The Diphtheria Toxin Channel-forming T Domain Translocates Its Own NH₂-Terminal Region Across Planar Bilayers

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ABSTRACT The T domain of diphtheria toxin, which extends from residue 202 to 378, causes the translocation of the catalytic A fragment (residues 1–201) across endosomal membranes and also forms ion-conducting channels in planar phospholipid bilayers. The carboxy terminal 57-amino acid segment (322–378) in the T domain is all that is required to form these channels, but its ability to do so is greatly augmented by the portion of the T domain upstream from this. In this work, we show that in association with channel formation by the T domain, its NH₂ terminus, as well as some or all of the adjacent hydrophilic 63 amino acid segment, cross the lipid bilayer. The phenomenon that enabled us to demonstrate that the NH₂-terminal region of the T domain was translocated across the membrane was the rapid closure of channels at *cis* negative voltages when the T domain contained a histidine tag at its NH₂ terminus. The inhibition of this effect by *trans* nickel, and by *trans* streptavidin when the histidine tag sequence was biotinylated, clearly established that the histidine tag was present on the *trans* side of the membrane. Furthermore, the inhibition of rapid channel closure by *trans* trypsin, combined with mutagenesis to localize the trypsin site, indicated that some portion of the 63 amino acid NH₂-terminal segment of the T domain was also translocated to the *trans* side of the membrane. If the NH₂ terminus was forced to remain on the *cis* side, by streptavidin binding to the biotinylated histidine tag sequence, channel formation was severely disrupted. Thus, normal channel formation by the T domain requires that its NH₂ terminus be translocated across the membrane from the *cis* to the *trans* side, even though the NH₂ terminus is >100 residues removed from the channel-forming part of the molecule.

KEY WORDS: histidine tag • nickel binding • streptavidin • trypsin • channel gating

INTRODUCTION

Diphtheria toxin, a 535 amino acid polypeptide, contains three domains: the amino terminal A fragment (catalytic C domain), the carboxy terminal receptor-binding R domain, and the translocation (or transmembrane) T domain lying between them (Fig. 1). The A fragment is connected to the T domain both by a readily reducible disulfide bridge and by an arginine-rich loop that is easily nicked by proteases. The A fragment catalyzes the ADP ribosylation of elongation factor 2 (EF-2), thereby inhibiting protein synthesis and resulting in the ultimate death of the target cell. To accomplish its nefarious purpose, the A fragment must gain access to the cytosol, where EF-2 resides, and this requires the action of the other two domains. The toxin first binds through its R domain to a specific receptor on the cell surface, is internalized via receptor-mediated endocytosis, and then, in an acidic vesicular compartment, undergoes a conformational change re-

sulting in the translocation of the A fragment to the cytosol; this last step is accomplished through the interaction of the T domain with the endosomal membrane. (For a general review of diphtheria toxin, see Madshus and Stenmark, 1992.) Experimentally, the endocytotic pathway can be bypassed by briefly exposing toxin-treated cells to a low pH environment, thereby resulting in translocation of the A fragment (via the T domain's action) directly across the plasma membrane (Draper and Simon, 1980; Sandvig and Olsnes, 1980).

How does the T domain promote the translocation of the A fragment across the endocytotic or plasma membrane? Indeed, can the T domain accomplish this task alone, or are other proteins in the plasma membrane (e.g., the toxin receptor) required? At low pH (≤6), the T domain forms channels in planar lipid bilayers (Kagan et al., 1981), as does the whole toxin (or just the combined T and R domains) in plasma membranes (Eriksen et al., 1994; Lanzrein et al., 1997). Whether these channels are directly involved in A fragment translocation or are an associated epiphenomenon is not clear, but there is a strong correlation between the ability of T domain mutants to form channels in lipid bilayers and of the corresponding mutant toxins to kill cells (Falnes et al., 1992; Cabiaux et al., 1993; Silverman et al., 1994*a*; vanderSpek et al., 1994).

A fragment (C-domain)   T-domain   R-domain

DT   1   186   201   386   535
⊢ S — S ⊣

T-domain   202   265   289 300   317 328   348 353   376 378

+   202   .   .   + . +   +   +   .   + .
M G S S H-H-H-H-H-H S S G L V P R G S H M I N L D W D V I R D K T K L T K I E S L K E H G P I

+   +   .   +   . . +   + ...   + 265
K N K M S E S P N K T V S E E K A K Q Y L E E F H Q T A L E H P E L S E L K *T V T G T N P V F*

A G A N A A W A V N V A Q V I D S E T A D N L E K *T T A A L S I L P G I G S V M G I A* D G A V

322   . .   .   .   .   .   + 378
H H N T E E *I V A Q S I A L S S L V A Q A I P L V G* E L V D *I G F A A Y N F V E S I I N L F Q V V H N S Y N* R P

