Polylysine-Mediated Translocation of the Diphtheria Toxin Catalytic Domain through the Anthrax Protective Antigen Pore

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ABSTRACT: The protective antigen (PA) moiety of anthrax toxin forms oligomeric pores in the endosomal membrane, which translocate the effector proteins of the toxin to the cytosol. Effector proteins bind to oligomeric PA via their respective N-terminal domains and undergo N- to C-terminal translocation through the pore. Earlier we reported that a tract of basic amino acids fused to the N-terminus of an unrelated effector protein (the catalytic domain diphtheria toxin, DTA) potentiated that protein to undergo weak PA-dependent translocation. In this study, we varied the location of the tract (N-terminal or C-terminal) and the length of a poly-Lys tract fused to DTA and examined the effects of these variations on PA-dependent translocation into cells and across planar bilayers *in vitro*. Entry into cells was most efficient with ~12 Lys residues (K12) fused to the N-terminus but also occurred, albeit 10−100-fold less efficiently, with a C-terminal tract of the same length. Similarly, K12 tracts at either terminus occluded PA pores in planar bilayers, and occlusion was more efficient with the N-terminal tag. We used biotin-labeled K12 constructs in conjunction with streptavidin to show that a biotinyl-K12 tag at either terminus is transiently exposed to the *trans* compartment of planar bilayers at 20 mV; this partial translocation *in vitro* was more efficient with an N-terminal tag than a C-terminal tag. Significantly, our studies with polycationic tracts fused to the N- and C-termini of DTA suggest that PA-mediated translocation can occur not only in the N to C direction but also in the C to N direction.
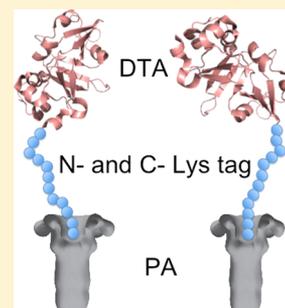
Anthrax toxin is a tripartite system consisting of two catalytic moieties, lethal factor (LF) and edema factor (EF),[1,2] and a receptor binding/pore forming moiety, protective antigen (PA; MW = 83 kDa).[3−6] These three individually nontoxic proteins combine to elicit many of the disease manifestations caused by *Bacillus anthracis*. After release from the bacteria, PA binds to its cellular receptors[7−12] and is cleaved by cell-surface furin to a 63 kDa form (PA_{63}).[13,14] PA_{63} self-assembles to form a heptameric[4,5,10] or octameric prepore,[15] which then binds the enzymatic LF and EF moieties, yielding a series of complexes at the cell surface.[15−18] These complexes are endocytosed,[19,20] and exposure to acidic conditions of the endosomal compartment causes the PA prepore to undergo a conformational change to the pore state.[3,21,22] The pore, inserted into the endosomal membrane, translocates the LF and EF moieties to the cytoplasm,[23−25] where they modify their respective intracellular targets to the benefit of the bacterium.

The anthrax toxin system has been studied extensively to learn how a proteinaceous toxin pore is able to translocate a protein across a phospholipid bilayer. Certain heterologous proteins may be potentiated to undergo PA-dependent translocation by fusion with the PA binding[26] N-terminal domain of LF (LFn, residues 1−263) or the corresponding domain of EF. Thus, for example, fusing LFn to the N-terminus allows heterologous proteins and peptides (e.g., the catalytic domain of *Pseudomonas* exotoxin A,[27] diphtheria toxin,[28] ricin[29] or shiga toxin,[30] and the cytotoxic T lymphocyte epitope from *Listeria monocytogenes*[31]) to be delivered to the cytosol via PA. The ability of LFn to potentiate proteins for PA-dependent

translocation stems from its ability to bind to the mouth of the PA pore and orient its disordered, highly charged leader into the lumen of the pore.[32] N- to C-terminal translocation occurs *in vitro* in planar bilayers in the presence of a transmembrane pH gradient corresponding to that between the acidic lumen of the endosome and the neutral cytosol,[33] and a charge state-dependent Brownian ratchet mechanism has been proposed.[34]

