Role of N-Terminal His6-Tags in Binding and Efficient Translocation of Polypeptides into Cells Using Anthrax Protective Antigen (PA)

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Abstract

It is of interest to define bacterial toxin biochemical properties to use them as molecular-syringe devices in order to deliver enzymatic activities into host cells. Binary toxins of the AB7/8-type are among the most potent and specialized bacterial protein toxins. The B subunits oligomerize to form a pore that binds with high affinity host cell receptors and the enzymatic A subunit. This allows the endocytosis of the complex and subsequent injection of the A subunit into the cytosol of the host cells. Here we report that the addition of an N-terminal His6-tag to different proteins increased their binding affinity to the protective antigen (PA) PA63-channels, irrespective if they are related (C2i) or unrelated (gpJ, EDIN) to the AB7/8-family of toxins. His6-EDIN exhibited voltage-dependent increase of the stability constant for binding by a factor of about 25 when the trans-side corresponding to the cell interior was set to ~70 mV. Surprisingly, the C. botulinum toxin C2i-channel did not share this feature of PA63. Cell-based experiments demonstrated that addition of an N-terminal His6-tag promoted also intoxication of endothelial cells by C2i or EDIN via PA63. Our results revealed that addition of His6-tags to several factors increase their binding properties to PA63 and enhance the property to intoxicate cells.

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Introduction

Gram-positive bacteria such as Bacillus anthracis and Clostridium botulinum synthesize as most crucial virulence factors protein toxins of the AB7/8 type. These toxins are composed of two components which are nontoxic by themselves when added to the external media of target cells [1]. One or more A-components of the toxins feature intracellular enzymatic activity and are responsible for the toxicity. The B-component binds to cellular receptors or directly to the membrane and transports the enzymatic component into the cell. Anthrax-toxin from B. anthracis belongs to the AB7/8-type of toxins classified by a pore forming B-component, protective antigen (PA) and two enzymes, edema factor (EF) and lethal factor (LF). PA is an 83 kDa water soluble precursor, which has to be activated by cleavage of a 20 kDa N-terminal part to form the functional PA63-heptamers/octamers [2–5]. The proteolytic activation is performed in vivo by cell bound furin. It allows pore formation and injection of both enzymatic components EF and LF into cells [6–9]. LF is an Zn2+-binding metalloprotease that cleaves mitogen-activated protein kinase kinases (MAPK-kinases).

C. botulinum, well known for the production of potent neurotoxins, also produces other protein toxins such as the binary C2-toxin and the single-component C3 exoenzyme [10–12]. The homologous pore forming component to PA63 of C2-toxin is C2II. After proteolytic cleavage with trypsin (60 kDa) it forms heptamer/octamers that insert into biological and artificial membranes at an acidic pH and promotes the translocation of the 45 kDa enzymatic component C2I. Similar to anthrax-toxin a receptor-mediated endocytosis of C2 is required for intoxication of the cell [13,14]. C2I acts as an ADP-riboseyltransferase on arginine177 of monomeric G-actin, causing disruption of the actin cytoskeleton of eukaryotic cells [15,16].

The toxins of the AB-type represent simple but sophisticated molecular syringes for protein delivery into target cells. This means that they could be important systems for development of new strategies for efficient injection of polypeptides into target cells. Possible Trojan Horses could be binary toxins of the AB7/8 type such as anthrax- and C2-toxin [1]. The binding of the N-terminal ends of the enzymatic components to the heptameric/octameric channel formed by the binding components is followed.
His-Tag Promoted Protein Delivery by PA into Cells

by receptor-mediated endocytosis, acidification of the endosomes and final release of the enzymatic components into the cytosol of target cells, where they exert their enzymatic activities [3,17]. Interestingly, the amino-terminal part of LF is sufficient to confer the ability to associate with PA63-heptamer/octamer on LF. It can be used to drive the translocation of unrelated polypeptides fused to LF1-254 into target cells in a PA63-dependent manner [18,19]. Although the enzymatic components of anthrax- and C2-toxin differ considerably in their enzymatic activity and in their primary structures as well, the binding components PA and C2II share a significant overall sequence homology of about 35%, which means that they are closely related in structure and probably also in function [5,8,20,21]. There exist several structural features that are important for the binding of channel blockers and enzymes to be delivered into the target cells. One is the so-called F(phenylalanine)-clamp - F427 in PA and F428 in C2II – which is combined with two rings of seven negatively charged amino acids - E399 and D428 in PA and E398 and D427 in C2II, respectively [22,23]. These negatively charged amino acids seem to interact with the positively charged N-terminal ends of the enzymatic components [19,24,25]. Another interesting feature involved in binding and transport of truncated and full-length effectors is the alpha-clamp in PA [26,27]. This represents a big amphipathic cleft on the surface of the PA63 heptamers/octamers, which plays an important role in oligomer formation of PA63 and unfolding and translocation of effectors [26,27].