Histidine Tag   Minimal Pore-Forming Region   *Hydrophobic Regions*
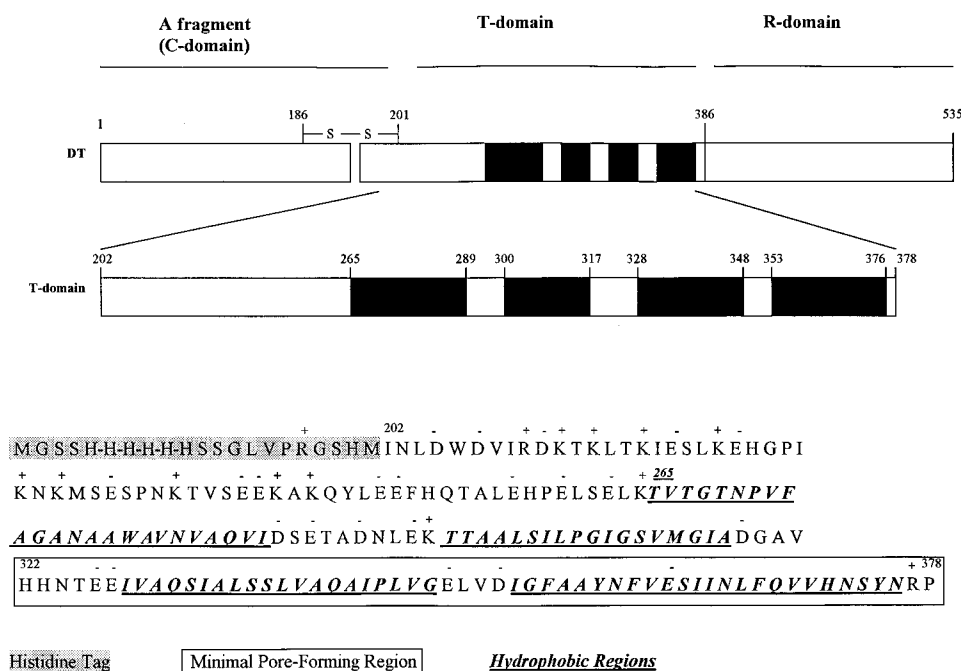
FIGURE 1. (*top*) Linear diagram of the whole diphtheria toxin (*DT*) molecule (protease nicked), with an expanded view of the channel-forming T domain. Black regions denote hydrophobic stretches within the T domain. (*bottom*) Sequence of the histidine-tagged T domain of diphtheria toxin.

In its water soluble form, the T domain consists of 10 α-helices, designated TH1–TH9 and TH5′, and their connecting loops (Bennett and Eisenberg, 1994). All that is required to form channels in planar bilayers is the 57 amino acid carboxy terminal stretch of the T domain, residues 322–378 (Silverman et al., 1994*b*; see footnote 1 in Huynh et al., 1997), which in the crystal structure of the water-soluble form of the toxin forms an α-helical hairpin designated TH8-9. However, although this minimal piece of the T domain forms channels that are indistinguishable in their transport properties from those formed by the whole domain, its ability to form channels is orders of magnitude less than that of the entire T domain. Thus, the region of the T domain upstream of TH8-9 plays an important role in the insertion of channels into the membrane. In this paper, we report that part of this region, including its NH$_2$ terminus, is translocated across planar lipid bilayers in association with channel formation, and we discuss the implications of this for the translocation of the A fragment by the T domain.

MATERIALS AND METHODS

The details of T domain expression and purification are given by Zhan et al. (1995). The T domain extends from residue 202 to 378 and, when expressed and purified this way, which is basically the method described in the pET system manual from Novagen, Inc. (Madison, WI), contains a histidine tag at its amino terminus. For some experiments, this tag was removed (as described in the manual), leaving four additional residues at the amino terminus: Gly-Ser-His-Met; for other experiments, the histidine tag was retained, leaving 20 additional residues at the amino terminus: Gly-Ser-Ser-(His)$_6$-Ser-Ser-Gly-Leu-Val-Pro-Arg-Gly-Ser-His-Met. (As

determined by NH$_2$-terminal analysis and confirmed by mass spectrometry, the NH$_2$-terminal methionine present in the construct was found to have been removed during the expression of the protein.) The numbering system used in this paper is the same as that described for native diphtheria toxin (Greenfield et al., 1983) and does not include the additional 4 or 20 residues. Mutagenesis of the second serine after the (His)$_6$ sequence to a cysteine and its biotinylation with *N*-[6-(biotinamido)hexyl]-3′-(2′-pyridyldithio) propionamide (Pierce Chemical Co., Rockford, IL) were accomplished by the methods described previously (Qiu et al., 1996; Jakes et al., 1998). Proteins at a concentration of ∼1 mg/ml were kept at −80°C in 20 mM Tris, pH 8.0. Aliquots and their dilutions used in experiments were stored at −20°C and could undergo numerous freeze–thaw cycles while still maintaining good channel-forming activity. Streptavidin was from Calbiochem Corp. (La Jolla, CA); biotin, *N*-tosyl-L-lysine chloromethyl ketone (TLCK), and soybean trypsin inhibitor were from Sigma Chemical Co. (St. Louis, MO); tosyl-L-phenylalanine chloromethyl ketone (TPCK)-treated trypsin was from Worthington Biochemical Corp. (Freehold, NJ); and tris(2-carboxyethyl)-phosphine (TCEP)[1] was from Pierce Chemical Co.

Planar lipid bilayer membranes made from asolectin (lecithin type IIS; Sigma Chemical Co.) from which neutral lipids were removed (Kagawa and Racker, 1971) were formed at room temperature across a hole ∼100 μm in diameter by the folded film method of Montal (1974) as described by Qiu et al. (1994), or by a modification of that method (Wonderlin et al., 1990) as previously described (Silverman et al., 1994*b*). In the former case, the volume of solution on each side of the membrane was ∼1 ml, whereas in the latter case it was ∼0.5 ml on one side and ∼1 ml on the other. The solutions on both sides of the membrane contained 1 M KCl, 2 mM CaCl$_2$, and 0–1 mM EDTA; in addition, the *cis* solution (the solution to which T domain was added) contained 30 mM Mes, pH 5.3, and the *trans* solution contained 50 mM HEPES, pH 7.2. Both solutions could be stirred by small

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[1]*Abbreviation used in this paper:* TCEP, tris(2-carboxyethyl)-phosphine.

magnetic stir bars. After a given amount of T domain was added to the *cis* solution, known voltages were applied across the membrane and the resulting current was monitored as previously described (Jakes et al., 1990). Voltages are that of the *cis* solution with respect to that of the opposite *trans* solution, whose potential is defined as zero.

