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# Membrane translocation assay based on proteolytic cleavage: Application to diphtheria toxin T domain

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ABSTRACT

The function of diphtheria toxin translocation (T) domain is to transfer the catalytic domain across the endosomal membrane upon acidification. The goal of this study was to develop and apply an *in vitro* functional assay for T domain activity, suitable for investigation of structure–function relationships of translocation across lipid bilayers of various compositions. Traditionally, T domain activity *in vitro* is estimated by measuring either conductance in planar lipid bilayers or the release of fluorescent markers from lipid vesicles. While an *in vivo* cell death assay is the most relevant to physiological function, it cannot be applied to studying the effects of pH or membrane lipid composition on translocation. Here we suggest an assay based on cleavage of the N-terminal part of T domain upon translocation into protease-loaded vesicles. A series of control experiment was used to confirm that cleavage occurs inside the vesicle and not as the result of vesicle disruption. Translocation of the N-terminus of the T domain is shown to require the presence of a critical fraction of anionic lipids, which is consistent with our previous biophysical measurements of insertion. Application of the proposed assay to a series of T domain mutants correlated well with the results of cytotoxicity assay.



## 1. Introduction

Diphtheria toxin consists of three covalently linked domains: receptor, translocation and catalytic. After the receptor domain binds to the EGFR-like receptor on the cell surface, the toxin is endocytosed into the cell. The subsequent acidification of the endosome induces refolding and membrane insertion of the translocation (T) domain and translocation of the catalytic domain into the cytosol. The translocated catalytic domain is cleaved off of the T domain at furin site (RVRR, position 190–193) and acts as an inhibitor of EF1 translation factor, leading to termination of protein synthesis and cell death [1,2]. Certain mutations in T domain are known to cause partial or complete loss of its activity, thus producing nontoxic protein species [3,4]. The cell death assay is commonly used to determine the activity of the full-length toxin, where decrease in incorporation of radioactive amino acids into the acid-insoluble fraction reflects cell death after toxin administration. This method is an ultimate assay for determination of T domain species activity but requires a live cell culture and radioactive isotopes [5,6]. The isolated T domain (residues 202–378) is known to form pores in the artificial membranes and to translocate its N-terminus across the bilayer in response to acidification. This pore formation can be utilized to study the effects of mutations and the influence of membrane lipid composition on the T domain activity *in vitro* [7,8]. The following two methods are used to study the properties of isolated T domain: leakage assay, in which activity is estimated by the release of fluorescent dye from the vesicles [9], and electrophysiological assay, in which the increase of conductance due to pore formation by T domain is measured [10]. Although both methods are relatively simple and reproducible, several studies have demonstrated that their results are not always consistent with those of cell death assay [5]. Therefore there is a need to develop a new method for T domain activity assay where the translocation of the N-terminal protein portion across the membrane can be observed directly.

It has been suggested that upon channel formation by the T domain, its N-terminus crosses the lipid bilayer. Also, 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 N-terminal segment of the T domain was translocated to the *trans* side of the membrane [8]. Here, we follow up on these findings to establish a proteolysis-based assay, where, upon translocation of T domain N-terminus across the lipid bilayer, the link between N-terminal His-tag and T domain body is cleaved at a specific site (PRG) by thrombin located on the *trans* side of the membrane (inside the vesicles).

Proteases are widely used to study membrane protein structure via two general approaches: (I) external protease addition, where potential proteolysis sites are protected by the membrane after incorporation of the protein into it, and (II) internal proteolysis, where protease-loaded LUV are used and the parts of the protein expanded into the membrane become accessible for proteolysis [11]. In some cases, artificial tags for protease cleavage were inserted into the protein sequence at locations of interest in order to study the accessibility of certain parts of the