Blank et al. [19] have established that addition of His6-tag and other polycationic presequences to diphtheria toxin chain (DTA) allows its injection into cells by PA63 binding component. The observation that polycationic peptides at the N-terminal end of DTA facilitates its import into the cytosol of a CHO-K1 cell line is of particular interest [19]. Here we aimed at establishing whether this effect of His6-tag could be generalized to other toxin enzymatic components and quantify the involvement of His6-tag on these toxin component interactions with PA63 in vivo and in vitro. To study this we have investigated the influence of additional charges on the N-terminal end on binding of the enzymatic factors to the channels formed by PA63 and C2II. First results in the field were found with polycationic peptides fused to EF, LF, LFN, LF and DTA [19,21]. Our results suggested that the binding of LF and EF to C2II is possible and that C2II binds to PA63 in the black lipid bilayer assay as well. The most significant result that was observed was a preferential binding of His6-C2I, as compared to C2I, to PA63. Interestingly, PA63 is able to transport His6-C2I into target cells with a higher efficiency than C2I. We extended these finding by demonstrating that a His6-tag fused to the N-terminal end of the epidermal cell differentiation inhibitor (EDIN) of Staphylococcus aureus also increases its affinity to PA63 and allows an efficient PA63-dependent delivery of His6-EDIN in target cells. In addition, the stability constant for binding of His6-EDIN and not that of EDIN to PA63-channels was found to be highly voltage-dependent, which could be one important factor for efficient delivery of effectors via PA63 into target cells.

Experimental Procedures

Materials

Protective antigen encoding gene was cloned with BamHI-SacI restriction sites into pET22 (Novagen) as previously described [28]. The translocation-defective PA mutant F427A [22,29] was constructed by site-directed mutagenesis using the Quick-Change™ kit (Stratagene) according to the manufacturers instructions. The PA-gene cloned in the plasmid pET19 (Novagen) [30,31], was used as a template. The construct was confirmed by DNA sequencing. The protein was expressed with an N-terminal His6-tag in BL21 (DE3) (Novagen) and purified by HiTrap chelating (Pharmacia) charged with Ni2+ ions.

C2I and C2II genes were PCR-amplified from genomic DNA of Clostridium botulinum D strain 1073 and cloned into pET22 (Novagen) and pQE30 (Qiagen) expression plasmids with BamHI-SacI restriction sites.

The plasmid coding for the chimera protein MBP-gpJ (maltose-binding-protein attached to amino acids 684–1132 of Lambda phage tail protein J) was a kind gift of Alain Charbit, Necker Enfants Malade, Paris, France. Expression and purification of MBP-gpJ was performed as described previously [32]. gpJ was obtained by treatment of MBP-gpJ bound to starch column beads (amylose-Sepharose, New England Biolabs) with factor Xa (Invitrogen). His6-gpJ (684–1132) was obtained as described previously [33].

The DNA encoding EDIN (NCBI M63917) was cloned into pET28a vector using BamHI-EcoRI restriction site as described previously [34]. Recombinant toxins containing His6-tags were expressed in E. coli BL21 (DE3) and purified on a Chelating Sepharose Fast Flow column previously chelated with nickel (Amersham Biosciences) as recommended by the manufacturer. Fractions containing toxin were pooled and dialyzed over night against 250 mM NaCl and 25 mM Tris-HCl, pH 8. The N-terminal His6-tag was removed by incubation with thrombin. Nicked anthrax PA63 from B. anthracis was obtained from List Biological Laboratories Inc., Campbell, CA. One mg of lyophilized protein was dissolved in 1 ml 5 mM HEPES, 50 mM NaCl, pH 7.5 complemented with 1.25% trehalose. Aliquots were stored at −20°C. Channel formation by PA63 was stable for months under these conditions.

Cell Culture and Biochemical Products

HUVECs [human umbilical vein endothelial cells, a human primary cell line obtained from PromoCell] were grown in serum-free medium (SFM) supplemented with 20% FBS (Invitrogen), 20 ng/ml basic bFGF (Invitrogen), 10 ng/ml EGF (Invitrogen) and 1 μg/ml heparin (Sigma-Aldrich) as described previously [35]. Monoclonal antibodies used were: anti-RhoA (BD Biosciences, clone 26C4); anti-β-actin (SIGMA, clone AC9–74); anti-His-tag (Qiagen, [Penta-His]). Primary antibodies were visualized using goat anti-mouse horseradish peroxidase-conjugated secondary antibodies (DakoCytomation), followed by chemiluminescence detection ECL (GE Healthcare). Levels of active Rho were determined by GST-rhotekin RBD pull-down that was modified as described previously [35].

Immunofluorescence Studies

The experiments were performed on cells fixed in 4% paraformaldehyde (SIGMA). Actin cytoskeleton was labelled using FITC-conjugated phalloidin (SIGMA), as described in [36]. Immunosignals were analyzed with inverted microscope (EVOSfl, AMG) using a 20X object lens.