In cellular assays of PA-dependent translocation, we have often used DTA, the catalytic domain of diphtheria toxin, as a heterologous effector protein, as its delivery to the cytosol may be readily detected by measuring the inhibition of protein synthesis. Many years ago, we observed that DTA with a hexa-His tag at the N-terminus undergoes significant PA-dependent entry into cells, and we then showed that short N-terminal tracts of Lys or Arg, as well as His, also fostered translocation of DTA via the PA pore.[35] Consistent with this finding, it has been reported that an N-terminal His_6 tag can promote PA-dependent entry of an active domain of the C2 toxin and epidermal cell differentiation inhibitor of *Staphylococcus aureus*.[36] In the study presented here, we fused tracts of up to 15 Lys residues to the N- or C-terminus of DTA and examined their ability to promote translocation of DTA into cells. These studies, together with experiments performed in parallel in planar bilayers, suggest that the PA pore is able to translocate appropriately tagged proteins across membranes in
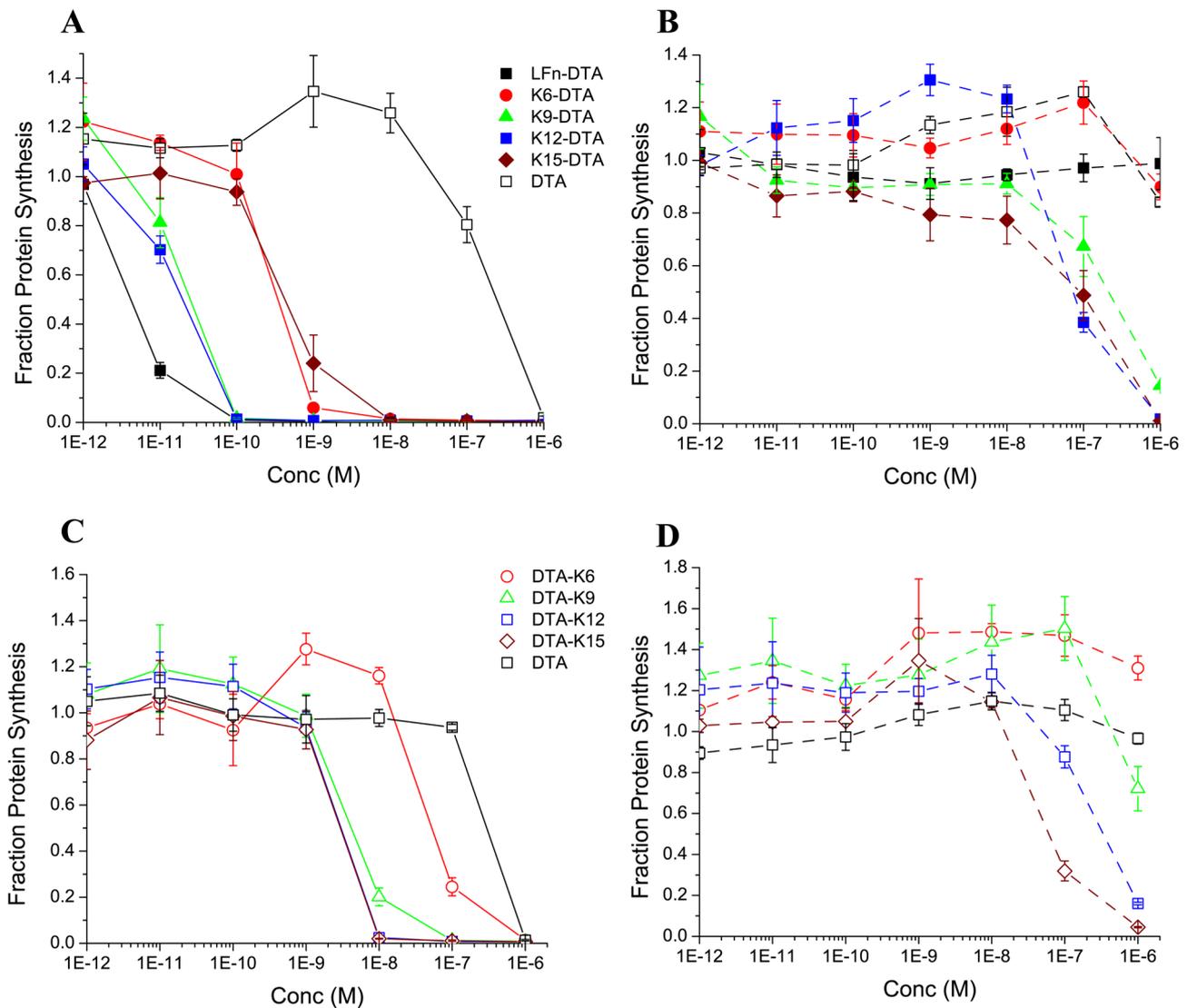
Figure 1. PA-mediated translocation of DTA constructs as measured by protein synthesis inhibition in CHO K1 cells. (A and B) Cytotoxicity of N-terminal lysine fusions in the presence and absence of PA, respectively. (C and D) Cytotoxicity of C-terminal lysine fusions in the presence and absence of PA, respectively. Solid lines show data with PA (20 nM) and dashed lines data without PA.