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protein for specific proteolysis [12–14]. Here we introduce the assay for determination of the activity of the isolated T domain, based on the proteolytic cleavage of the N-terminal His-tag translocated into lipid vesicles. The T domain was cloned into pET15b vector, which contained the following features: 18 amino acid N-terminal His-tag and a thrombin cleavage site, located 3 amino acids upstream from the beginning of canonical T domain sequence. We demonstrate that the cleavage of the His-tag in the course of T domain interaction with thrombin-loaded vesicles occurs only when the N-terminal part had been translocated across the bilayer into the vesicle. We applied this method to studying dependence of LUV translocation efficiency on lipid composition of the artificial membrane; the results confirm our previous statement that a high content of acidic phospholipids is important for the formation of the final inserted state [15]. We have also studied the activity of several T domain mutants carrying replacements of certain histidine residues with neutral or positively charged ones, and results obtained are in good agreement with cell death assay data.

## 2. Materials and methods

### 2.1. Materials

Palmitoyloleoylphosphatidylcholine (POPC), palmitoyloleoylphosphatidylglycerol (POPG), and palmitoyloleoylphosphatidylserine (POPS) were obtained from Avanti Polar Lipids (Alabaster, AL). Bovine thrombin was from Fisher Scientific (Pittsburgh, PA). The CLY3 construct containing the fusion of enhanced cyan and enhanced yellow fluorescent proteins was provided by Addgene (Cambridge, MA)[16]. The mutations were introduced using Site-Directed Mutagenesis Kit (Stratagene, Santa Clara, CA) according to manufacturer's protocol. 8-aminonaphtalene-1,3,6-trisulfonic acid, disodium salt (ANTS) and *p*-xylene-bis-pyridinium bromide (DPX) were obtained from Molecular Probes (Eugene, OR).

### 2.2. Preparation of T domain

Diphtheria toxin T domain (segment 202–378 of the full-length toxin) was prepared as described in [2]. Briefly, BL23DELysS cells transformed with pET15b plasmid containing T domain gene were grown to $OD_{600} = 0.6$, induced with 0.8 mM IPTG and grown overnight at 24 °C. After harvesting, cells were lysed by sonification, cell debris spun down and soluble T domain was bound to Ni-NTA (Qiagen, MA). After several washes the protein was eluted from the resin with 0.5 M imidazole in the binding buffer and additionally purified by size-exclusion FPLC on a Superose 12 column in 50 mM sodium-phosphate buffer, pH 8. T domain-containing fractions were stored at −80 °C until use.

### 2.3. Vesicle permeabilization (leakage) assay

LUV with entrapped ANTS and DPX were prepared as previously described [9,17]. Briefly, lipids from chloroform stocks were mixed in desired proportions and dried overnight, resuspended in 50 mM phosphate buffer, pH 8 containing 1 mM of ANTS and 10 mM of DPX. The lipid solutions were then frozen and thawed 10 times followed by extrusion. Lipid solutions were prepared at 20 mM concentration. Separation of the untrapped solutes was achieved by size exclusion chromatography on the Sepharose 6 column 1 × 30 cm, in 50 mM phosphate buffer pH 8, flow rate 0.25 mL/min. The fraction of LUV was eluted at the free volume of the column (retention 6 mL). The release of markers was assayed fluriometrically using a SPEX Flurolog FL 3-22 steady state fluorescence spectrometer (Jobin Yvon, Edison, NJ) equipped with double grating excitation and emission monochromators. ANTS fluorescence was excited at 353 nm and measured at 520 nm wavelength. The concentration of the T domain WT and mutants was 0.2 μM, and the concentration of LUV was 0.2 mM in the sample total volume of 0.4 mL. The reaction was initiated by decrease of pH to 5.5 by addition of an aliquot of 2.5 M sodium acetic buffer. The kinetics of T domain-induced dye release were measured until ANTS fluorescence reached the plateau, normally after 1 h of incubation. In order to determine the level of ANTS fluorescence after complete release of LUV content, the vesicles were completely dissolved by addition of 10 μl of 25% Triton X-100.