Lipid Bilayer Experiments

Black lipid bilayer measurements were performed as described previously [37]. The instrumentation consisted of a Teflon chamber with two aqueous compartments connected by a small circular hole. The hole had a surface area of about 0.4 mm2. Membranes were formed by painting a 1% solution of diphytanoyl phosphatidylcholine (Avanti Polar Lipids, Alabaster, AL) in n-decane onto the hole. The aqueous salt solutions (Merck, Darmstadt, Germany) were buffered with 10 mM MES-KOH to pH 5.5 to pH 6. Control experiments revealed that the pH was
stable during the time course of the experiments. The binding components of the binary toxins were reconstituted into the lipid bilayer membranes by adding concentrated solutions to the aqueous phase on one side (the cis-side) of a black membrane. The temperature was kept at 20°C throughout. Membrane conductance was measured after application of a fixed membrane potential with a pair of silver/silver chloride electrodes inserted into the aqueous solutions on both sides of the membrane. Membrane current was measured using a homemade current-to-voltage converter combined with a Burr Brown operational amplifier. The amplified signal was monitored on a storage oscilloscope and recorded on a strip chart recorder.

**Binding Experiments**

The binding of the His-tagged proteins to the C2II-channel and the binding component PA63 was investigated with titration experiments similar to those performed previously to study the binding of 4-aminoquinolones to the C2II- and PA63-channels and EF and LF to the PA63-channel in single- or multi-channel experiments [38–40]. The aqueous phase contained always 150 mM KCl, buffered with 10 mM MES-KOH to pH 5.5 to 6. The C2II- and PA63-channels were reconstituted into lipid bilayers, where channel formation is obtained under mildly acidic conditions [44]. 60 minutes after the addition of the protein to the cis-side of the lipid bilayer, the rate of conductance increase had slowed down considerably at an applied membrane potential of 20 mV. At that time, small amounts of a concentrated protein solution were added to the cis-side of the membrane and the PA63-induced membrane conductance decreased in a stepwise manner.

Figure 1A shows an experiment of this type in which increasing concentrations of His6-C2I (arrows) were added to the cis-side of a membrane containing about 300 PA63-channels. The membrane conductance decreased as a function of the His6-C2I concentration. The data of Figure 1A and of similar experiments were analyzed assuming Langmuir isotherms for binding (equation (1)) [39,41,45] Lineweaver-Burk (double reciprocal) plots were used to calculate the stability constant for binding as shown in Figure 1B for the data of Figure 1A. The resulting straight corresponded to a stability constant of (3.93±0.39)×10^7 M^-1 for His6-C2I binding to PA63-pores (half saturation constant Ks = 25 nM).

At least three individual experiments were used to calculate the half saturation constants Ks of C2II- and His6-C2I binding to the PA63-channel. The average of the half-saturation constant Ks was 150 nM for C2I, whereas for His6-C2I binding to PA63-channels was 16 nM] in 150 mM KCl. This means that the half-saturation constant Ks for binding of His6-C2I was roughly ten times lower than that for C2I without His6-tag (Table 1). Titration experiments with artificial bilayer membranes of the wildtype A-B components C2II and C2I of C2-toxin revealed a half saturation constant Ks of 27 nM. Interestingly, a His6-tag attached to the N-terminal end had no obvious effect on binding of C2I to C2II-pores (Ks = 29 nM; Table 1).

**Addition of His6-tag to C2I Potentiates its Transfer via PA63**

In further experiments we tested if addition of His6-tag to C2I triggers its entry into cells via PA63-channels in vitro. C2I acts as an ADP-ribosyltransferase, targeting cellular G-actin [46]. Therefore, successful delivery of this enzymatic component into target cells can be detected by disruption of the cytoskeleton followed by rounding up and detachment of target cells from the extracellular matrix, defined as intoxicated cells [15]. HUVECs were intoxicated with C2I and His6-C2I driven by PA63 as indicated, and the number of intoxicated cells was directly assessed by counting (Fig. 2A). These results were compared to that of native toxin combination C2I and C2II. We observed a cytotoxic effect with the combination of His6-C2I and PA63. No effect could be detected for C2I and PA63 under the same conditions. The specificity of this internalization was verified by using a mutant of PA63: PA F427A. This mutant is competent for receptor binding and internalization, but defective in the pH dependent functions: pore formation and ability to translocate bound ligands [47]. Intoxication of cells with His6-C2I and PA F427A did not induce any cellular effect (Fig. 2B). Thus, the increase of affinity between PA63 and

\[
\text{Fraction of blocked channels} = \frac{(G_{\text{max}}-G(c))}{G_{\text{max}}} = \frac{K \cdot c}{(K \cdot c + 1)}
\]

\[
1/(\text{Fraction of blocked channels}) = \frac{1}{K \cdot c} = 1 + \frac{1}{K \cdot c}
\]

\[K \text{ is the stability constant for binding of the enzymatic components of the binary toxins to the PA63- or C2II-channels. The half saturation constant } K_c \text{ is given by the inverse stability constant } 1/K.\]

**Statistical Analysis**

When assessing multiple groups, one-way ANOVA was used followed by Bonferroni post hoc test with *p<0.05. Data are presented as means ± SEM. The statistical software used was Prism 5.0 b.

