Concerted Protonation of Key Histidines Triggers Membrane Interaction of the Diphtheria Toxin T Domain*

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The translocation domain (T domain) of the diphtheria toxin contributes to the transfer of the catalytic domain from the cell endosome to the cytosol, where it blocks protein synthesis. Translocation is initiated when endosome acidification induces the interaction of the T domain with the membrane of the compartment. We found that the protonation of histidine side chains triggers the conformational changes required for membrane interaction. All histidines are involved in a concerted manner, but none is indispensable. However, the preponderance of each histidine varies according to the transition observed. The pair His223–His257 and His251 are the most sensitive triggers for the formation of the molten globule state in solution, whereas His322–His323 and His251 are the most sensitive triggers for membrane binding. Interestingly, the histidines are located at key positions throughout the structure of the protein, in hinges and at the interface between each of the three layers of helices forming the domain. Their protonation induces local destabilizations, disrupting the tertiary structure and favoring membrane interaction. We propose that the selection of histidine residues as triggers of membrane interaction enables the T domain to initiate translocation at the rather mild pH found in the endosome, contributing to toxin efficacy.

The function of a number of proteins is regulated by acidic pH. This is particularly the case for a series of bacterial protein toxins with intracellular targets (1). These include, for instance, Pseudomonas exotoxin A (2), anthrax toxin (3), botulinum toxins (4) and diphtheria toxin (5). After binding to a cell surface receptor, these toxins are internalized into the endosome. The receptor, these toxins are internalized into the endosome. The exotoxin A (2), anthrax toxin (3), botulinum toxins (4) and diphtheria toxin (5). After binding to a cell surface receptor, these toxins are internalized into the endosome. The exotoxin A (2), anthrax toxin (3), botulinum toxins (4) and diphtheria toxin (5). After binding to a cell surface receptor, these toxins are internalized into the endosome. The

acidic pH found in this compartment triggers the insertion of a translocation moiety into the membrane, which assists the passage of a catalytic domain into the cytoplasm. There, the catalytic domain modifies a substrate, leading to perturbation of a physiological process. The purpose of the present study is to determine how pH controls the conformational changes leading to the interaction of the translocation domain (T domain) of the diphtheria toxin with membranes.

In its soluble form, the T domain of the diphtheria toxin is composed of 10 α helices named TH1–TH9 and TH5, organized in a globular shape (6) (Fig. 1). Hydrophobic helices TH8 and TH9 are sandwiched by two layers of amphiphilic helices, TH1–TH4 and TH5–TH7. In solution, the acidification of pH between 6 and 5 induces a conformational change, leading to a molten globule (MG) state prone to interact with membranes (7, 8). In the presence of membranes however, the T domain binds to the phospholipid bilayer between pH 7 and pH 6. This interaction involves the C terminus of the domain, whereas the N terminus is more exposed to the solvent (9). Between pH 6 and pH 4, the protein rearranges its structure and penetrates deeply inside the membrane (8–10). The pH ranges at which these transitions occur suggest that the protonation of His side chains may be involved. Indeed, it has been shown that His residues may function as pH sensors for many molecular mechanisms involving protein-membrane interaction (11, 12) and/or MG formation (13, 14).

The T domain of diphtheria toxin contains six His residues (Fig. 1). Unfortunately, the protein aggregates around its isoelectric point at high concentration (15). This precludes probing the role of these His residues by NMR. To overcome this experimental problem, we substituted the His by Phe residues using site-directed mutagenesis and studied the behavior of the mutant T domains by a combination of biophysical approaches. The data showed that MG formation and membrane interaction involve the concerted protonation of key His side chains. However, different His residues have a preponderant role in the different steps studied: the pair His223–His257 and His251 are important for the formation of the MG state in solution, whereas the pair His322–His323 and His251 are the most sensitive triggers of the membrane binding step.