RESULTS

Voltage Gating of Channels Formed by T domain with and without the NH₂-Terminal Histidine Tag

When a step of positive voltage (30–60 mV) was applied across a membrane containing T domain in the *cis* compartment, the current rose linearly over the several seconds of the voltage step, representing the continuous opening (or insertion) of T domain channels. This response to positive voltage steps was essentially the same whether or not the T domain had the attached NH₂-terminal histidine tag; when the sign of the voltage step was reversed, however, the current responses were dramatically different for T domain with and without the histidine tag. Channels formed by T domain lacking the histidine tag did not close over many seconds at −30 mV (Fig. 2 *A*) and closed very slowly, if at all, at −60 mV. In contrast, channels formed by T domain having the NH₂-terminal histidine tag closed very rapidly at −30 mV (Fig. 2 *B*). The rate of closure was voltage dependent, with the rate increasing with the magnitude of the negative voltage. Even at −10 mV, the rate of channel closure was rapid (Fig. 2 *B*). When the voltage was switched back from −30 to +30 mV, the current rapidly increased to its value before the switch from +30 to −30 mV, and then resumed its linear rise (Fig. 2 *B*). Thus, the pre-existing channels that had been closed at −30 mV rapidly reopened at +30 mV, and the subsequent linear rise in current represents the resumption of the continuous opening (or insertion) of new channels. Despite the difference in voltage gating behavior of the channels formed by histidine-tagged and -untagged T domain, there was no difference in their single-channel conductance or in the reaction of their cysteine mutants in the TH8-9 region with methanethiosulfonate derivatives (Huynh et al., 1997).

Translocation of the Histidine Tag Across the Membrane

How does the presence of the NH₂-terminal histidine tag lead to rapid closure of the T domain channels at negative voltages? We believe that the histidine tag is translocated to the *trans* side and from there acts to close the channels, either by directly plugging them or by an allosteric effect upon binding to a T domain region on the *trans* side. We shall comment further on the mechanism of channel closure in the DISCUSSION, but at this point we present the experimental evidence that leads us to our assertion that the histidine tag has been translocated across the membrane to the *trans* side.
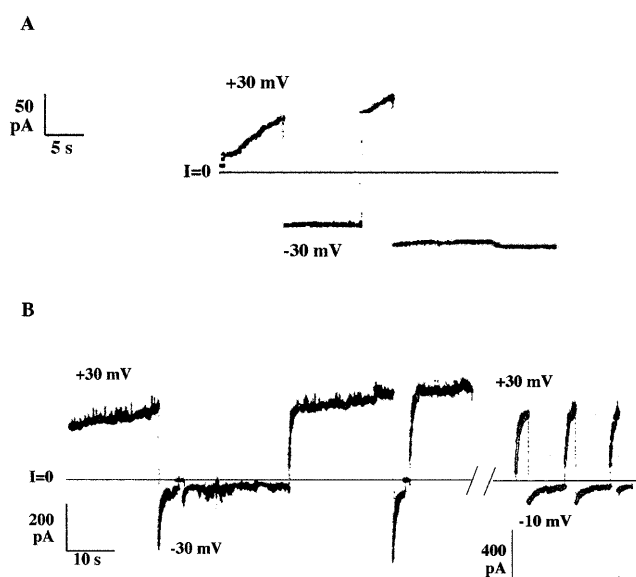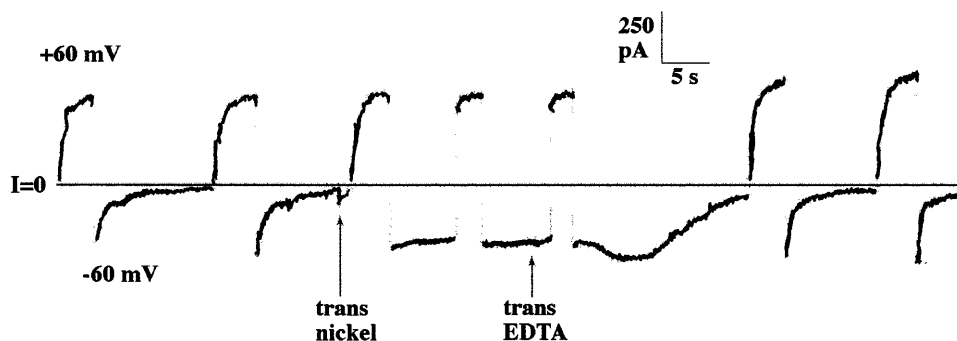

FIGURE 2. Differences in voltage gating properties between T domain channels that have and do not have an NH₂-terminal histidine tag. Before the start of each record, either untagged (*A*) or histidine-tagged (*B*) T domain was added to the *cis* solution to a concentration of 40 ng/ml, and the current across the membrane began to rise linearly with the voltage held at +30 mV. (*A*) T domain-induced conductance is seen rising linearly at +30 mV; when the voltage was switched to −30 mV, the conductance remained on. When the voltage was switched back to +30 mV, the conductance again continued to rise, and again remained on when the voltage was switched back to −30 mV. (*B*) Histidine-tagged T domain-induced conductance turned on at +30 mV as in *A*, but in contrast turned off rapidly at −30 mV. When the voltage was switched back to +30 mV, conductance rapidly recovered, and then continued to increase. The rapid turn off of conductance is seen (after the 1-min break) to occur even at −10 mV, although it is slower at that voltage than at −30 mV. Again, returning the voltage to +30 mV resulted in a rapid turn on of conductance. The solutions on both sides of the membrane contained 1 M KCl, 2 mM CaCl₂, and 1 mM EDTA; the *cis* solution contained 30 mM Mes, pH 5.3, and the *trans* contained 50 mM HEPES, pH 7.2. The record was filtered at 10 Hz by the chart recorder.