**Results**

**Interaction of PA63-pores with His6-C2I in Artificial Black Lipid Bilayer Membranes**

We compared the binding affinity of different proteins with and without a His6-tag to the PA63- and C2II-channels. Taking into account that positive charges seem to have a huge influence in binding to the PA63-pore but only less to the C2II-pore [40,42], we chose the enzymatic component C2I as the first substrate. In a previous study we could show that it binds to PA63-pores and could even be translocated into cells albeit with very low efficiency at high C2I concentration [43]. We now addressed the question, if binding and translocation are enhanced by addition of a His6-tag to C2I.

The stability constants K (and the half-saturation constant Ks) for the binding of His6-C2I to the PA63-channel were measured in multi-channel experiments, performed as described previously [39]. A receptor is required for the binding and oligomerization of PA63 on the surface of mammalian cells [9]. However, this is not necessary for reconstitution of PA63-channels in artificial lipid bilayers, where channel formation is obtained under mildly acidic conditions [44]. 60 minutes after the addition of the protein to the cis-side of the lipid bilayer, the rate of conductance increase had slowed down considerably at an applied membrane potential of 20 mV. At that time, small amounts of a concentrated protein solution were added to the cis-side of the membrane and the PA63-induced membrane conductance decreased in a stepwise manner.

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C2I, upon addition of His6-tag to C2I allows His6-C2I to efficiently intoxicate cells via PA63-channels.

His6-tags do not Facilitate Binding of EF and LF to C2II-channels

To examine whether the N-terminal His6-tag of EF and LF have a similar effect on binding kinetics to the C2II-channel, as previously shown for His6-EF and His6-LF and PA63 [21], we omitted the cleavage of the His6-tag after the affinity purification and studied binding to C2II-channels. Interestingly, His6-EF and His6-LF did not exhibit any significant changes of their affinity to C2II-channels as compared to EF and LF (see Table 1). The half saturation constants $K_S$ of the interactions between His6-EF and His6-LF and the C2II-channels were 19 nM for His6-EF and 29 nM for His6-LF (Table 1).

Binding of His6-gpJ and gpJ Proteins to PA63- and C2II-channels

The His6-tag had a remarkable influence on binding of enzymatic components to the PA63-channel but not to the C2II-channel. To check if this interaction was specific for the presence of the His6-tag we performed titration experiment with a His-tagged protein that is not related to the effectors EF, LF or C2I. gpJ is a 447 amino acids C-terminal fragment of protein J (amino acids 684–1131), which is responsible for binding of bacteriophage Lambda to LamB on the surface of E. coli K-12 [31]. His6-gpJ exhibited high affinity binding (block) to the PA63-channel. The half saturation constant $K_S$ for binding of His6-gpJ to PA63 was calculated to be 5.0 nM in 150 mM KCl, 10 mM MES-KOH, pH 6.0 (mean of three measurements) (Table 1). Similar experiments with gpJ did not exploit any
binding of gpJ to the PA63-channel. This implies half saturation constants \( K_s \) of gpJ-binding to PA63 were much higher than 100 \( \mu\)M. We could not detect any substantial binding of His6-gpJ nor of gpJ to the C2II-channel (Table 1). Our results reveal the substantial role of the His6-tag at the N-terminal end of C2I and gpJ for their binding to the PA63- but not to the C2II-channel.

### Binding of EDIN and His6-EDIN to PA63- and C2II-channels

Next, we tested whether PA63-pores bind and transport EDIN of *Staphylococcus aureus*, as well as His6-EDIN. EDIN normally enters host cells inefficiently by nonspecific macropinocytosis and not by delivery systems considered in this study [20]. Previously, it has been shown that LF1-254-EDIN can enter cells via PA63 [20]. EDIN is a *Staphylococcus aureus* exoenzyme with ADP-ribosylating activity on RhoA. EDIN targets RhoA in cells for inactivation producing actin cable disruption in target cells [34]. Interestingly, we found that PA63-pores bound both EDIN and His6-EDIN with half saturation constants that were considerably lower than those reported before for the crossing of the \( \Delta R_{625} / 70\) types of toxin [43]. The half saturation constant \( K_s \) for EDIN binding to PA63-channels was on average 2.7 \( \mu\)M in 150 mM KCl, whereas this constant decreased to 0.7 \( \mu\)M for His6-EDIN. The results of these experiments are summarized in Table 1 and demonstrate that EDIN without His6-tag binding at low transmembrane voltage (5 mV) with a roughly four-fold lower affinity than the PA63-channels. At higher voltages, we applied a noticeable effect of voltage on His6-EDIN binding (see below). The affinity of EDIN to the C2II-channels \( K_s = 23 \mu\)M was by a factor of about eight-fold lower as compared to binding to the PA63-channels. Surprisingly, we observed a considerable effect when the His6-tag was attached to the N-terminal end of EDIN. The half saturation constant dropped in this case to 0.9 \( \mu\)M for its binding to C2II-pores (Table 1).