### 2.4. Preparation of thrombin-loaded LUV

Phospholipid mixtures with desired compositions from chloroform stocks were dried overnight and suspended in 50 mM sodium-phosphate buffer, pH 8, in the final concentration of 20 mM, with addition of 100 U thrombin per mL. After 5 cycles of freezing in liquid nitrogen/thawing, followed by extrusion through 100 nm filter (Whatman, PA), LUV were stored overnight at 4 °C and subjected to FPLC on a Superose 6 column to remove non-trapped thrombin (we typically repeated this procedure 2–3 times, at the low elution rate of 0.25 mL/min, which appeared key to preserving LUV integrity). To confirm the absence of untrapped thrombin we incubated the mixture of LUV with T domain or CLY3 (0.1 μM of both proteins and 0.1 mM of lipid) at pH 8 for 1 h at room temperature. If we did not observe cleavage of T domain, the batch was considered suitable for activity determination.

### 2.5. Translocation assay

Wild type (WT) or mutant T domain was mixed with LUV in the volume 50 μL in 50 mM sodium-phosphate buffer pH 8 at room temperature. The final concentrations were: 0.1 μM of protein and 0.1 mM of lipid. The samples were incubated for 10 min after which pH was adjusted to the desired level by the addition of appropriate amounts of 2.5 M sodium acetate buffer, pH 4. After 1 hour incubation at desired pH, the reaction was stopped by addition of Laemmli sample buffer and boiling, the results of proteolysis were analyzed by SDS-polyacrylamide gel electrophoresis in 4–20% gels and quantified by densitometry using Molecular Imager Gel Doc XR + image system (BioRad, CA). Each experiment was repeated in triplicate. The degree of translocation is calculated as a fraction of cleaved T domain (*−His-tag* band on SDS-PAGE) of the total T domain (*−His-tag* and *+His-tag* bands on SDS-PAGE).

### 2.6. Control experiment

The objective for the control experiment was to determine that cleavage of His-tag was achieved via N-terminus translocation but not by acidification or membrane disruption caused by T domain. We prepared the mutant of CLY3 dimer of fluorescent proteins described in [16], where Ile was mutated to Pro in the linker between cyan and yellow fluorescent proteins, thus creating the site for thrombin cleavage (sequence IRG to PRG, cleavage occurring between R and G). This construct was incubated with thrombin-loaded LUV (0.1 mM) at pH 8 and 5.6, in a final concentration of 100 nM in the presence and absence of T domain, for 1 h at room temperature. The cleavage was examined using SDS-polyacrylamide gel.

## 3. Results

The principal scheme of the translocation assay is shown in Fig. 1. T domain was added to thrombin-loaded vesicles at pH 8, and after short co-incubation pH was adjusted to the desired level. Under acidic conditions T domain was bound to the membranes and translocated its N-terminal part across the lipid bilayer. When His-tag was inserted into the vesicle, the thrombin site RG (indicated by arrow) becomes accessible to the protease and the 18-residue N-terminal segment is cleaved. The cleaved and uncleaved forms have sufficiently different electrophoretic mobility to be distinguished on SDS-polyacrylamide gels (Fig. 1).