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The abbreviations used are: T domain, translocation domain; ANTS, 8-amino-naphthalene-1,3,6-trisulfonic acid; DPX, p-xylene-bis-pyridinium bromide; LUV, large unilamellar vesicle(s); MG, molten globule; WT, wild type.
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**EXPERIMENTAL PROCEDURES**

**Recombinant Proteins**—The recombinant T domain containing mutation C201S (native diphtheria toxin numbering) and referred to as “wild type” (WT) has been described previously (15, 16). His to Phe mutations were introduced by site-directed mutagenesis in plasmid pA/T C201S and checked by DNA sequencing. Protein expression was performed as described previously (15, 16) except that yields were increased by growing overnight precultures at 25 °C. The T domains were purified from the soluble cytoplasmic fraction as described (9, 15). Proteins were subjected to immobilized nickel affinity, size exclusion, and ion exchange chromatography. The purification buffer of the T domain was finally exchanged with NH4HCO3 on a G25SF column before lyophilization and storage at −20 °C. The molar epsilon used was 17,780 M−1 cm−1 at 280 nm, and the molecular mass was 21,860 g mol−1.

**Lipid Vesicles**—Suspensions of large unilamellar vesicles of anionic lipid bilayers at a lipid concentration of 10 mM were prepared in 5 mM citrate buffer at pH 7.5 with egg phosphatidylcholine and egg phosphatidic acid (Avanti Polar Lipids, Alabaster, AL) at a 9:1 molar ratio by reverse phase evaporation and filtration on a G25SF column before lyophilization and storage at −20 °C. The molar epsilon was 17,780 M−1 cm−1 at 280 nm, and the molecular mass was 21,860 g mol−1.

**Fluorescence Spectroscopy in Solution**—Fluorescence measurements were performed with an FP-750 spectrofluorimeter (Jasco, Tokyo, Japan) as described previously (9). Proteins were diluted in 5 mM sodium citrate buffer at the indicated pH 2 h before measurements. Protein concentration and pH were checked after measurements. For near UV and far UV measurements, each spectrum is the average of 20 scans and 10 scans, with proteins at concentrations of 20 and 5 μM, respectively. The spectra were corrected for the blank and were not smoothed. Spectra were treated as described previously (15).

**CD experiments** were performed on a J-815 spectropolarimeter (Jasco, Tokyo, Japan) as described previously (15). Proteins were diluted in 5 mM sodium citrate buffer at the indicated pH 2 h before measurements. Protein concentration and pH were checked after measurements. For near UV and far UV measurements, each spectrum is the average of 20 scans and 10 scans, with proteins at concentrations of 20 and 5 μM, respectively. The spectra were corrected for the blank and were not smoothed. Spectra were treated as described previously (15).

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**Circular Dichroism Spectropolarimetry**—CD experiments were performed on a J-815 spectropolarimeter (Jasco, Tokyo, Japan) as described previously (15). Proteins were diluted in 5 mM sodium citrate buffer at the indicated pH 2 h before measurements. Protein concentration and pH were checked after measurements. For near UV and far UV measurements, each spectrum is the average of 20 scans and 10 scans, with proteins at concentrations of 20 and 5 μM, respectively. The spectra were corrected for the blank and were not smoothed. Spectra were treated as described previously (15).

**Fluorescence Spectroscopy in Solution**—Fluorescence measurements were performed with an FP-750 spectrofluorimeter (Jasco, Tokyo, Japan) as described previously (9). Proteins were diluted in 5 mM sodium citrate buffer, 200 mM NaCl buffer at pH 7.5 containing 20 mM ANTS (Molecular Probes, Eugene, OR). Nonencapsulated ANTS was removed by gel filtration on a Sephadex G-25 column (Amersham Biosciences). Concentrated protein samples at pH 7.5 were diluted in a suspension of LUV in 5 mM sodium citrate, 200 mM NaCl, and 20 mM DPX (Molecular Probes) at pH 4 at 22 °C. Protein concentration was 9 nm at a lipid/protein molar ratio of 1000. The fluorescence decrease, due to the leakage of ANTS out of the LUV and its quenching by DPX, was recorded. Fluorescence measurements were performed by setting the excitation at 516 nm and the excitation at 360 nm.