### His6-Tag Promotes EDIN Internalization via PA63-pores

We next verified the role of His6-tag in the uptake of EDIN into cells. After purification the His6-tag was cleaved as described in the material and methods section. We verified the cleavage by immunoblotting the purified proteins using an antibody against the His6-tag (Fig. 3A). The efficiency of RhoA targeting by EDIN was assessed by GST-Rhotekin pull down of active RhoA (GTP-bound RhoA). No effect was measured on cells challenged with His6-EDIN alone up to 10 \( \mu\)g/ml (Fig. 3B). We then intoxicated cells with His6-EDIN in the presence and absence of PA63. Strikingly, this revealed that the addition of PA63 together with His6-EDIN (10 \( \mu\)g/ml) increased the capacity of EDIN to intoxicate cells. This led us to decipher the role of His6-tag. Cells were intoxicated with PA63 together with EDIN or His6-EDIN. This clearly established that addition of His6-tag to EDIN in presence of PA63 produced a 70% decrease of RhoA activation specifically (Fig. 3C). In conclusion, addition of His6-tag to EDIN promotes its internalization via PA63 for cell intoxication.

### Immunofluorescence Studies of HUVECs and PA63 with EDIN and His6-EDIN

We next analyzed the actin cytoskeleton phenotype of cells intoxicated with PA63+His6-EDIN and PA63+LFN-EDIN, as well as EDIN alone or in combination with PA63 (Figure 4A). In all cases we observed that intoxicated cells displayed a disruption of filamentous actin and actin cables, as well as a undergo spreading, as previously described (Figure 4A) [34,36]. In addition, intoxicated cells displayed large transendothelial cell macropore tunnels (TEM) [see Figure 4A]. Induction of TEMs results from the dose dependent inhibition of RhoA [34,36]. We thus further determined the efficiency of cell intoxication, using different combinations of toxin components by measure of the percentage of cells displaying TEMs (Figure 4B). Most importantly, this showed that addition of His6-tag to EDIN increases its capacity to intoxicate cells in combination with PA63 at a concentration of

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### Table 1. Stability constants \( K \) and half saturation constants \( K_s \) for binding of proteins with and without His6-tags to membrane channels formed by anthrax PA63 and C2II.

| Channel | \( K \) [10^7 1/(Ms)] | \( K_s \) [nM] | \( K \) [10^7 1/(Ms)] | \( K_s \) [nM] | Ratio \( K_s \) without and with His6-tag |
|---------|---------------------|-------------|---------------------|-------------|-----------------------------------|
| PA63    | with EF** 7.7         | 13.0        | His6-EF** 5.2       | 13.0        | 0.68                              |
|         | LF** 3.7             | 20.0        | His6-LF** 3.4       | 19.0        | 1.7                               |
| C2II    | gpJ <0.001           | >100,000    | His6-gpJ 20.0       | >20,000     | 0.42                              |
| EDIN    | 0.040 ±0.011         | 2,700       | His6-EDIN 0.14 ±0.015 | 700         | 3.9                               |
| C2II    | with EF** 7.7         | 13.0        | His6-EF** 5.2       | 13.0        | 0.68                              |
|         | LF** 3.7             | 20.0        | His6-LF** 3.4       | 19.0        | 1.7                               |
| C2II**  | gpJ <0.001           | >100,000    | His6-gpJ 20.0       | >20,000     | 0.42                              |
| EDIN    | 0.0043 ±0.0007       | 23,000      | His6-EDIN 0.11 ±0.03 | 900         | 26                                |

Stability constants \( K \) and half saturation constants \( K_s \) for the binding of His6-tagged and untagged EF, LF, C2I, gpJ or EDIN to PA63- or C2II-channels in lipid bilayer membranes. The membranes were painted from 1% (w/v) diphytanoyl phosphatidylcholine dissolved in n-decane. The aqueous phase contained 150 mM KCl, buffered to pH between 5.5 and 6 using 10 mM MES-KOH; \( T = 20 \) C. Measurements were performed at a membrane potential of 20 mV. The data represent the means (± SD) of at least three individual titration experiments. \( K_s \) is the half saturation constant, i.e. \( K_s = 1/K \). Stability constants given in bold were adjusted to the voltage dependent behavior of binding. († taken from [21] ** taken from [43].)