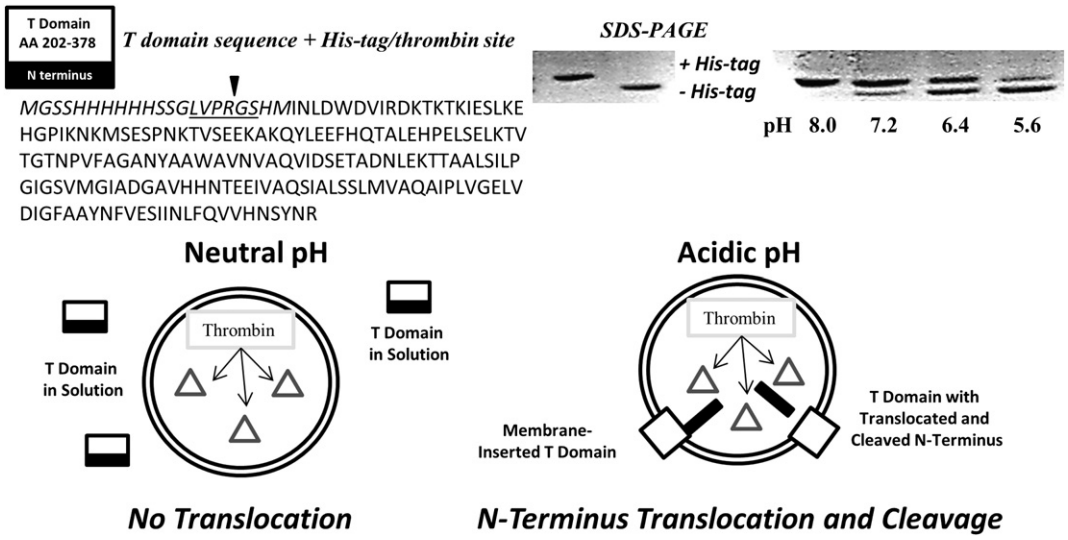


**Fig. 1.** Schematic representation of the functional assay of diphtheria toxin T domain based on the cleavage of the translocated N-terminal segment. T domain is presented by two rectangles (top left), with the open one representing the bulk of the structure, and solid one representing cleavable N-terminus. Cleave site is identified on the sequence (top center), while cleaved (*−His-tag*, MW 19.7 KDa) and uncleaved T domain (*+His-tag*, MW 21.6 KDa) can be distinguished by SDS-PAGE (top right). When T domain is mixed with lipid vesicles, pre-loaded with thrombin, at neutral pH (bottom left), no translocation occurs and no cleavage is observed (only *+His-tag* species is detected by SDS-PAGE). Membrane insertion of the T domain, triggered by acidic pH, results in the translocation of its N-terminus across the bilayer (bottom right), which exposes the thrombin cleavage site to the thrombin and results in cleavage of the His-tag (increase in *−His-tag* species at decreasing pH values is detected by SDS-PAGE). A series of specially designed control experiments (see Fig. 2 and text) confirms that the cleavage occurs indeed inside the vesicle.

In order to determine whether thrombin remains active under acidic conditions relevant to translocation conditions, we first examined the pH dependence of cleavage without LUV. The results show that in the pH range between 8 and 5.6, the protease exhibited 100% activity against T domain. The thrombin activity, however, was reduced strongly below pH 5.5, and no cleavage was observed at pH 5 (see Supplemental Materials). Thus, the working pH range was sufficient for application of thrombin to studies of diphtheria toxin.

Next we explored the effect of LUV composition on translocation in the membranes consisting of the mixtures of neutral (POPC) and acidic (POPG or POPS) phospholipids. The quality of the thrombin-loaded vesicles appears to be a key factor in translocation assay. The most important aspect of vesicle preparation was removal of external thrombin from the samples. We have checked different columns for size-exclusion chromatography in our initial attempts and obtained the best results using a Superose 6 1 × 30 cm column, where LUV were eluted in the free volume while untrapped thrombin was retained on the column (elution at 8 and 12 mL, respectively). Despite good peak resolution, we have found that some LUV samples prepared this way still contained considerable amounts of untrapped thrombin. To eliminate this problem, we subjected the preparations to additional gel filtration steps through the same column under the same conditions (50 mM Na-phosphate buffer, pH 8, flow rate 0.25 mL/min). The vesicles remained intact at a flow rate of 0.25 mL/min or less, while higher flow rates caused apparent vesicle disruption, as evidenced by the altered elution profiles showing the reduction in peak corresponding to LUV. The thrombin loaded into LUV remained active for at least 2 weeks when stored at 4 °C.