**RESULTS**

**Secondary and Tertiary Structures of the Mutant T Domains in the Native and MG States**—The T domain contains six His residues at positions 223, 251, 257, 322, 323, and 372 (Fig. 1). In order to investigate the effect of the absence of protonation of His residues on the behavior of the protein, 16 mutants of the T domain were produced in which single or multiple His residues were replaced by Phe (Table 1). The mutant T domains are numbered thereafter according to the mutated position(s). Phe was chosen because the shape and volume of its side chain are similar to those of a neutral His. Thus, it is the best amino acid to mimic a nonprotonable form of His. Among the six His residues of the T domain (Fig. 1), two pairs are located in loops. His223 between helices TH1 and TH2 is stacked with His257 located between helices TH3 and TH4. His322 and His323 are between helices TH7 and TH8. His321 and His372 are located in helices TH5 and TH9, respectively.

We studied by CD spectropolarimetry the secondary and tertiary structures of the mutant T domains at pH 7.5 at which the WT is in the native state (8, 15). The far-UV CD spectra of all
mutants (Fig. 2A and data not shown) were nearly identical to that of the WT T domain. They all indicated a secondary structure mainly composed of $\beta$-helices, in agreement with the crystal structure of the diphtheria toxin (6, 19). The near UV CD spectra of the mutants were also very similar to that of the WT T domain (Fig. 2C) (data not shown). They all exhibited a band at 292 nm attributed to a Trp constrained in an organized tertiary structure (8, 15). The spectra of the mutants containing the mutation at 251 had an additional negative band around 280 nm. His251 is positioned at about 4 Å of Tyr278 in the tertiary structure. Its replacement by a Phe, which may induce constraints on Tyr278, may explain the appearance of this band.

Then we studied by CD the secondary and tertiary structures of the mutant T domains at acidic pH at which the WT T domain is in the MG state (8, 15). Measurements were all done at pH 3.5, which was necessary for some of the mutants to complete their transition (see below for the fluorescence results). The far-UV CD spectra of all the mutants were similar to that of the WT T domain (Fig. 2B) (data not shown). They indicated that all of the proteins retained most of their helical secondary structure. Near-UV CD spectra showed that the signal at 292 nm disappeared, indicating a loss of the tertiary constraints on the Trp (Fig. 2D) (data not shown). As shown previously, this loss of tertiary structure combined with the persistence of the secondary structure corresponds to the MG state of the T domain (8, 15).

Overall, the data suggested that all mutants were folded in a native-like conformation at neutral pH and adopted an MG state at acidic pH, similar to that of the WT T domain.

His to Phe Mutations Shifted the Transition to the MG State toward Lower pH Values in an Additive Manner—The acid-induced conformational change of the mutant T domains was monitored by Trp fluorescence spectroscopy (Fig. 3). The results were compared with those obtained with the WT T domain (Fig. 3A, closed circles). As described previously (8, 15), the spectra of the WT T domain in solution showed a red shift of the maximum emission wavelength ($\lambda_{\text{max}}$) from 336 nm in the native state to 341 nm in the MG state. All mutants followed a transition toward higher $\lambda_{\text{max}}$. The end of the transition corresponded to the MG state, in agreement with the results of the
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FIGURE 3. Effect of His to Phe mutations on the transition of the T domain from the native state to the MG state monitored by Trp fluorescence. A, \( \lambda_{\text{max}} \) as a function of pH. Closed circles, WT; cross, T251; open triangle, T223-257; inverted open triangle, T322-323; open circles, TΔHis. B, pK_{1/2}^\text{MG} as a function of the number of mutations.

FIGURE 4. Effect of His to Phe mutations on the interaction of the T domain with anionic LUV as a function of pH. A–E, partition between the buffer and the LUV measured by ultracentrifugation (closed circles) and \( \lambda_{\text{max}} \) of Trp fluorescence (open circles) as a function of pH. The traces are the best fits to the experimental data according to the model applied (see “Experimental Procedures” and “Results”). F, pK_{1/2}^\text{MG} as a function of the number of mutations. All mutant T domains carrying the pair of mutations 322-323 as well as T322 and T323 are shown as squares. All other mutant T domains carrying the pair of mutations 223-257, as well as T223 and T257 are shown as circles. Triangle, T372; diamonds, T251 and T251-257. Mutations had a cumulative effect on membrane binding. However, mutation of the pair 322-323 (and of histidine 251) had an effect on membrane binding stronger than that of the pair 223-257, as shown by the correlation lines. Correlation coefficients were as follows: \( r = 0.95 \) for the squares; \( r = 0.38 \) for the circles.