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10 µg/ml, whereas intoxication of HUVECS with LFN-EDIN saturated already at 1 µg/ml (Figure 4B).

The Voltage Dependency of PA63-channels is Changed when His6-EDIN is Bound to the Pore

PA63-channels exhibit a well described voltage dependency [21]. If only added to the cis-side, PA63-induced conductivity decreases when applied voltage is higher than +50 mV or lower than −20 mV at the cis-side. It is also known that His6-EF bound to the channel changes the voltage dependency [21]. When different potentials were applied to membranes after the titration of PA63-pores with EDIN, there was only little change in voltage dependency of the channel (Fig. 5A). On the other hand, His6-EDIN bound to PA63-channels induced dramatic responses even at low positive voltages (Fig. 5B). Starting at +10 mV, the conductivity decreased exponentially immediately after the onset of the voltage with a voltage-dependent exponential relaxation time. Its time constant decreased with higher positive potentials at the cis-side (negative at the trans-side). This result indicated that

Figure 2. Efficiency of HUVEC intoxication by C2I and His6-C2I using PA63 compared to PA F427A mutant. HUVECs (5 × 10⁵ cells/100 mm well) were intoxicated during 24 hours and the number of intoxicated cells (round cells) was assessed by counting floating cells. A: PA63 and C2I at 5 µg/ml and C2I and His6-C2I at 2 µg/ml. One representative experiment showing mean values of 5 independent counting for each condition. ± SEM *p<0.05 versus control condition. B: PA63 and PA F427A at 50 µg/ml and His6-C2I at 2 µg/ml. Data correspond to mean values of n = 5 experiments ± SEM, *p<0.05 versus control condition. The control corresponds to conditions without PA63. All experiments were performed with the same batch of cells at the same time. The 3-fold increase of toxicity using 10-fold more PA63 was repeatedly measured.

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channels, which were not blocked before by His6-EDIN at low voltage bound this compound and closed as a result of the higher voltage. This result suggested an increase of the stability constant of binding up to very high voltages an effect that has already been observed with full length EF [21].

The increase of the stability constant for binding could be calculated from the data of Figures 5A and 5B and similar experiments by dividing the initial current (which was a linear function of voltage) by the stationary current after the exponential relaxation and multiplying the ratio with the stability constant derived at 5 mV. Figure 6 summarizes the effect of the positive membrane potential on the stability constant $K$ for EDIN and His6-EDIN binding as a function of the voltage. Starting already with $-10$ mV at the trans-side the stability constant $K$ for His6-EDIN binding started to increase and reached with about 60 to 70 mV a maximum. At that voltage $K$ was roughly 25 times greater than at 5 mV. For higher voltages the stability constant saturated probably because of secondary effects of the high voltage on the PA63-channel or on His6-EDIN binding. Figure 6 shows also the effect of the positive membrane potential at the cis-side on the stability constant $K$ for EDIN binding to PA63- pores as a function of the voltage. Interestingly, EDIN binding was only little affected by voltage as Figure 6 clearly indicated.

**Discussion**

**His6-tag Addition to Several Bacterial Factors Increased the Protein Binding Affinity to PA63- but not to C2II-channels**

Recent studies demonstrated that negatively charged amino acids in the vestibule of the PA63-channel play a crucial role in binding of effector molecules [40,42]. Thus, it is possible that a His6-tag, which adds positive charges under mildly acidic conditions to the N-terminal end of His6-EF and His6-LF affects binding and transport. This has indeed been shown for the native combinations of EF+PA63 or LF+PA63 and the potential ion-ion interaction discussed with EFN [21,39,48,49]. Recently, we could...
Figure 4. Immunofluorescence studies of HUVECs treated with EDIN and His6-EDIN and PA63. A: HUVECs were intoxicated for 24 h with a combination of PA63 3 \( \mu \)g/ml, His6-EDIN 10 \( \mu \)g/ml and LF1–254–EDIN (LFN-EDIN) 1 \( \mu \)g/ml, as indicated. Cells were fixed and actin cytoskeleton was labelled using FITC-conjugated phalloidin. Bar=10 \( \mu \)m. Arrows indicate transendothelial cell macroaperture tunnels (TEMs, transcellular tunnels). B: Graph shows percentage of cells with toxin-induced transendothelial cell macroaperture tunnels (TEMs, transcellular tunnels). HUVECs were intoxicated for 24 h with a combination of PA63 3 \( \mu \)g/ml, His6-EDIN or EDIN 10 \( \mu \)g/ml and LF1–254–EDIN (LFN-EDIN) 1 \( \mu \)g/ml, as indicated on the graph legend. Data correspond to means \( \pm \) SEM (n = 3, 400 cells per condition). doi:10.1371/journal.pone.0046964.g004