both N to C and C to N direction, although translocation is less efficient in the C to N direction.

MATERIALS AND METHODS

Cell Culture. CHO-K1 and A431 cells were grown in Ham's F-12 and DMEM media, respectively, each supplemented with 10% FBS, 100 units/mL penicillin, 100 μg/mL streptomycin, and 2 mM glutamine. Cells were maintained as monolayers and grown at 37 °C and 5% CO_2 in a humidified incubator.

Cloning and Mutagenesis. DTA C186S was amplified via polymerase chain reaction using Taq polymerase and cloned into the pET-Sumo vector (Invitrogen, Grand Island, NY) using the "T" overhangs so that an in-frame fusion construct of sumo protein with DTA is generated. This was then used as the DNA template for addition of lysines in all other constructs, which was done using the QuikChange site-directed mutagenesis kit (Stratagene, Santa Clara, CA). The N-terminal lysine constructs had a serine residue added in front of the lysines to allow for efficient cutting by sumo protease. Site-directed mutagenesis was used to introduce a cysteine in the K12-DTA and DTA-K12 moieties to generate the C-K12-DTA

(with a serine residue before the cysteine for optimal cleavage by sumo protease) and DTA-K12-C constructs.

Protein Synthesis. DTA C186S (and its Lys-tagged variants) were transformed in *Escherichia coli* BL21(DE3) cells (Invitrogen). The proteins were overexpressed using isopropyl β-D-1-thiogalactopyranoside (1 mM), and the proteins were purified using Ni-charged metal affinity chromatography. The proteins were then subjected to another round of purification by separating them on a High Performance Q column (GE). Sumo protease was then used to cleave the sumo-DTA construct. Finally, the His_6-sumo protease and His_6-sumo protein were separated from DTA by Ni-charged metal affinity chromatography.

Biotin Labeling. Site-directed mutagenesis was used to generate cysteine mutants C-K12-DTA and DTA-K12-C. As mentioned above, the N-terminal construct has a serine preceding the cysteine in C-K12-DTA to allow efficient sumo protein cleavage. These proteins were labeled using EZ link Biotin-HPDP (pyridyldisulfide-biotin, Thermo Scientific, Rockford, IL) as the labeling agent following the manufacturer's protocol. Briefly, DTA constructs were mixed with a 10-fold

excess of Biotin HPDP (dissolved in DMF), and the reaction was allowed to proceed for 2 h at room temperature. Unbound HPDP biotin was separated from protein using a desalting column. The labeled protein was further separated from any unlabeled protein by purifying biotin-labeled protein using a monomeric avidin column. Addition of biotin was confirmed by matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) analysis of biotinylated constructs.

Planar Lipid Bilayer Experiments. Planar phospholipid bilayer experiments were performed in a Warner Instruments Planar Lipid Bilayer Workstation (BC 525D, Warner Instruments, Hamden, CT). Planar bilayers were formed by painting a 35 mM solution of 1,2-diphytanoyl-*sn*-glycero-3-phosphocholine (DPhPC) in *n*-decane (Avanti Polar Lipids, Alabaster, AL) on a 200 μm aperture of a Delrin cup in a Lucite chamber. One milliliter of buffer containing 100 mM KCl, 1 mM ethylenediaminetetraacetic acid (EDTA), 10 mM sodium oxalate, 10 mM potassium phosphate, and 10 mM 2-(*N*-morpholino)-ethanesulfonic acid (MES) (pH 5.5) was added to the cup (*trans*) and the chamber (*cis*), and both compartments were stirred continuously.