Fig. 2 shows the control experiment in which we checked whether acidification of environment itself or acid-induced pore formation by T domain could cause the release of protease from the vesicles. For these experiments we used a protein chimera construct—a fusion of eCFP and eYFP fluorescent proteins (CLY3) connected through flexible linker [16]. In order to introduce thrombin site we substituted original Ile in the linker with Pro: Pro-Arg-Gly. The experiments in solution have shown that this construct was readily cleaved by thrombin in all the ranges of pH suitable for the translocation assay. We did not observe the cleavage of the construct at either pH 8 or at pH 5.6 in the presence of thrombin-loaded LUV (75% POPG:25% POPC). The addition of T domain and its acid-induced insertion did not cause the digestion of the control CLY3 protein mutant (concentration of either protein was 100 nM), confirming that thrombin is not released from the vesicles under T domain insertion conditions.

We measured the translocation activity of T domain WT at pH 8, 7.2, 6.4, and 5.6 in membranes with a high content of acidic phospholipid POPG (75%), which are known to be optimal for T domain insertion [18]. The results show a monotonic increase of the cleaved (*−His-tag*) T domain form in the course of pH decrease (Fig. 1, top right panel), which is consistent with acid-driven insertion and translocation. Next, we characterized the dependence of translocation at pH 5.6 on the fraction of anionic lipids in the bilayer. Translocation was decreased with the lower acidic phospholipid content, and at POPG content below 15% it was practically undetectable (Fig. 3, squares). Similar threshold dependence was observed for POPS with virtually no activity observed below 20% of anionic lipid (triangles). In contrast, no threshold behavior is seen for the leakage activity of the T domain, measured by fluorescence changes associated with the release of dye-quencher pair ANTS/DPX (circles).

We have also studied the translocation activity of the following T domain mutants: two single mutants, H257R andH257Q, and two triple mutants, H322R/H323R/H372R (3H-to-R) and H322Q/H323Q/H372Q (3H-to-Q). Previously we have examined the biophysical properties of these mutants [5,15]. Data in Fig. 4A show the results of a translocation assay in highly acidic LUV (75%POPG:25%POPC). In these experiments the levels of activity for both H257R and H257Q mutants were comparable to those of WT T domain, while both triple mutants (especially 3H-to-R) showed significantly reduced levels of activity at pH 5.6. These data confirm the important role of the group of three C-terminal histidines for T domain translocation, supporting the results we obtained earlier using the set of biophysical methods [5]. The activity of both triple mutants was almost undetectable when translocation assay was performed using LUV with the lower content of acidic phospholipids (25%POPG:75%POPC). In contrast, WT and H257 mutants, when measured with 25% POPG, preserved more than half of translocation activity relative to that observed with 75% POPG membranes (data not shown).