Effect of trans Ni++ on channel gating.

Nickel readily binds to the histidine tag; this, in fact, is the basis for the purification of histidine-tagged proteins on nickel columns. We reasoned that if the histidine tag was on the *trans* side of the membrane and we added Ni++ to that side, its binding of Ni++ might alter its effect on channel gating. This indeed proved to be the case. The rapid closure of histidine-tagged T domain channels at negative voltages was prevented or drastically reduced by micromolar concentrations of *trans* Ni++ and was restored by subsequent *trans* EDTA (Fig. 3). *Cis* Ni++ even at 8 mM concentration had no such effect. That the Ni++ effect was the result of its specific binding to the protein and not of nonspecific binding to the bilayer was evidenced by the similar effect of *trans* 5 μM Cu++, another metal

FIGURE 3. Nickel added to the *trans* side prevents rapid turn off by histidine-tagged T domain channels. Before the start of the record, histidine-tagged T domain was added to the *cis* solution to a concentration of 40 ng/ml, and the voltage across the membrane was held at +60 mV. After channel activity appeared, the voltage was switched from +60 to −60 mV several times. Conductance turned off rapidly at −60 mV. (The rather slow turn off at the beginning of the record probably results from trace multivalent cation contamination that is not completely complexed by the 5 μM EDTA present in the *trans* solution.) At the first arrow, NiSO$_4$ was added to the *trans* solution to a concentration of 25 μM. After the nickel addition, channel conductance failed to turn off at −60 mV. At the second arrow, EDTA was added to the *trans* solution to a concentration of 2 mM. With the voltage held at −60 mV, the conductance decreased nearly to zero over a time course of several seconds. Subsequent switching of voltage between −60 and +60 mV produced the rapid turn on and off of conductance seen before the nickel addition. The solutions on the two sides of the membrane were the same as in Fig. 2, except the *trans* solution contained only 5 μM EDTA instead of 1 mM. The record was filtered at 10 Hz by the chart recorder.

chelated by histidines, and the absence of any effect by *trans* La^{+++} (10 μM), which binds strongly to phospholipid bilayers.

Effect of trans streptavidin on the gating of channels formed by T domain biotinylated in the histidine tag sequence. The above *trans* Ni^{++} experiments are good evidence that the NH$_2$-terminal histidine tag has been translocated to the *trans* side of the membrane, but there are at least two other (although unlikely) interpretations of those experiments that do not require the histidine tag to be on the *trans* side of the membrane. One is that the effect of *trans* Ni^{++} on channel gating is due to its binding to other histidines or other residues in the T domain. The second is that the histidine tag has not completely traversed the membrane to the *trans* solution, but is instead somewhere within the channel where Ni^{++}, because of its small size, can gain access. To eliminate these possibilities, we mutated one of the serines (see MATERIALS AND METHODS) in the histidine tag sequence to a cysteine and biotinylated it. We hypothesized that if the histidine tag was on the *trans* side, the biotinylated cysteine would be bound by *trans* streptavidin, and this in turn would interfere with the histidine tag's effect on channel gating. Again our expectation was fulfilled. The addition of streptavidin to the *trans* solution completely prevented the rapid closure at negative voltages of biotinylated, histidine-tagged T domain channels, and this voltage gating was restored by the subsequent addition of the disulfide reducing agent TCEP (which reduces the disulfide bond that links the biotin to cysteine, thereby removing the biotin moiety along with its bound streptavidin from the protein; Fig. 4 A). The effect of *trans* streptavidin on channel gating was not seen if free biotin was added to the *trans* solution before the addition of streptavidin, thereby con-

firming that the streptavidin effect is the result of its binding to the biotin moiety attached to the histidine tag.

Interestingly, while the channels were in the histidine tag-induced closed state, *trans* streptavidin did not open them, but once they were opened by positive voltage, subsequent negative voltage pulses did not close them, because the histidine tag was now bound by streptavidin (Fig. 4 A). Moreover, that binding did not occur while the channels were closed, but only after they had been re-opened by the positive voltage step. This is demonstrated by the experiment shown in Fig. 4 B. When streptavidin was added to the *trans* solution while the channels were in the histidine tag-induced closed state, and then biotin was added to the *trans* solution to tie up all the free streptavidin-binding sites, the subsequent channel gating by positive and negative voltages was the same as if streptavidin had never been added to the *trans* solution. Thus, the biotinylated cysteine in the histidine tag is not accessible to *trans* streptavidin while the channels are in the histidine tag-induced closed state.