Upon formation of a bilayer membrane, up to 5 μg of PA prepore (25 pM) was added to the *cis* compartment held at 20 mV with respect to the *trans* compartment. After incorporation of PA pores as monitored by conductance across the membrane, the *cis* compartment was perfused to remove any free PA. Once the current had stabilized, 1 μg of a DTA construct (except where mentioned) was added to the *cis* compartment, and interaction with PA channels was monitored by the decrease in conductance.

Protein Synthesis Inhibition Assay. CHO-K1 cells were plated in a 96-well plate at a density of 10000 cells/well approximately 16 h before the start of an experiment. DTA toxin constructs diluted to various concentrations in Ham's F-12 medium and PA$_{83}$ (20 nM) supplemented with 10% FBS were added to the plates. The plates were then incubated at 37 °C for 24 h, after which toxin-containing medium was removed and replaced with L-leucine-deficient F-12 medium supplemented with L-[4,5-^{3}H]leucine. The plates were incubated for an additional 1 h at 37 °C and washed with ice-cold PBS. Liquid scintillation cocktail was added, and radioactivity incorporated by the cells was measured. The results were normalized and expressed as a fraction of the radioactivity incorporated into the CHO-K1 cells that were not treated with toxin.

■ RESULTS

PA-Dependent Translocation of DTA Containing Fused N- or C-Terminal Lys Tracts. DTA constructs with tracts of 6, 9, 12, or 15 Lys residues fused to the N- or C-terminus were tested for translocation into CHO K1 cells in the presence or absence of PA (Figure 1). In the presence of PA, N-terminal tracts of 9 or 12 Lys residues (K9-DTA or K12-DTA, respectively) were effective potentiators of DTA translocation, with half-maximal inhibition of protein synthesis occurring at concentrations of ~20 pM (Figure 1A). Consistent with our earlier findings, LFn-DTA was ~10-fold more potent than the most active of these poly-Lys-tagged constructs. In the absence of PA, constructs containing 9, 12, or 15 N-terminal Lys residues blocked protein synthesis, but only at ~10000-fold higher concentrations (≥0.1 μM) than in the presence (Figure 1B). Untagged DTA showed cytotoxicity at concentrations

approaching 1 μM in the presence of PA and was inactive at the highest concentration tested (1 μM) in the absence of PA.

Lys tracts fused to the C-terminus of DTA were less active in potentiating PA-dependent delivery than those of a corresponding length fused to the N-terminus (Figure 1C,D). In the presence of PA, C-terminal tracts of 9, 12, and 15 Lys residues (DTA-K9, DTA-K12, and DTA-K15, respectively) all showed about the same level of activity, with half-maximal inhibition in the low nanomolar range. In the absence of PA, these adducts were also active, but only at 10- to 100-fold higher concentrations, with DTA-K15 being the most potent and DTA-K9 the least.

K12-DTA and DTA-K12 were chosen for further studies because each had the highest PA-dependent cytotoxicity (among the N- and C-terminal tags, respectively) for CHO-K1 cells, and they efficiently occluded PA channels in planar bilayers, as shown below. ADP ribosylation activities of K12-DTA, DTA-K12, and untagged DTA were measured *in vitro* and found to show little variation.