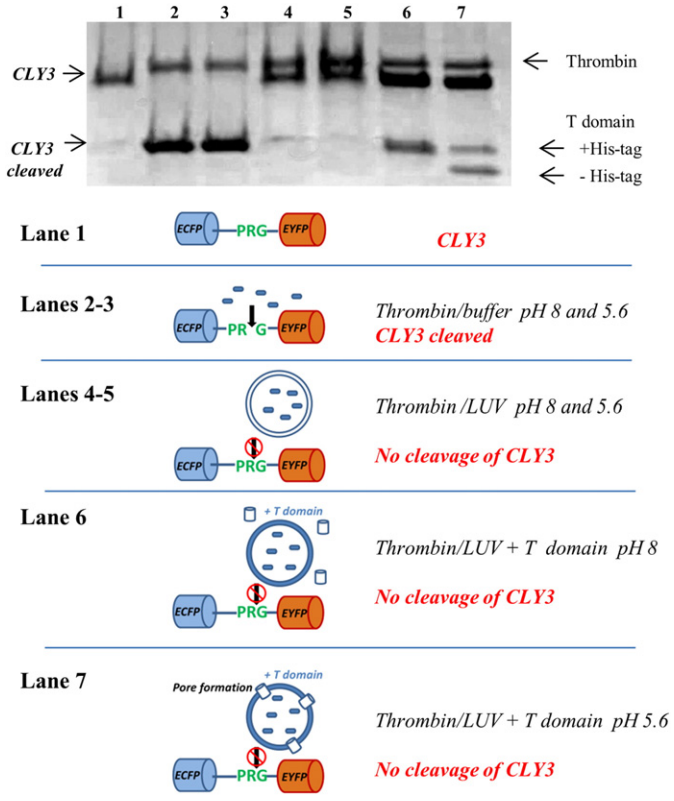


**Fig. 2.** Summary of control experiment confirming that the cleavage by thrombin does not occur as a result of release of thrombin from the vesicles. These experiments utilize a model protein CLY3 (MW 57 KDa), consisting of two fluorescent proteins connected by a flexible linker into which a site cleavable by thrombin is introduced. Cleavage of this construct into two monomers (MW 28 KDa each) can easily be detected by SDS-PAGE at either neutral or mildly acidic pH (lanes 1–3) and the experiments can be repeated either in the absence or presence of vesicles or T domain. No cleavage of CLY3 is detected in the presence of vesicles pre-loaded with thrombin (lanes 4, 5), nor when T domain is added (lines 6, 7). While the T domain inserted at pH 5.6 is cleaved (line 7), the CLY3-dimer, residing outside of the vesicles, is not. This confirms that under conditions of our assay the cleavage occurs only inside the vesicles, and therefore truly represents the translocation of T domain's N-terminus.

We have compared our translocation results for various mutants with those of the cell death assay (Fig. 4B), reproduced from our previous publications [5,15]. The cell death assay is performed using a weakened strain of the full-length protein and is based on monitoring the inhibition of protein synthesis [6]. Reduction in cell death activity is reflected in an increased concentration of the T domain, necessary to achieve a particular reduction in protein synthesis. The intercepts of the activity curves with the dotted line, corresponding to a 50% activity level, are highlighted in Fig. 4B and indicate the following rank order of activity: WT = H257R = H257Q > 3H-to-Q > 3H-to-R. This is exactly the rank order observed in our translocation experiments at pH 5.6 (highlighted in Fig. 4A). In contrast, leakage-based assay (Fig. 4C) shows no correlation with either cell death or translocation data. The time traces corresponding to changes in fluorescence dye ANTS, co-entrapped with the quencher DPX into LUV containing (75%POPG:25%POPC), caused by T domain-induced leakage of vesicle content are shown in Fig. 4C. For all of the mutants, the changes triggered by lowering the pH to 5.5 result in increased intensity which reaches the same level as for the WT (albeit with different rates). Note that under these conditions the release caused by T domain is not complete and complete disruption of LUV with Triton causes further increase of fluorescence intensity. Thus, all mutants exhibit identical levels of ANTS/DPX release after 1 h of triggering the insertion.

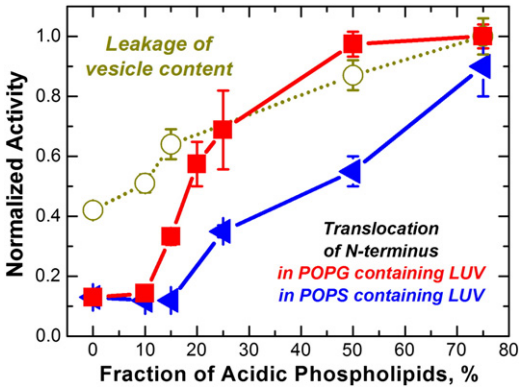


**Fig. 3.** Lipid-dependent translocation activity of the T domain (solid symbols) is compared to its membrane permeabilization activity, measured in fluorescent dye ANTS and its quencher DPX leakage assay (open symbols). For better visualization, both translocation and leakage activity data are normalized to the corresponding activity value observed in 75%POPG:25%POPC LUV. The levels of leakage in the vesicles with different composition were determined after 1 h of incubation at pH 5.5 (the details of leakage and translocation experiments are provided in Materials and methods section.) The translocation activity observed in both POPG-containing (red squares) and POPS-containing vesicles (blue triangles) exhibits a clear threshold effect. No activity is observed at low content of anionic lipids, which correlates with our published observation that T-domain at such conditions populates a non-active interfacial intermediate state [18]. In contrast, leakage data show no such threshold dependence, suggesting that membrane disruption allowing for the leakage of small markers is caused by the transient bilayer perturbation, due to interfacial binding, and not translocation.