Effect of cis streptavidin on the gating of channels formed by T domain biotinylated in the histidine tag sequence. The results obtained upon addition of streptavidin to the *cis* solution are complicated by the presence of two populations of T domain molecules: (*a*) those that have formed channels, and hence have their histidine tag on the *trans* side, and (*b*) those that have not yet formed channels, and therefore have their histidine tag on the *cis* side. The complications arise from the possibility, suggested in some of our experiments, that the histidine tag-induced closing of the channels can eventually lead to the return of the histidine tag to the *cis* side (possibly through the channels that it has blocked). The description of these experiments is reserved for a
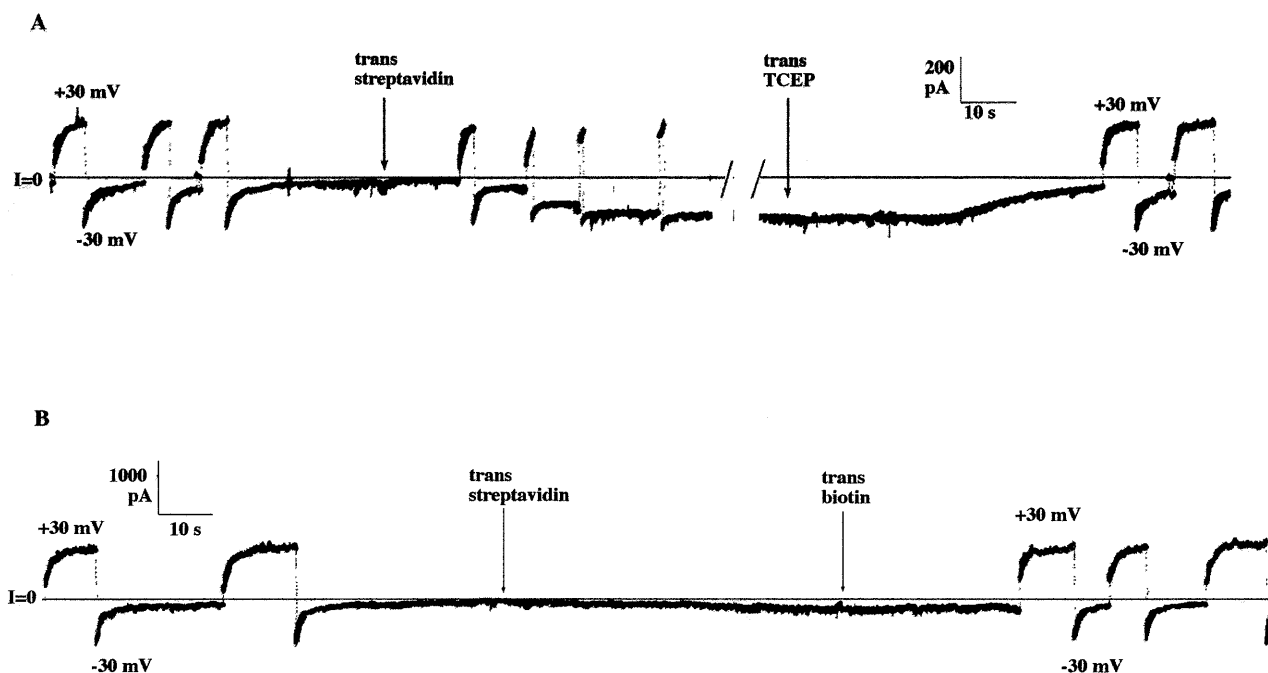
A

B

FIGURE 4. Streptavidin added to the *trans* side prevents rapid turn off by biotinylated histidine-tagged T domain channels. A serine residue within the histidine tag sequence was mutated to a cysteine, and then biotinylated as described in MATERIALS AND METHODS. Before the start of each record, biotinylated histidine-tagged T domain was added to the *cis* solution to a concentration of 540 ng/ml, and the voltage across the membrane was held at +30 mV. After channel activity appeared, the voltage was switched from +30 to −30 mV several times. (*A*) Biotinylated histidine-tagged T domain-induced conductance turned off rapidly at −30 mV. At the first arrow, 40 μg of streptavidin was added to the *trans* solution. The voltage was switched from +30 to −30 mV a few times. Within a minute, the rapid turn off of conductance at −30 mV disappeared. At the second arrow, the disulfide reducing agent TCEP was added to the *trans* solution to a concentration of 30 mM. (TCEP can reduce the disulfide bond in the linker of biotin to cysteine, thereby removing the biotin moiety along with its bound streptavidin from the protein.) With the voltage held at −30 mV, the conductance decreased nearly to zero over a time course of several seconds. Subsequent switching of voltage between −30 and +30 mV produced the rapid turn on and off of conductance seen before the streptavidin addition. The break in the record is 4.5 min. (*B*) The biotinylated cysteine in the histidine tag sequence is not accessible to *trans* streptavidin while the channels are held in the histidine tag-induced closed state. Biotinylated histidine-tagged T domain-induced conductance turned off rapidly at −30 mV. At the first arrow, 40 μg of streptavidin were added to the *trans* solution. The voltage was held at −30 mV to maintain channels in their histidine tag-induced closed state. At the second arrow, after 70 s at −30 mV, 5 μg of biotin were added to the *trans* solution to prevent any further reaction of streptavidin with the channels. Subsequent switching of voltage between −30 and +30 mV produced the rapid turn on and off of conductance seen before the addition of the streptavidin and biotin, indicating a failure of streptavidin to react with the channel, as it did in *A*. The solutions on the two sides of the membrane were the same as in Fig. 2. The records were filtered at 100 Hz by the chart recorder.

future communication in which the mechanism of histidine tag-induced closing of T domain channels is analyzed. Of relevance to us now is the result obtained when biotinylated histidine-tagged T domain that had been preincubated with streptavidin was added to the *cis* solution. In this case, the current records obtained in response to positive voltage steps were very noisy and ragged, and remained so at negative voltage steps (data not shown). The membranes frequently broke after several voltage pulses. We have not attempted any analyses of these records, but what they appear to indicate is that if the amino terminus of the T domain is prevented from reaching the *trans* side (in this case by streptavidin), the normal formation and opening of channels is severely compromised. The implication is that the amino terminus of the T domain is moved to the *trans* side in normal channel formation, and if it is constrained to remain on or near the *cis* side, then a part of the NH₂-terminal region that normally resides on the *trans* side when channels open ends up within the membrane and disrupts the channel.