Comparative Inhibition of LFn-Mediated and Poly-Lys-Mediated Translocation. A key determinant of LFn-DTA translocation by PA is the Phe clamp, a structure formed by the F427 side chains within the lumen of the PA pore.[34,37,38] We tested K12-DTA and DTA-K12 for cytotoxicity in the presence of PA variants containing the F427H or F427S mutation, each of which blocks translocation of LFn-DTA without affecting pore formation. Both mutations completely blocked LFn-DTA translocation, as expected, but we found an unanticipated difference between F427H and F427S in inhibiting translocation of the K12-tagged DTA constructs (Figure 2). Whereas F427H completely blocked translocation
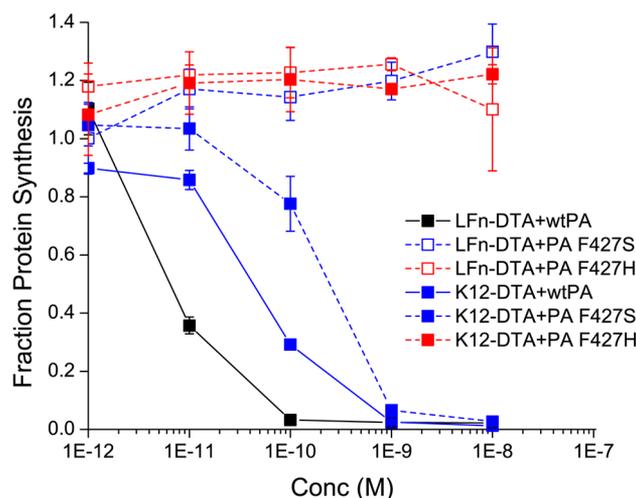
Figure 2. Effect of PA Phe clamp mutations, F427H and F427S, on the cytotoxicity of LFn-DTA and K12-DTA constructs in CHO-K1 cells.

of K12-DTA and DTA-K12, F427S inhibited their translocation ≤10-fold. These results support the notion that translocation promoted by Lys tracts occurs via the pore lumen but also indicate differences in the mechanisms by which F427H and F427S inhibit translocation.

Free LFn (1 μM) was a strong competitive inhibitor of the PA-dependent cytotoxic activity of LFn-DTA, but not of K12-DTA, consistent with earlier findings.[35] We found a similar lack of an effect of LFn on DTA-K12.

Interaction of Lys-Tagged DTA with PA Pores in Planar Bilayers. Binding of LFn to PA pores in planar bilayers results in pore occlusion, as measured by ion conductance.[39,40] We monitored occlusion of pores in DPhPC bilayers for 60 s following addition of various constructs (final concentration of 1 μg/mL) to the *cis* compartment (Figure 3). Free LFn blocked
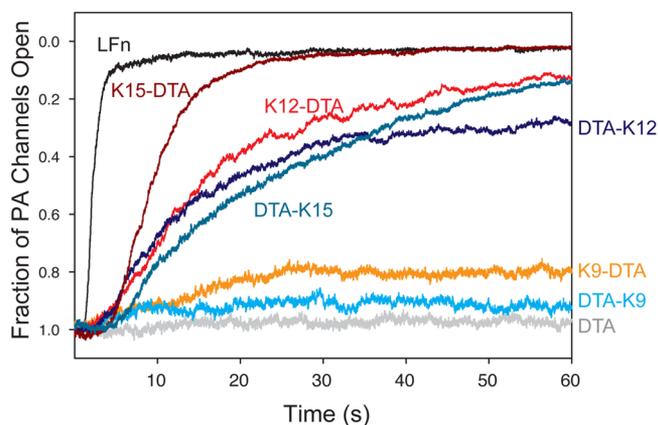
Figure 3. Kinetics of occlusion of PA pores in planar bilayers by LFn, DTA, and Lys-tagged DTA constructs. Up to 5 μg of PA63 prepore was added to the *cis* compartment. After insertion of PA pores, 1 μg of a DTA construct was added to the *cis* compartment and the fraction of PA channels occluded in the presence of *cis* 20 mV after 60 s was calculated.

conductance rapidly (within 10 s) and almost completely (99%), whereas occlusion by Lys-tagged DTA constructs was slower and strongly dependent on the number of Lys residues in the tag. The greater the number of Lys residues, the more rapid and more complete the occlusion (at 60 s). N-Terminally tagged constructs were more effective than the corresponding C-terminally tagged ones. Constructs with K9 tracts caused weak occlusion, and constructs with K6 caused almost none.

Translocation of Lys-Tagged Constructs across Planar Bilayers. To probe the translocation of Lys-tagged constructs across planar bilayers, we appended a Cys residue to the N-terminus of K12-DTA and to the C-terminus of DTA-K12 and labeled both constructs with a Cys-reactive biotinylation reagent. The resulting biotin-C-K12-DTA and DTA-K12-C-biotin constructs occluded PA channels with efficiencies comparable to those of the corresponding K12-DTA and DTA-K12 constructs. Addition of streptavidin to the *cis* compartment strongly hindered occlusion by both of the biotinylated constructs (Figure 4A,B) but had no effect on occlusion by DTA constructs lacking the biotin label. Thus, the Lys tract initiated blockage of the PA pore irrespective of the terminus of DTA to which it was fused, and binding of a bulky protein at the terminus adjacent to the Lys tract prevented the blockage.