## 4. Discussion

The goal of this study was to develop and test a simple functional assay for membrane translocation achieved by the isolated T domain of diphtheria toxin, which would be applicable for screening various mutants as well as lipid compositions. The need for such an assay is critical not only for deciphering the molecular mechanisms of bacterial infection but also for developing T domain-based cellular delivery systems with potential of targeting cancer cells [19–22]. The traditional functional assays for the isolated T domain are based either on measurements of conductance in artificial bilayers [10] or on measurements of release of vesicular contents [9]. While the latter can be used to assay various lipid compositions, it is rather indirect, and, as our published results indicate, non-specific [5].

Here we have developed a new method for determination of T domain activity, which specifically addresses the translocation of hydrophilic N-terminal extension of the cloned T domain across the bilayer of phospholipid vesicles. This functional assay appears to be no more complicated than traditional methods used previously [2,4,5], and it accommodates easy manipulation of the target membrane's lipid composition, as illustrated in Fig. 3. The results obtained for both POPG (red) and POPS (blue) clearly indicate that a certain fraction of anionic lipids is needed for translocation activity. This threshold behavior is consistent with our previous biophysical observations, indicating that anionic lipids decrease the energy barrier for the transmembrane insertion transition and that the T domain is kinetically trapped in the interfacial intermediate state at low anionic lipid content [15,18]. This intermediate state is not functionally relevant and lacks the transmembrane orientation of the helices.

Markedly, the leakage of low molecular weight markers ANTS/DPX caused by T domain interaction with the vesicles does not show a threshold effect (Fig. 3) and exhibits a 40% activity in pure POPC LUV. It is likely that the leakage is caused during the transient initial binding event by the redistribution of the mass between the leaflets due to lipid flip-flop, and not by the formation of the stable pore. Therefore, while leakage studies can be very useful for detecting initial membrane interactions and for comparing various mutants and bilayer lipid compositions [5,15], their results should always be verified with other more