The Effect of trans Trypsin on Channel Gating

When trypsin was added to the *trans* solution to a concentration of 30–50 μg/ml, the histidine tag-induced closing of the T domain channels was abolished within a few minutes (Fig. 5). This effect was clearly attributable to the enzymatic effect of trypsin, since (*a*) the trypsin used was tosyl-L-phenylalanine chloromethyl ketone treated (see MATERIALS AND METHODS), thereby excluding chymotrypsin activity; (*b*) the effect was elim-
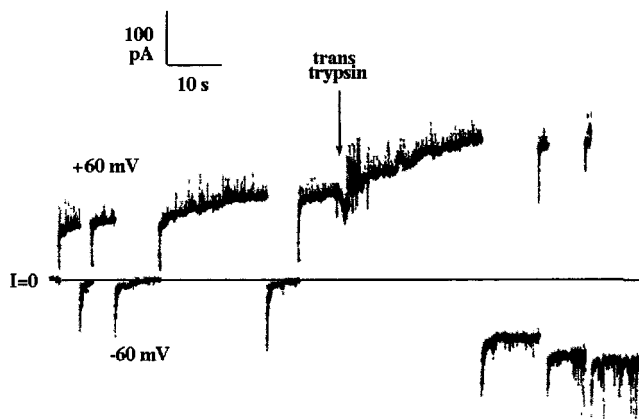
FIGURE 5. Trypsin added to the *trans* side prevents rapid turn off by histidine-tagged T domain channels. Before the start of the record, histidine-tagged T domain was added to the *cis* solution to a concentration of 18 ng/ml, and the voltage across the membrane was held at +60 mV. After channel activity appeared, the voltage was switched from +60 to −60 mV several times. (The membrane was reformed once.) Channel conductance turned off rapidly at −60 mV. At the arrow, 50 μg of trypsin were added to the *trans* solution. Within 1 min, the rapid turn off of conductance at −60 mV essentially disappeared. The solutions on the two sides of the membrane were the same as in Fig. 2. The record was filtered at 100 Hz by the chart recorder.
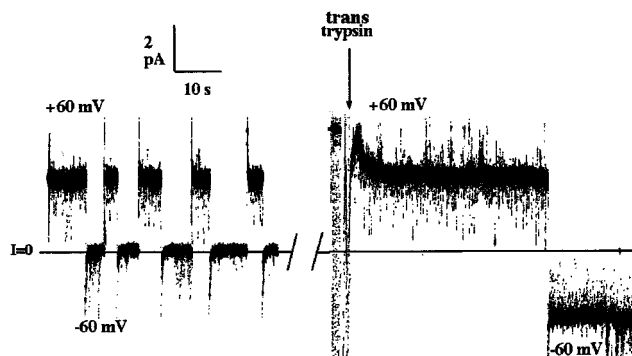

FIGURE 6. Reaction of histidine-tagged T domain channels with *trans* trypsin at the single channel level. Before the start of the record, histidine-tagged T domain was added to the *cis* solution to a concentration of 0.5 ng/ml, and the voltage across the membrane was held at +60 mV. After a single channel appeared, the voltage was switched from +60 to −60 mV several times. The channel conductance turned on with each pulse to +60 mV, remained on at that voltage, and then turned off immediately when the voltage was switched to −60 mV. During the 3-min break, a second channel appeared. At the arrow, 20 μg of trypsin were added to the *trans* solution. While the trypsin was being added, the second channel disappeared, leaving a single channel (there is also a transient artifact just after trypsin addition). The channel conductance stayed on at +60 mV, but when the voltage was switched to −60 mV, the channel failed to turn off. Note that no effect of trypsin was seen (at +60 mV) on single channel conductance or flickering behavior; the only indication that trypsin had acted was the failure of the channel to close rapidly at −60 mV. The solutions on the two sides of the membrane were the same as in Fig. 2. The record was filtered at 100 Hz by the chart recorder.

inated if the trypsin was preincubated for 3 h with the trypsin inhibitor *N*–tosyl-L-lysine chloromethyl ketone; (*c*) if soybean trypsin inhibitor was first added to the *trans* compartment to a concentration of 60 μg/ml, the subsequent addition of trypsin (60 μg/ml) had no effect on channel gating. The obvious interpretation of the trypsin effect is that it resulted from cleavage, upstream of the channel-forming TH8-9, of the histidine tag from the T domain. If trypsin was added to the *trans* solution before the channels were opened by a positive voltage stimulus, and then soybean trypsin inhibitor was added to inhibit further trypsin enzymatic activity, the channels that were subsequently opened by positive voltage stimuli were gated normally by the histidine tag. Thus, the trypsin cleavage site was not accessible to *trans* trypsin before channel opening. Interestingly, *trans* trypsin had no discernible effect on the channel per se. With the voltage held at +60 mV, for example, there was no indication in terms of single-channel conductance or flickering behavior that trypsin had done anything; only the failure of the channel to close rapidly when the voltage was switched to −60 mV revealed that trypsin had indeed acted (Fig. 6).