To explore the possibility that the positively charged K12 tracts fused to DTA translocated through the PA pore under an applied voltage of 20 mV, we added K12-DTA and DTA-K12-C-biotin constructs to the *cis* compartment and streptavidin to the *trans* compartment to bind to any biotin-tagged termini of biotin-C that became accessible (Figure 4C,D). Biotin-C-K12-DTA and DTA-K12-C-biotin constructs were allowed to occlude PA channels; streptavidin was added to the *trans* compartment, and the applied transmembrane potential was repeatedly cycled between 20 and −20 mV, allowing repeated

blocking and unblocking of PA pores by the DTA constructs. This led to the biotin-C-K12-DTA and DTA-K12-C-biotin constructs causing ∼50 and ∼20%, respectively, of the PA pores to be permanently blocked. Streptavidin alone had no effect on the occlusion capabilities of DTA constructs in the absence of biotin.

DISCUSSION

The N-terminal domain of LF recognizes the prepore and pore formed by PA63 and initiates protein translocation by a well-described sequence of events.[6,32,33] LFn binds at the junction of adjacent PA63 subunits, with each subunit making unique contacts with the ligand.[16,18] LFn partially unfolds in the process of binding, such that the first α-helix and β-strand become separated from the bulk of the domain and occupy an amphipathic cleft, termed the α-clamp, between two PA subunits.[32] The residual, folded portion of LFn makes multiple additional contacts with the prepore or pore. The highly charged and disordered N-terminal leader sequence of LFn is thereby positioned within the lumen of the pore, and several residues at the extreme N-terminus have been shown by an electron paramagnetic resonance study to come into contact with the Phe clamp,[41] a structure whose integrity is essential for translocation. These interactions poise LFn bound to the pore to undergo translocation from the N- to C-terminus[33] by a charge state-dependent Brownian ratchet mechanism, driven primarily by the transmembrane proton gradient.[34] This translocation mechanism is based largely on data from planar bilayer studies; other results suggest that accessory proteinaceous factors facilitate translocation *in vivo*.[42]

We discovered several years ago that simply fusing a stretch of basic amino acids to the N-terminus of DTA can functionally replace LFn by potentiating PA-dependent entry of the protein into cells.[35] In the study presented here, we compared the PA-dependent cytotoxicity values of DTA constructs with various numbers of Lys residues fused to the N- or C-terminus. A tract of 9 or 12 N-terminal Lys residues gave cytotoxicity values within 1 order of magnitude of that of LFn-DTA, while the levels of cytotoxicity were much lower with 6 or 15 Lys residues. PA-dependent cytotoxicity was also seen with Lys tracts fused to the C-terminus (tracts of 9, 12, and 15 Lys residues proved to be approximately equally effective), but the maximal cytotoxicity levels observed were 10−100-fold lower than that of the most active N-terminal poly-Lys tracts and thus 100−1000-fold lower than that of LFn-DTA.

What is the mechanism of translocation through the pore mediated by a polycationic tract of amino acids, and how does it compare with that mediated by LFn? Free LFn competitively inhibits the cytotoxicity of LFn-DTA, but not of poly-Lys-DTA, suggesting that little overlap exists between the binding sites. However, like LFn, the poly-Lys-tagged DTA constructs blocked ion conductance by PA pores in planar bilayers, and streptavidin bound to a biotinyl group placed at the beginning of an N-terminal 12K tag or the end of a C-terminal K12 sterically hindered pore occlusion. These results strongly suggest that the Lys tag at either terminus physically enters the pore and interacts directly with the Phe clamp and/or with the nearby acidic residues.

