Modulation of botulinum neurotoxin A catalytic domain stability by tyrosine phosphorylation

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Abstract Botulinum neurotoxin A (BoNT A) is a substrate of the Src family of tyrosine kinases. Here, we report that the BoNT A light chain (LC) is phosphorylated in the tyrosine-71 located at N-terminus. Covalent modification of this residue notably increases the thermal stability of the endopeptidase activity, without affecting its catalytic efficacy. Similarly, mutation of this residue specifically affected the protein stability but not its endopeptidase function. Fusion of the Tat-translocating domain to the N-terminus of the enzyme produced a cell permeable, functional enzyme, as evidenced by immunocytochemistry and by the cleavage of cytosolic SNAP25 in intact PC12 cells. Noteworthy, truncation of cellular SNAP25 was reduced in cells when the Src kinase activity was inhibited with a specific antagonist, implying that tyrosine phosphorylation of BoNT A LC modulates the in vivo proteolytic activity of the neurotoxin. Taken together, these findings substantiate the tenet that tyrosine phosphorylation of BoNT A LC could be an important modulatory strategy of the neurotoxin stability and suggest that the phosphorylated neurotoxin may be a relevant molecule in vivo.

Keywords: Clostridial; Exocytosis; Protease; Neurotransmission; SNAP25; Excitability

1. Introduction

Botulinum neurotoxins (BoNTs) are a family of substrate-specific, Zn^{2+}-dependent endopeptidases that abrogate regulated exocytosis [1]. These potent neurotoxins target the key molecular components of the SNARE complex, thus impeding the fusion of neurotransmitter-loaded vesicles with the presynaptic membrane. For instance, BoNT A, C and E truncate SNAP25, while BoNT B, D, F and G cleave VAMP2, and BoNT C also proteolyzes syntaxin [1,2]. Biochemically, BoNTs are proteins composed of a heavy chain (HC) of 100 kDa, and a light chain (LC) of 50 kDa that contains the catalytic domain that truncates the SNARE proteins [1,2].

We previously demonstrated that both the HC and the LC of native BoNTs are substrates of the Src tyrosine-specific protein kinase [3]. Tyrosine phosphorylation of the BoNT A LC occurred in vitro as well as in vivo. Covalent modification of native BoNT A LC gave rise to prominent augmentation of their catalytic efficacy and thermal stability, which correlated with a disorder-to-order transition promoted by phosphorylation [3,4]. Similar results were obtained for native BoNT E [3,4]. Surprisingly, however, covalent modification of the recombinant BoNT E LC resulted in a modest increase in the protein thermal stability without altering its catalytic efficacy [5], implying differences between the native and recombinant proteins. This apparent discrepancy led to the notion that stimulation of the protease function may be due, at least partly, to enhancement of LC dissociation from the holotoxin, rather than direct modulation of the catalytic activity [5]. Nonetheless, additional studies are necessary to understand these aspects of BoNTs modulation by tyrosine phosphorylation.

Here, we have used recombinant, purified His-BoNT A LC to further understand the role of tyrosine phosphorylation modulating the protein structure–function relationships. We report that His-BoNT A LC is phosphorylated by the Src quinase at Y71, a tyrosine residue located at the N-terminus of the neurotoxin. Tyrosine phosphorylation of His-BoNT A LC enhanced its thermal stability without altering its catalytic efficacy, akin to the modulation exerted in the recombinant catalytic domain of BoNT E [5]. Mutation of Y71 to phenylalanine produced a mutant protein that was stable and virtually identical to the non-phosphorylated form of the neurotoxin. Replacement of Y71 by tryptophan, however, decreased the thermal stability of the protein without affecting its endopeptidase activity. We also report that inhibition of Src activity in PC12 cells notably reduced His-BoNT A LC-mediated truncation of SNAP25. Therefore, these results imply that tyrosine phosphorylation of the neurotoxin by Src plays a role in modulating the stability of the protein in vitro and in vivo.

2. Materials and methods

2.1. Cloning, mutagenesis, expression and purification of recombinant His-BoNT A LC species and SNAP25

The full-length of His-BoNT A LC (strain 62A) cloned into the pQE3 vector was kindly provided by Dr. Blasi [6,7]. This cDNA was inserted into the pTat-HA plasmid (kindly provided by Dr. Dowdy) to obtain the Tat-His-BoNT A LC. Site-specific mutagenesis was carried out by PCR amplification as described [5]. Mutants were confirmed by DNA sequencing.