Although we have not yet identified the trypsin cleavage site (or sites), our elimination of the two most obvious possibilities is revealing. One such possibility is the arginine in the histidine tag sequence itself. Mutation of this to glutamine did not affect the normal histidine tag-induced closing of the channels nor its abolishment by *trans* trypsin; thus, the trypsin cleavage site is within the T domain proper.[2] Similarly, the mutation of Lys 299 to Ala did not alter the channel gating nor the effect of trypsin on it. This result is particularly significant. Lys 299 is the only potential trypsin cleavage site from Thr 265 to the carboxy terminal Pro 378 of the T domain that can account for trypsin's removal of the histidine tag. (The one other trypsin cleavage site in this entire 104 amino acid segment is the penultimate Arg 377.) The elimination of this site, along with the arginine in the histidine tag sequence, means that the trypsin cleavage site must lie somewhere in the segment beginning at the start of the T domain at Ile 202 and ending at Lys 264. This 63 amino acid segment contains 26 charged residues: 10 lysines, 1 arginine (making 11 potential trypsin cleavage sites), 10 glutamates, 2 aspartates, and 3 histidines. Thus, in addition to the

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[2]On the off chance that trypsin could be acting on the polyhistidine in the histidine tag, we incubated synthetic histidine tag peptide (1 mg/ml) with trypsin (30 μg/ml) for 30 min at room temperature in the pH 7.2 solution used in our bilayer experiments, and looked for reaction products or loss of original peptide by mass spectrometry and HPLC. No discernible cleavage of the peptide was seen.

histidine tag, some or all of this extremely hydrophilic segment must be translocated across the membrane from the *cis* to the *trans* side when the channels are formed (or inserted).

DISCUSSION

The T domain of diphtheria toxin, which extends from residue 202 to 378, causes the translocation of the catalytic A fragment of the toxin across endosomal membranes and also forms channels in planar bilayers (and cell membranes). The relation of these two actions of the T domain to each other is unclear, but there is a strong correlation between them (Falnes et al., 1992; Cabiaux et al., 1993; Silverman et al., 1994*a*; vanderSpek et al., 1994). It has not yet been unambiguously demonstrated that the T domain alone, unassisted by membrane proteins or cellular components, can translocate the A fragment across a lipid bilayer, but, if it can, then at least the NH_2 terminus of the T domain must also be translocated. In this paper, we have shown that not only the NH_2 terminus, but also some or all of the hydrophilic 63 amino acid NH_2-terminal segment (residues 202–264) (see Fig. 1) is translocated from the *cis* to the *trans* side of the membrane in association with channel formation. The phenomenon that enabled us to demonstrate that the NH_2-terminal region of the T domain was translocated to the *trans* side of the membrane was the rapid closure of channels at *cis* negative voltages when the T domain contained a histidine tag at its NH_2 terminus. The inhibition of this effect by *trans* Ni^{++}, and by *trans* streptavidin when the histidine tag sequence was biotinylated, clearly established that the histidine tag was present on the *trans* side of the membrane.

Mechanism of Histidine Tag-induced Channel Closure

The most likely mechanism by which the *trans* histidine tag closes T domain channels is by inserting into their lumen and blocking them, analogous to the "ball and chain" mechanism of inactivation of *Shaker* K^+ channels (Zagotta et al., 1990). Unlike the inactivation of the *Shaker* K^+ channels, however, the blockage of the T domain channels by the histidine tag is voltage dependent; i.e., the more negative the voltage the faster the rate of channel closure ($\sim$e-fold per 25 mV in preliminary experiments). It is of course possible that the *trans* histidine tag does not act as a channel blocker, but instead binds to a region of the T domain outside of the channel lumen and thereby produces a conformational change that closes the channel. It is difficult to rigorously exclude this possibility, but preliminary experiments with a synthetic histidine tag peptide argue for a blocking mechanism.

NH_2-Terminal Translocation and Channel Formation

Earlier work has shown that the only part of the T domain needed to form channels having the same conductance, ion selectivity, and pH dependence of conductance as those formed by the entire T domain is the 57 amino acid carboxy terminal stretch, residues 322–378 (Silverman et al., 1994*b*; see footnote 1 in Huynh et al., 1997). Although the rest of the T domain, residues 202–321, is not essential to channel structure, it makes a major contribution to channel formation; that is, the ability of the whole T domain to form channels is orders of magnitude greater than that of the carboxy terminal segment alone (Silverman et al., 1994*b*). The capacity of the rest of the T domain to promote channel formation is apparently dependent on the translocation of its NH_2 terminus to the *trans* side, as evidenced by our observation that when biotinylated histidine tag sequence was prebound to streptavidin, channel formation was severely disrupted. Thus, normal channel formation requires that the NH_2 terminus of the T domain be moved to the *trans* side. If it is held on the *cis* side (in this case by streptavidin) and prevented from crossing the membrane, channel stability is perturbed. An analogous phenomenon occurs when normally translocated residues of colicin Ia are held on the *cis* side by streptavidin (Qiu et al., 1996).

The Extent of the Translocated Region

The 177-residue T domain can be subdivided into an amino terminal third (residues 202–264) that is very polar and a carboxy terminal two thirds (265–378) that contains four hydrophobic stretches of $\sim$20 residues each (Fig. 1). The minimal piece required to form a channel consists of the carboxy terminal hydrophobic pair and its polar four-residue connecting segment, which corresponds to the α-helical hairpin TH8-9 in the water-soluble form of diphtheria toxin. The other hydrophobic pair and its polar 10-residue connecting segment corresponds to the α-helical hairpin TH5-7. (The correspondence in both these instances is not exact with respect to where the helices begin and end, and what constitutes the loop of the hairpin.) We have identified the existence of a site in the polar amino terminal third of the T domain that is sensitive to *trans* trypsin (Fig. 5). Given the very polar nature of this segment (26 of its 63 residues are charged) and our finding that its NH_2 terminus is also on the *trans* side, this suggests that the entire segment has been translocated to the *trans* side. If this is correct, why shouldn't the A fragment in the whole toxin also be on the *trans* side? There is no obvious nonpolar region in the A fragment to insert in the membrane and hinder its translocation. We therefore suggest that the entire A fragment of diphtheria toxin, along with the polar amino terminal

third of the T domain (i.e., residues 1–264), is translocated across the membrane in association with channel formation. (Preliminary experiments with whole toxin containing a histidine tag at the A fragment's NH$_2$ terminus are consistent with this suggestion.) The structure created by the carboxy terminal two thirds of the T domain (residues 265–378) that effects this translocation remains to be determined.