Our understanding of the mechanism of translocation of protein through the PA pore mediated by a polylysine tract centers around a model in which the Lys tag, regardless of the terminus to which it is appended, is drawn by electrostatic attraction into the negatively charged, cation-selective pore.
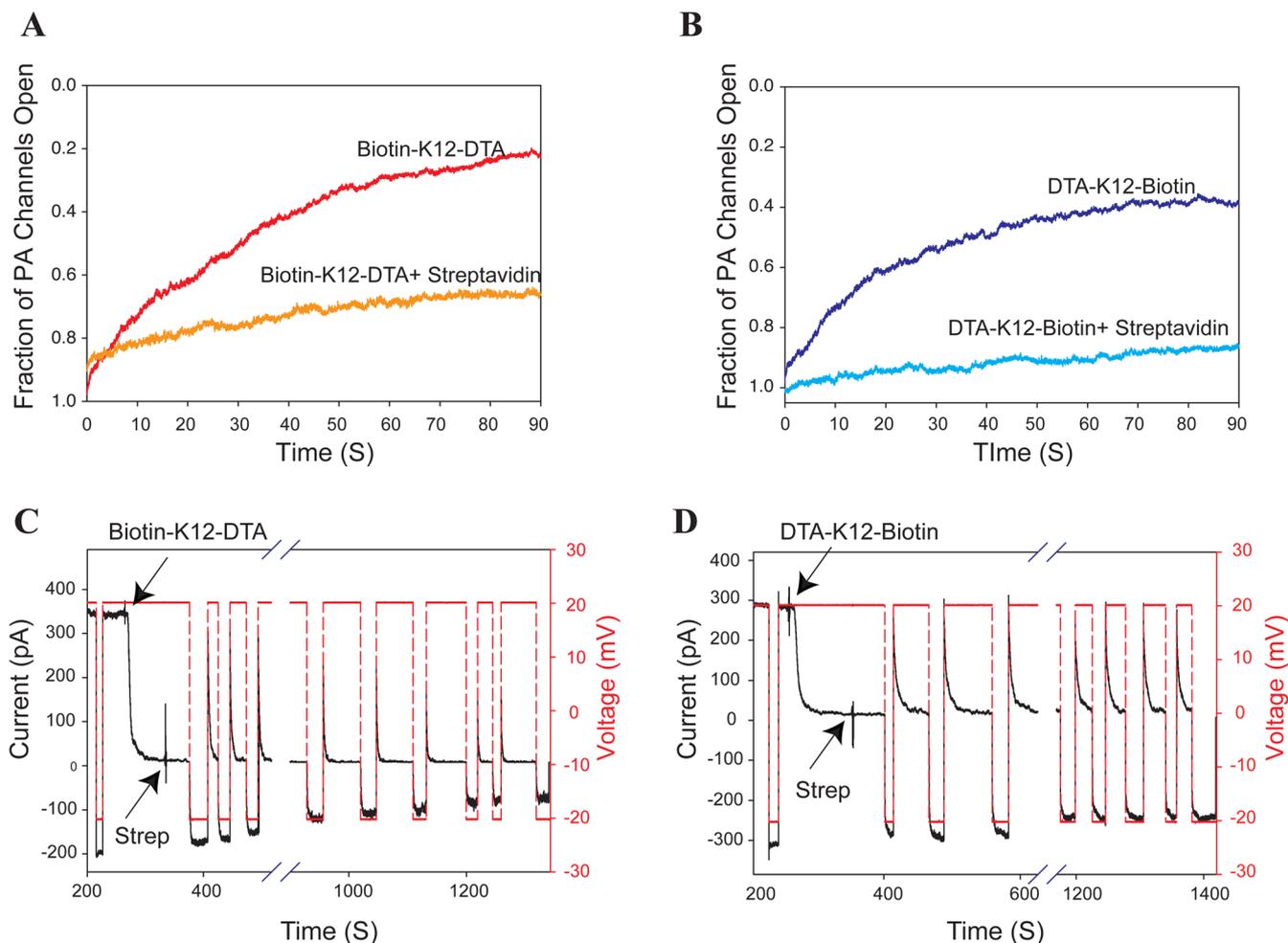
Figure 4. Experiments with biotinylated constructs. The PA63 prepore was added to the *cis* compartment. Upon insertion of PA pores, the DTA construct was added to the *cis* compartment and occlusion of PA channels in the presence of *cis* 20 mV was monitored. (A and B) Interaction of biotin-C-K12-DTA (0.5 μg) and DTA-K12-C-biotin (1 μg) constructs, respectively, with PA pores incorporated into planar bilayers in the presence of a 5-fold (A) or 2.5-fold (B) molar excess of tetrameric streptavidin added to the *cis* compartment. (C and D) Interaction of biotin-C-K12-DTA and DTA-K12-C-biotin constructs, respectively, with PA pores incorporated into planar bilayers in the presence of a molar excess of tetrameric streptavidin added to the *trans* compartment.