CD experiments described above. However, this transition occurred at lower pH for practically all mutants to various extents (Fig. 3 and Table 1). In the case of TΔHis, in which all His residues were mutated, this transition was strongly altered with a shift of \( \lambda_{\text{max}} \) from 332 to 336 nm. This suggested that the Trp remained in a rather apolar environment despite the release of tertiary constraints revealed by the near UV CD spectra.

Mutations 223, 251, and 257 had the strongest effect on the formation of the MG state. The pH values of midtransition from the native to the MG state (pK_{1/2}^\text{MG}) found for the corresponding mutants were 4.8, 4.75, and 4.8, respectively, whereas the pK_{1/2}^\text{MG} was 5.3 for the WT T domain. Mutations 322 and 372 had moderate effects with pK_{1/2}^\text{MG} of 5 and 5.2, respectively. The pK_{1/2}^\text{MG} of the mutant T322 was the same as for the WT T domain.

The combination of mutations within the T domain had cumulative effects (Fig. 3 and Table 1). A correlation was observed between the number of mutations carried by the protein and the pK_{1/2}^\text{MG}, the maximum effect being observed for the mutant TΔHis with pK_{1/2}^\text{MG} = 3.8. However, as for the proteins bearing a single mutation, the addition of any of the mutations 223, 251, and 257 had an effect on the pK_{1/2}^\text{MG} stronger than that of the mutations 322, 323, and 372. Remarkably, the triple mutant T223-251-257 had a pK_{1/2}^\text{MG} similar to that of TΔHis.

These results indicated that the protonation of all of the His residues of the T domain were implicated in the formation of the MG state in solution, with a preponderant role for His^{223}, His^{251}, and His^{257}.

His to Phe Mutations Shifted Membrane Binding, but Not Membrane Penetration, toward Lower pH Values in an Additive Manner—We studied the interaction of the mutant T domains with anionic LUV as a function of pH. The interaction was monitored by centrifugation and Trp fluorescence. All mutants interacted with the membrane according to the two-step process found for the WT T domain (8, 9) (Fig. 4). A first transition corresponding to membrane binding was detected by centrifugation (Fig. 4, closed circles) and by a change of fluorescence \( \lambda_{\text{max}} \) (Fig. 4, open circles), from 336 to 344 nm for the WT T domain (Fig. 4A). A second transition corresponding to penetration into the bilayer was characterized by a decrease of \( \lambda_{\text{max}} \) (Fig. 4, open circles), from 344 to 332 nm for the WT T domain (Fig. 4A). LUV permeabilization was found to accompany membrane penetration (8), a property retained by the mutants (Fig. 5).

For almost all mutants, membrane binding appeared to be shifted to various extents toward lower pH values, whereas membrane penetration seemed not to be (Fig. 4 and Table 1). If this were the case, the alteration of the fluorescence transition pattern found for these mutants could be explained by the only shift of the first transition, which would mask the second tran-
sition. We designed a fitting model to evaluate the validity of this interpretation. For all mutants, we fitted the data of the second transition with the same parameters (pK and pH) taken from the WT T domain fitting curve (see “Experimental Procedures”). Then, for each mutant, we determined the value of these parameters for the first transition. These values (Table 1) reproduced well the experimental data (Fig. 4). Thus, the model strongly supported the observation suggesting that the mutations affected membrane binding but not membrane penetration. We propose that as soon as the membrane-bound state started to be populated (i.e. at a pH below 6), the second transition occurred simultaneously and reached the level of membrane penetration found for the WT T domain at this pH value. In other words, the membrane-bound state could not be observed in these steady-state experiments.

The shift of the pK_{1/2}^D (the pH of midtransition from the soluble to the membrane-bound state) was strongest for mutations 251, 322, and 323, with pK_{1/2}^D of 6.2, 6.1, and 6.2, respectively, whereas the pK_{1/2}^D of the WT T domain was 6.5 (Fig. 4F and Table 1). Mutations 223 and 372 led to shifts of pK_{1/2}^D of 6.3 and 6.4, respectively. Mutation 257 had no effect. Thus, the data clearly indicated that the protonation of His^{251}, His^{322}, and His^{323} was important for triggering the binding of the protein to the membrane.