REFERENCES

Bennett, M.J., and D. Eisenberg. 1994. Refined structure of monomeric diphtheria toxin at 2.3 Å resolution. *Prot. Sci.* 3:1464–1475.

Cabiaux, V., J. Mindell, and R.J. Collier. 1993. Membrane translocation and channel-forming activities of diphtheria toxin are blocked by replacing isoleucine 364 with lysine. *Infect. Immun.* 61:2200–2202.

Draper, R.K., and M.I. Simon. 1980. The entry of diphtheria toxin into the mammalian cell cytoplasm: evidence for lysosomal involvement. *J. Cell Biol.* 87:849–854.

Eriksen, S., S. Olsnes, K. Sandvig, and O. Sand. 1994. Diphtheria toxin at low pH depolarizes the membrane, increases the membrane conductance and induces a new type of ion channel in Vero cells. *EMBO (Eur. Mol. Biol. Organ.) J.* 13:4433–4439.

Falnes, P.O., I.H. Madshus, K. Sandvig, and S. Olsnes. 1992. Replacement of negative by positive charges in the presumed membrane-inserted part of diphtheria toxin B fragment: effect on membrane translocation and on formation of channels. *J. Biol. Chem.* 267:12284–12290.

Greenfield, L., M.J. Bjorn, G. Horn, D. Fong, G.A. Buck, R.J. Collier, and D.A. Kaplan. 1983. Nucleotide sequence of the structural gene for diphtheria toxin carried by corynebacteriophage b. *Proc. Natl. Acad. Sci. USA.* 80:6853–6857.

Huynh, P., C. Cui, H. Zhan, K.J. Oh, R.J. Collier, and A. Finkelstein. 1997. Probing the structure of the diphtheria toxin channel: reactivity in planar lipid bilayer membranes of cysteine-substituted mutant channels with methanethiosulfonate derivatives. *J. Gen. Physiol.* 110:229–242.

Jakes, K., C.K. Abrams, A. Finkelstein, and S. Slatin. 1990. Alteration of the pH-dependent ion selectivity of the colicin E1 channel by site-directed mutagenesis. *J. Biol. Chem.* 265:6984–6991.

Jakes, K., P. Kienker, S. Slatin, and A. Finkelstein. 1998. Translocation of inserted foreign epitopes by a channel-forming protein. *Proc. Natl. Acad. Sci. USA.* 95:4321–4326.

Kagan, B.L., A. Finkelstein, and M. Colombini. 1981. Diphtheria toxin fragment forms large pores in phospholipid bilayer membranes. *Proc. Natl. Acad. Sci. USA.* 78:4950–4954.

Kagawa, Y., and E. Racker. 1971. Partial resolution of the enzymes catalyzing oxidative phosphorylation. XXV. Reconstitution of vesicles catalyzing ^{32}Pi-adenosine triphosphate exchange. *J. Biol. Chem.* 246:5477–5487.

Lanzrein, M., P.O. Falnes, O. Sand, and S. Olsnes. 1997. Structure-function relationship of the ion channel formed by diphtheria toxin in Vero cell membranes. *J. Membr. Biol.* 156:141–148.

Madshus, I., and H. Stenmark. 1992. Entry of ADP-ribosylating toxins into cells. *Curr. Top. Microbiol. Immunol.* 175:1–26.

Montal, M. 1974. Formation of bimolecular membranes from lipid monolayers. *Methods Enzymol.* 32:545–554.

Qiu., X.-Q., K. Jakes, P. Kienker, A. Finkelstein, and S. Slatin. 1996. Major transmembrane movement associated with colicin Ia channel gating. *J. Gen. Physiol.* 107:313–328.

Sandvig, K., and S. Olsnes. 1980. Diphtheria toxin entry into cells is facilitated by low pH. *J. Cell Biol.* 87:828–832.

Silverman, J.A., J. Mindell, A. Finkelstein, W.H. Shen, and R.J. Collier. 1994a. Mutational analysis of the helical hairpin region of diphtheria toxin transmembrane domain. *J. Biol. Chem.* 269: 22524–22532.

Silverman, J.A., J.A. Mindell, H. Zhan, A. Finkelstein, and R.J. Collier. 1994b. Structure–function relationships in diphtheria toxin channels: determining a minimal channel-forming domain. *J. Membr. Biol.* 137:17–28.

vanderSpek, J.C., D. Cassidy, F. Genbauffe, P. Huynh, and J.R. Murphy. 1994. An intact transmembrane helix 9 is essential for the efficient delivery of the diphtheria toxin catalytic domain to the cytosol of target cells. *J. Biol. Chem.* 269:21455–21459.

Wonderlin, W.F., A. Finkel, and R.J. French. 1990. Optimizing planar lipid bilayer single-channel recordings for high time resolution. *Biophys. J.* 58:289–297.

Zagotta, W.N., T. Hoshi, and R.W. Aldrich. 1990. Restoration of inactivation in mutants of *Shaker* potassium channels by a peptide derived from ShB. *Science.* 250:568–571.

Zhan, H., K.J. Oh, Y.K. Shin, W.L. Hubbell, and R.J. Collier. 1995. Interaction of the isolated transmembrane domain of diphtheria toxin with membranes. *Biochemistry.* 34:4856–4863.