Figure 5. Voltage dependency of PA63-channels in the presence of EDIN and His6-EDIN. A: Current response of PA63-channels in presence of EDIN. Voltage pulses between +20 and +70 mV were applied to a diphytanoyl phosphatidylcholine/n-decane membrane in the presence of PA63 pores and EDIN (both added only to the cis side of the membrane). The aqueous phase contained 150 mM KCl, 10 mM MES-KOH, pH 6. The temperature was 20°C. B: Current response of PA63 channels in the presence of His6-EDIN. Voltage pulses between +10 and +90 mV were applied to a diphytanoyl phosphatidylcholine/n-decane membrane in the presence of PA63 pores and His6-EDIN (both added only to the cis side of the membrane). The aqueous phase contained 150 mM KCl, 10 mM MES-KOH, pH 6. The temperature was 20°C. Note the change of the scale (Arrow). doi:10.1371/journal.pone.0046964.g005
show that C2I binds to PA63 and may even be transported into
target cells albeit at high PA63 concentration and with very low
efficiency compared with the native combination of C2I with C2II [43].
Here we studied the cross reactivity of anthrax- and C2-toxin
in more detail and found a strong relation between binding affinity
and the presence of a His6-tag at the N-terminal end of the
enzymatic components. The addition of positive charges at the N-
terminal end of C2I (due to the partially charged histidines)
enhanced binding to and translocation into target cells via PA63-
pores and agreed very well with the findings previously reported
for His6-tags attached to EF and to LF [21,39]. Binding to PA63-
channels was found to be strongly enhanced for the three
enzymatic components EF, LF and C2I when they contained a
His6-tag at the N-terminal end.

Interestingly, we did not observe major effects if these His6-
tagged proteins were combined with C2II-channels. The results of
binding experiments with His6-EF and His6-LF to C2II-channels
suggested that the increased positive charge at the N-terminal end,
due to the partially charged histidines, did not increase the binding
of these enzymatic components to the C2II-channels. These results
definitely imply that binding of the enzymatic components to
PA63-channels occurs in a different way than binding to C2II-
channels.

To gain deeper insight in the influence of N-terminal His6-tags
on binding of proteins to PA63-channels we choose a protein, gpJ,
that was not related to any of the enzymatic components used in
this study [33]. As expected, we did not observe any binding of gpJ
to PA63- or C2II-channels (Ks >100 µM). However, binding was
observed when a His6-tag was attached to the N-terminal end of
gpJ. This protein had a half-saturation constant for binding to the
PA63-channels of 5 nM, which suggested that the affinity of His6-
gpJ did accordingly not interact with C2II-channels; revealing
again a somewhat different process for binding of His6-tagged
proteins to PA63-channels than to C2II-channels. However, it is
not clear if His6-gpJ could also be imported via PA63 into target
cells because this protein has no intracellular enzymatic activity
[320].

Influence of the His6-tag on Uptake of EF, LF and C2I into
Cells

The binding step is a prerequisite, but not sufficient for the
delivery of enzymatic subunits into target cells. Thus, in order to
complement the results of binding studies we went on to
investigate the translocation by analyzing the enzymatic activity
in a cellular system. We verified that a His6-tag attached to the N-
terminal end of C2I increased its transport by PA63-channels,
which correlates with the difference of 10-fold measured between
the stability constants for binding of C2I and His6-C2I. Some
difference in transport was observed by using EF or LF with or
without a His6-tag in combination with PA63-channels. In
particular, we could demonstrate that addition of a His6-tag
promoted uptake of LT (PA + His6-LF) into HUVECs (data not
shown). This result is in agreement with the increased affinity of
EF and LF to PA63-heptamer/octamers when they contain a
His6-tag at the N-terminus [21,23].

Although the binding of EF N and LF N (truncated forms of EF
and LF) to the PA63-channel is substantially weaker as compared
to wild-type enzymatic components [42], these proteins interact
with high affinity with the PA63-channels and are accordingly
transported into the cell [48,50,51]. Similarly, short stretches of
positively charged amino acids attached to the N-terminal end of
foreign proteins can lead to a PA63-dependent delivery as it has
been demonstrated for the addition of polycationic peptides to the
N-terminus of the enzymatic A chain of diphtheria toxin (DTA;
residues 1–193) or for LF1–254-EDIN [19,28].