The combination of mutations had cumulative effects (Fig. 4F and Table 1). Correlations were observed between the number of mutations carried by the protein and the pK_{1/2}^D, the maximum effect being observed for the mutant TΔHis with pK_{1/2}^D = 5.2. The proteins combining the mutations 322 and 323 (Fig. 4F, squares) or the mutation 251 (Fig. 4F, diamonds) exhibited a shift of pK_{1/2}^D stronger than that of proteins combining mutations 223 and 257 (Fig. 4F, circles) and 372 (Fig. 4F, triangle). As illustrated in Fig. 6, a correlation was found between the pK_{1/2}^D values for proteins carrying the mutations 322 and 323 (Fig. 6, squares). In contrast, the mutations 223 and 257, which had an effect on MG formation had no effect on membrane binding (Fig. 6, circles). This suggested that the protonation of these His residues was not required for membrane binding but rather for the stabilization of the MG state in solution.

Altogether, these results indicated that the protonation of the His residues of the T domain was involved in its binding to the membrane, with a preponderant role for His^{251}, His^{322}, and His^{323}. However, penetration into the membrane, revealed by the second fluorescence transition (Fig. 4) and by membrane permeabilization (Fig. 5), was mainly unaffected by the His mutations. Even TΔHis was able to permeabilize LUV.

**Effect of Mutations on MG State Formation and Membrane Binding Is Not Linked with a Change of Stability**—Two explanations may account for the effect of the mutations on MG state formation and membrane interaction. The protonation of His residues was critical to induce the conformational change, and/or the Phe substituting for the His increased the stability of the protein in the native state. To rule out these two possibilities, we studied the effect of the mutations on the equilibrium unfolding transition of the T domain in the native state (i.e. at pH 7.5).

The guanidinium hydrochloride-induced unfolding reaction of the T domain was monitored with Trp fluorescence (Fig. S1 and Table S1). For all mutants, an unfolding intermediate state was detected, as characterized previously for the WT T domain (15). The free energy values calculated from the first unfolding transition (ΔG_{01}) were used to compare the stability of the WT and mutant T domains. Besides T251, T251–257, and TΔHis, which appeared more stable than the WT T domain, the stability of the other mutants was unchanged or diminished (Table S1). We plotted the ΔG_{01} of each mutant against its pK_{1/2}^{MG} and
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pK_a (Fig. S2). No correlation was found linking stability and pK_a values (a similar absence of correlation was found using ΔG_{θ1} + ΔG_{θ2}). For instance, the decrease of pK_a of T223–257 was not a consequence of the stabilization of the native state, the mutant being less stable than the WT T domain. This lack of correlation suggested that the effect of mutations was mainly due to the loss of protonable residue(s) rather than to a stabilizing effect of the mutation(s). This is less clear, however, for His^{251}.

**DISCUSSION**

All His Residues of the T Domain Act as pH-sensitive Triggers, with Various Sensitivities—We studied the relative implication of the His residues of the diphtheria toxin T domain in the various steps by which it changes its conformation and interacts with the membrane as pH decreases. Our aim was to determine the involvement of His protonation in triggering these conformational changes, which are expected to occur in the endosome during cell intoxication. His residues were mutated to Phe in order to mimic a nonprotonable form of His. These mutations could also affect the stability of the protein, but guanidinium hydrochloride-induced unfolding experiments showed this was not the case, except perhaps for His^{251} (Figs. S1 and S2 and Table S1).

The major finding of this study is that the protonation of the His residues of the T domain participates in the pH-dependent transitions, leading to MG state formation in solution and to membrane binding. Although the accumulation of mutations shifted the transitions toward more acidic pH (Figs. 3B and 4F), the protonation of the remaining His residues or of acidic residues (Asp and Glu) could still trigger the conformational changes. In other words, all His residues are involved in a cooperative manner, but none is indispensable. Only the change of all His residues strongly shifts the pH of formation of the MG state and the capacity of the T domain to bind membranes. However, the importance of the protonation of each His side chain is different, depending on the step investigated (Fig. 7).