The stereospecific binding of LFn to PA places its N-terminus in the lumen, thereby initiating an exclusively N to C translocation. In contrast, because simple electrostatic charge attracts the Lys tag into the PA pore, the direction of translocation of a Lys-tagged protein is flexible and is determined by the location of the tag. Interaction of the positively charged tag with the phenyl side chains of F427, via cation−π interactions, and/or with nearby acidic residues, could foster Brownian motion-dependent contact of upstream acidic residues, which are essential for the ratcheting process, with the Phe clamp. Such contact would promote charge state-dependent Brownian ratcheting through the pore. According to this model, there would be no necessity that the Lys-tagged polypeptide interact with the α-clamp or any other site at the mouth of the pore to initiate the translocation process in the N to C or C to N direction.

The efficiency of the translocation process initiated by a polycationic tract would be expected to have a complex dependence on the length of the tract. Up to a point, increasing the positive charge would increase the level of electrostatic attraction and thus accelerate entry of the substrate protein into the pore and strengthen the interaction with the Phe clamp

region. Indeed, we found that increasing the number of Lys residues brought about a corresponding increase in the rate and steady state extent of occlusion of PA channels in planar bilayers. However, beyond a certain threshold, strengthening the electrostatic interaction with the pore would hinder transit through the lumen. Consistent with this notion, we found that an optimal range existed for the number of Lys residues in the N-terminal tag, with cytotoxic activity declining with numbers above or below that range.

The fact that Lys tracts on the C-terminus of DTA enhanced its PA-dependent cytotoxicity, although weakly, suggests that cargo proteins may be translocated through the PA pore in the C to N direction as well as in the N to C direction. In its simplest form, the charge state-dependent Brownian ratchet model of translocation would not dictate the direction of translocation, as it does not assume specific contacts with the pore that might serve as the basis of a directional bias. Supporting the hypothesis of C to N translocation, experiments in planar bilayers showed that binding of streptavidin to a biotinyl group at the extreme C-terminus of DTA-K12 in the *cis* compartment blocked pore occlusion by the Lys tag. Further, the C-terminal biotinyl group became transiently exposed to

streptavidin added to the *trans* compartment under conditions that promote translocation. The fact that exposure to *trans* streptavidin was less efficient with the K12-biotinyl tag at the C-terminus than a biotinyl-K12 tag at the N-terminus correlates with the lower cytotoxicity of C-terminally tagged DTA. The C-terminally tagged DTA variants tested were also significantly less effective at occluding PA pores in planar bilayers than their N-terminally tagged counterparts, suggesting that the C-terminal tags may be conformationally constrained and less accessible for penetration into the pore.

In the course of our studies, we encountered the unexpected result that, whereas the F427S mutation in PA completely blocked the cytotoxicity of LFn-DTA, this mutation caused <10-fold inhibition of the cytotoxicity of K12-DTA. This result is consistent with the proposed model in which the Lys tag is electrostatically attracted by the cation-selective PA pore. It is likely that a partial positive charge on histidines in PA F427H would repel the strong positively charged Lys tract, blocking translocation. However, in the case of PA F427S, the lack of charge on serine would allow translocation of Lys-tagged DTA protein across the PA pore, albeit less efficiently because of the loss of cation$-\pi$ interactions with the phenyl side chains of F427. In comparison, interaction with the Phe clamp is critical for LFn-mediated translocation, and both PA F427H and PA F427S mutants are unable to translocate LFn-DTA. We suggest that translocation of K12-DTA *in vivo* may be driven to a greater degree by $\Delta\Psi$ than by the ΔpH-driven Brownian ratchet.

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Notes
The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

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