fact that in addition to the originally identified adenine nucleotide-binding G-protein-coupled receptors, uracil nucleotide-binding receptors had been identified subsequently [29,30], it is very likely that ultimately, specific cUMP-binding effector proteins will be identified. In view of the fact that ExoY is effective at increasing cUMP levels in all cell types studied so far, it is likely that such putative cUMP-binding effector proteins are broadly expressed.

In conclusion, in this report, we have identified a novel and unexpected biochemical activity of the NC ExoY, i.e. the effective production of cUMP. This unique activity of ExoY calls for research on the catalytic properties of purified ExoY and the search for specific mammalian cUMP effector proteins. Furthermore, our present study will facilitate the identification of the elusive mammalian cofactor for ExoY [16]. Lastly, in view of the recently discovered pathological effects of ExoY in the lung [17], ExoY may constitute a novel promising target for the development of antibiotics against *P. aeruginosa*. This goal has very high priority because antibiotic resistance against this bacterium is a very serious medical problem [31].

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.06.088.

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