Figure 6. Correlation of affinity constant K and voltage dependence of PA63-channels in presence of EDIN and His6-EDIN. The
stability constants of EDIN and His6-EDIN binding to the PA63-channel are given as a function of the applied membrane potential taken from
experiments similar to that shown in Fig. 5 A/B. Means of three experiments are shown.
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Bound His6-EDIN or EDIN Causes a Difference in Voltage-dependency of PA63-pores. Experiments with EDIN of S. aureus were performed to gain deeper insight in the binding and translocation processes through PA63-channels and its His6-tag dependence. Surprisingly, black lipid bilayer experiments displayed that not only His6-EDIN but also EDIN itself bound to PA63-channels. The affinity of EDIN and His6-EDIN to the PA63-channels was in the same range at low trans-membrane potentials because His6-EDIN exhibited only a three times higher affinity for binding to the PA63-channels than EDIN. Under normal conditions the PA63-channels only close for higher negative voltages applied to the cis-side [21]. For positive potentials the channels are open and do not show a voltage-dependent closure until 100–150 mV [21]. However, His6-EDIN binding to the PA63-channels showed an extremely high voltage-dependence when the voltage was positive at the cis-side of the membrane indicating that the potential pulled His6-EDIN into the channels. As a result the stability constant for binding of His6-EDIN to the PA63-channels increased at voltages of +70 mV at the cis-side (corresponding to −70 mV at the trans-side) by a factor of roughly 25 as compared to zero voltage. Bound EDIN displayed an only minor voltage-dependence. This means that the His6-tag is responsible for the binding EDIN and gpJ to the PA63-channels. Binding is essential for translocation because it is the first step of the whole process (see below).

The PA63-channel Transports His6-C2I and His6-EDIN into the HUVECs. EDIN uptake into target cells can easily be detected because it decreases RhoA activity. Import of EDIN via PA63-channels could not be observed. Import was however, possible when EDIN contained a His6-tag. This finding demonstrated that His6-tag itself provides the ability for proteins to be transported into cells through PA63-pores. This effect was presumably promoted by the voltage-dependence of His6-EDIN binding to the PA63-channels. Biological membranes are polarized to about −60 mV to −70 mV (inside negative). This may explain the much higher effect of His6-EDIN compared to EDIN on cells described above. In any case it clearly indicates the potentiating effect of a His6-tag and applied voltage on binding and translocation of protein molecules to PA63-channels. Summarizing the results, there definitely exists a difference in the binding and translocation mechanism between the two very homologous binding components PA63 and C2II of anthrax- and C2-toxin. Obviously, this distinction is induced by unequal binding surroundings inside the head region of the two protein channels. The amino acids responsible for binding within the N-terminal end of the enzymatic components are still a matter of debate, although there is clear evidence that positively charged amino acids are involved in binding, forming salt bridges between the enzymatic components and the heptamers/octamers. In a recent study it has been shown that besides the Phi-clamp also the so-called alpha-clamp is also involved in effector binding, unfolding and translocation in combination with PA63 [26,27]. This alpha-clamp is composed of hydrophobic and aromatic residues, such as F202 and P205 and forms a deep amphipathic cleft on the surface of the PA63 oligomer [26,27]. It is also possible that R178 contributes to effector binding but not to translocation. However, the alpha-clamp does not seem to be very specific because of its broad substrate specificity and non-specific polypeptide binding activity [26]. It is noteworthy that amino acids of the alpha-clamp do not appear to be preserved in C2II because PA R178, PA F202 and PA P205 correspond to C2II T169, C2II W193 and C2II K196 [26,27]. This could mean that the design of the alpha-clamp in C2II if it existed has a different structure or is absent.

The positively charged N-termini of the enzymes play presumably a crucial role, because quaternary ammonium ions and 4-aminoquinolones show PA63 and C2II channel block in lipid bilayer experiments [38,40,44,52]. Both channels show a high selectivity for cations, i.e. cations have a strong influence on the single channel conductance as compared to anions [53,54]. This means that negative charged amino acids play a crucial role in the binding and constriction region of the PA63-channels, where they form two rings of seven putative negatively charged amino acids in the vestibule of this pore (E398 and D426). Similarly, the channel lumen contains additional three rings of seven possibly negatively charged groups (E302, E308 and D315). Some of these charges cannot be found in the C2II-channel lumen, resulting in minor effects of His6-tag on binding and transport. However, transport into cells seems to be possible with C2II-pores and when N-terminal parts of C2I are coupled to foreign proteins [1,55,56]. The most interesting result of this study was that we could use the anthrax PA63-channels to deliver into cells a polypeptide completely unrelated to the AB7/8-type of toxins. In fact, we here provide evidence that the His6-addition on EDIN allows its entry in target cells, in a PA63-dependent manner. On the other hand, we would like to stress the point that the natural uptake of EDIN occurs very slowly at very high EDIN concentration (100 μg/ml: see Figure 3C). Nevertheless, here our data support the idea that it seems possible to design a very simple transportation system using His6-tags on proteins unrelated to the AB7/8-family and PA63-channels for biological purpose.

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Author Contributions

Conceived and designed the experiments: CB CS AK MR EL RB. Performed the experiments: CB CS AK MR. Analyzed the data: CB CS AK MR EL RB. Contributed reagents/materials/analysis tools: CB CS AK MR GF EL RB. Wrote the paper: CB CS MR EL RB.

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