We found that the pair His^{223}–His^{257} and His^{251} are the most sensitive triggers for the formation of the MG state (Figs. 3, 6, and 7), whereas the pair His^{322}–His^{323} and His^{251} are the most sensitive triggers for membrane binding (Figs. 4, 6, and 7).

Mechanisms of Tertiary Structure Destabilization by Protonation of His Residues—The structure of the diphtheria toxin (6, 19) shows that at neutral pH, the side chain of the His residues of the T domain are partially buried within the protein. These side chains are neutral, slightly polar. The gain of a positive charge by protonation induces a strong gain of polarity. Hence, maintaining this side chain in the rather apolar environment of the protein becomes energetically unfavorable. This should induce the solvation of the protonated His together with local destabilization of the protein. In addition, some His side chains may be involved in hydrogen bond networks. This is the case for His^{257}, which shares hydrogen bonds with Glu^{259} and Ser^{219}. Disruption of hydrogen bond networks by protonation of the His side chain should also destabilize the protein.

Regional Role of His Protonation—The examination of the position of each His residue in the structure of the T domain (Fig. 1) may help to propose a functional role for each protonation event. Four His residues are arranged in two pairs: His^{223} and His^{257} are stacked together and located between helices TH1 and TH2 and between TH3 and TH4, respectively; His^{322} and His^{323} are vicinal and located in a loop forming a hinge between helices TH7 and TH8. In each case, the mutation of one or the other or both residues had almost the same effect (Fig. 3B for His^{223}_–His^{257} and Fig. 4F for His^{322}_–His^{323}). This suggests that each pair constitutes a functional unit, in which protonation (of one or both residues) plays a regional role (Fig. 7, native state).

The protonation of the pair His^{223}–His^{257} should destabilize the N-terminal region by disruption of the group of helices TH1–TH4. We found indeed that this pair is important for MG formation (Figs. 3 and 7). A similar mechanism involving His pair protonation has been proposed for the formation of the MG state of apomyoglobin (13). Moreover, London and coworkers (20) recently showed that the formation of the MG state of the T domain is characterized by a profound conformational rearrangement of its N-terminal region. The protonation of the pair His^{322}–His^{323} (and to a lesser extent His^{327}) should destabilize the C-terminal hydrophobic region, causing a partial disconnection of the layers of helices TH5–TH7 and TH8–TH9, enabling their partition from the solvent to the membrane. We found indeed that these residues are important for membrane binding (Figs. 4 and 7), which involves hydrophobic effects between the C-terminal region of the protein and the membrane (8, 9). Finally, the protonation of His^{251} located on helix TH3, facing helix TH5, should destabilize the central region of the protein by disconnecting the groups of helices TH1–TH4 and TH5–TH7. We found indeed that His^{251} is important for MG formation and membrane binding (Figs. 3, 4, and 7).
From our data, we propose a model describing the role of His protonation in inducing the interaction of the T domain with the membrane (Fig. 7). The protonation of His$^{322}$–His$^{323}$ and His$^{251}$ induces a destabilization of the tertiary structure. This gain of flexibility would favor membrane binding of the T domain, mainly via hydrophobic effects, involving its C-terminal part (9). The protonation of His$^{225}$–His$^{257}$ would be required to release the remaining local forces packing the N-terminal helices. Finally, a combination of hydrophobic and attractive electrostatic interactions between the lipid bilayer and the N-terminal region of the T domain allows its conformational reorganization and membrane permeabilization (8, 9).

His Residues as Triggers of Protein Conformational Changes at Physiological pH Values—When all six His residues were mutated, the $pK_{\text{1/2}}$ MG and $pK_{\text{1/2}}$ B dropped to pH 3.8 and 5.2, respectively (Figs. 3B and 4F). This suggests that the protonation of Glu and Asp residues may partially take over for the missing His residue(s) at lower pH. Hence, besides the particular behavior of His upon protonation (switching from a neutral, slightly polar, to a charged polar side chain), the disruption by protonation of the electrostatic network, involving Glu and Asp residues, may also contribute to the structural rearrangements of the T domain. Nevertheless, we propose that the selection of a number of His residues rather than Glu and Asp, located in hinges of the protein, enables the T domain to function at a rather mild pH, such as the pH of the endosome, contributing to toxin efficacy (21).

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