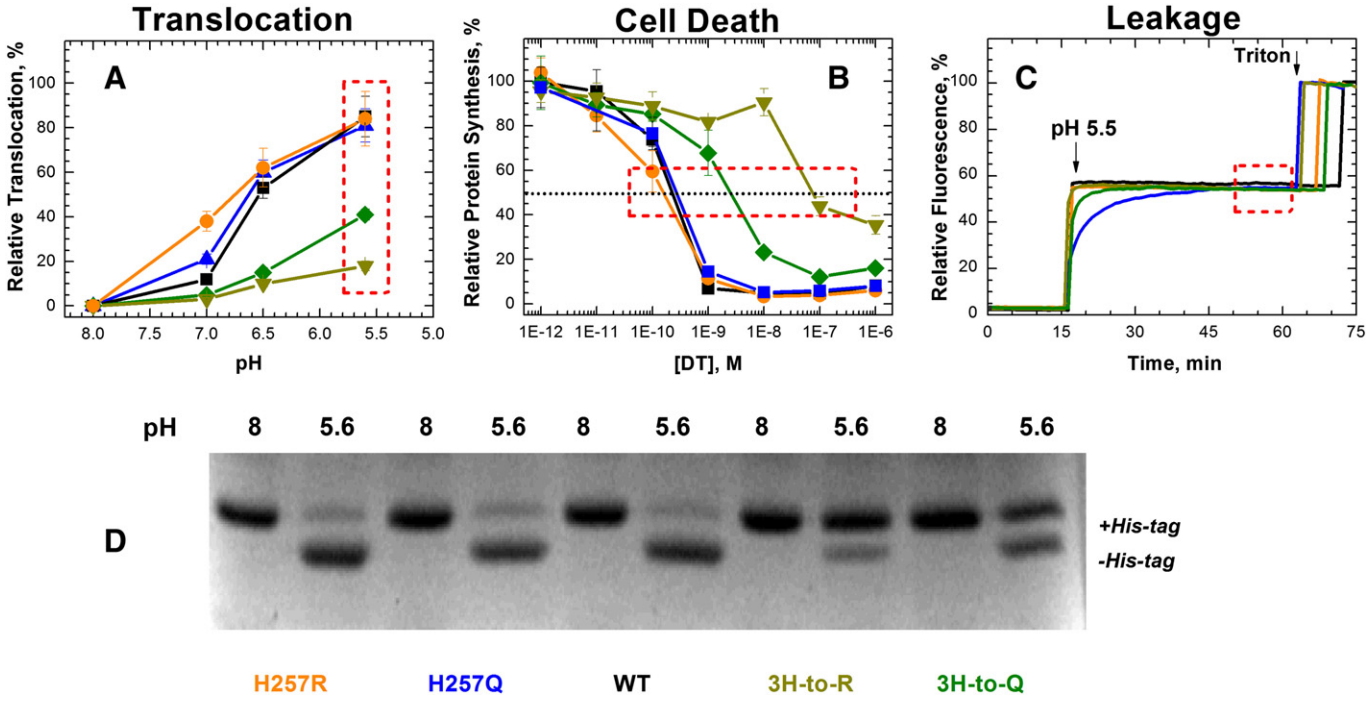


**Fig. 4.** Comparison of the activity of the T domain WT and four of its mutants determined by three independent assays: translocation assay (A) cell death assay (B) and fluorimetric leakage assay (C). The color coding of T domain mutants is as follows: WT, black; H257R, orange; H257Q, blue; H322R/H323R/H372R (3H-to-R), dark yellow; H322Q/H323Q/H372Q (3H-to-Q), olive. An example of the raw SDS-PAGE data used in determination of the translocation activity is shown in panel D (only the limiting pH points are presented for each mutant.). Both translocation and leakage assays were conducted in 75%POPG:25%POPC LUV. Cytotoxicity and leakage data for triple C-terminal histidine mutants 3H-to-R and 3H-to-Q are from [5]. The leakage data for H257R and H257Q mutants are from [15]. WT T domain and all mutants presented here induced identically high levels of leakage of ANTS/DPX from vesicles (compare relative fluorescence values at ~60 min highlighted on panel C). In contrast, a cytotoxicity assay revealed a substantial loss of activity for 3H-to-Q, and especially for 3H-to-R, as compared to the WT protein and the H257R/Q mutants (compare intersections with the dotted line corresponding to the 50% activity highlighted in panel B). The results of the translocation assay demonstrate identical high levels of translocation for the WT and H257 mutants, moderate translocation for 3H-to-Q and low translocation for 3H-to-R (compare data at pH 5.6 highlighted in panel A). This pattern is consistent with the results of the cytotoxicity assay (see text for details).

specific functional assays. For example, the leakage assay, when applied to histidine mutants of the T domain, does not demonstrate substantial differences from the WT protein, except for slightly slowed kinetics in the case of glutamine substitutions (Fig. 4, panel C). At the same time, the cell death assay revealed the highly decreased activity of the triple C-terminal mutants compared to the WT and H257R and H257Q mutants (panel B). The results of our T domain translocation assay (panel A) reveal low translocation capability of the triple mutants in model membranes, which again correlates well with the results of the cell death assay performed with the entire toxin [5]. Consistently, our previous biophysical studies indicate that these mutants can bind membranes and even insert a pair of the most hydrophobic helices TH8 and TH9 (and thus cause leakage), but they get trapped in this intermediate conformation and are unable to properly insert the rest of the protein [5].

The good correlation between the results of the cell death assay and our data for T domain mutants suggests that the mutants able to translocate His-tag in our assay are also capable of translocating the catalytic domain under *in vivo* conditions. We conclude that our assay is physiologically relevant as well as being suitable for investigation of translocation across the membranes of various lipid compositions by various mutants of diphtheria toxin T domain, and potentially by other binary toxins.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbamem.2014.09.013.

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