His-BoNT A LC species were affinity-purified on Ni–NTA columns (Qiagen) as described [5]. Briefly, His-BoNT A LC was expressed in the *Escherichia coli* strain M15pREP4 and protein expression was induced with 1 mM Isopropyl-β-thiogalactoside (IPTG) at 30 °C for 5 h. Bacteria were collected by centrifugation, resuspended in 10 mM phosphate, pH 7.4, 140 mM NaCl, and 3 mM KCl (PBS), digested with

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0.1 mg/ml lysozyme (10 min at 23 °C) supplemented with 10 mM imidazole, and 2 mM phenylmethylsulfonylfluoride, 5 mM iodoacetamide, 2 mM EDTA, and sonicated in a Branson Sonifier Cell Disruptor 250 at 4 °C. Bacterial extracts were clarified by centrifugation and incubated with Ni–NTA resin (1 ml/1 L culture) overnight at 4 °C. His-tagged BoNT A LC was extensively washed with PBS/imidazole (20 mM) and eluted from the column with PBS, pH 7.0, supplemented with 100 mM imidazol. Tat-His-BoNT A LC was eluted with 500 mM imidazole. Protein eluates were dialyzed against 20 mM Tris, pH 7.0, and 100 mM NaCl for 24 h at 4 °C. Purity of the protein was evaluated by SDS–PAGE and protein concentration was determined with the BCA kit (Pierce).

2.2. Expression and purification of SNAP25

Recombinant GST-SNAP25 was expressed in *E. coli* strain BL21(DE3) and affinity-purified on glutathione–agarose columns as described in detail elsewhere [5,8]. Resin-bound GST-SNAP25 was released by digestion with thrombin protease for 5 h at 23 °C. SNAP25 was dialyzed into 20 mM HEPES, pH 7.4, 80 mM KCl for 24 h at 4 °C. Protein concentration was assayed with the BCA kit (Pierce) and purity by SDS–PAGE analysis.

2.3. Phosphorylation of His-BoNT A LC

Src-mediated phosphorylation of His-BoNT A LC species was performed with recombinant Src kinase (Upstate Biotech. Inc.) as previously described [3–5]. Briefly, 0.5 μM His-BoNT A LC was incubated with 3 U of Src in 20 mM HEPES, pH 7.4, 20 mM $MgCl_2$, 1 mM EGTA, 2 mM EDTA and 0.5 mM ATP at 30 °C for the indicated times. Tyrosine phosphorylation was evaluated by Western immunoblot with an anti-phosphotyrosine monoclonal antibody (Sigma) [3,5].

2.4. Endopeptidase activity of His-BoNT A LC

The proteolytic activity was assayed in vitro using 6 μM SNAP25 as described [3,5]. Briefly, cleavage assays (20 μl) were performed in 20 mM HEPES, pH 7.4, 2 mM DTT, 10 μM $Zn(CH_3COO)_2$ at 30 °C for the indicated times. The extent of SNAP25 cleavage was evaluated by native PAGE. Gels were digitized and quantified as reported [3,5].

2.5. Circular dichroism spectroscopy

Circular dichroism (CD) was carried out in a JASCO J-810 Spectropolarimeter equipped with a computer-controlled temperature cuvette holder. CD data for the Far-UV CD spectra (region 195–260 nm) were recorded with a 1 mm path length cell containing 4 μM protein in 20 mM Tris–HCl, pH 7.0. All spectra were recorded at 25 °C and at 50 nm/min (response time of 1 s), averaged (5 scans), and corrected for the buffer contribution. CD signals (in mdegrees) were converted to mean ellipticity (θ, mdegrees cm^2 $dmol^{-1}$) [5]. Secondary structure elements were inferred by fitting the CD spectra as described [5,8,9]. Thermal denaturation was accomplished by recording the CD spectra at increasing temperatures of 20–80 °C.

2.6. Cell culture and immunocytochemistry

PC12 cells were cultured in DMEM plus 10% horse serum and 5% bovine serum as described [3]. Cell cultures were incubated with 300 nM Tat-His-BoNT A LC or His-BoNT A LC for 3 h at 37 °C. For immunocytochemistry, PC12 were extensively washed with cold PBS, fixed, blocked, permeabilized and, subsequently, incubated with anti-His monoclonal antibody (mAb) (1:2000) (Sigma) [9,10]. After washing, they were incubated with Alexa 488-coupled secondary antibody and propidium iodide, washed, embedded and analyzed by confocal microscopy (Leica TCS) [10].

The extent of cellular SNAP25 cleavage was determined in whole cell extracts of control and neurotoxin-intoxicated PC12 cells by Western immunoblots using an anti-SNAP25 antibody (Alomone Lab) [3].

3. Results

To study the functional and structural role of tyrosine phosphorylation of the BoNT A catalytic domain, we expressed and purified His-tagged BoNT A LC. IPTG induced the synthesis of a soluble protein with an apparent mobility of 58 kDa that was specifically retained on a Ni–NTA affinity chromatography column (Fig. 1A). The His-BoNT A LC was eluted with 100 mM imidazole (Fig. 1A, lane 5) and confirmed by Western immunoblotting using an anti-His mAb (data not shown). This experimental paradigm produced 7 mg protein/L of bacterial cultures of a fairly homogeneous ($\geqslant 90\%$) His-BoNT A LC fusion protein.

We next determined if the purified His-BoNT A LC exhibited proteolytic activity. For this task, we used recombinantly produced SNAP25 as a substrate [8]. Incubation of the substrate with increasing concentrations of the His-BoNT A LC at 30 °C for 60 min resulted in complete cleavage of SNAP25 at [His-BoNT A LC] $\geqslant$ 5 nM (Fig. 1B, top). The estimated concentration of recombinant neurotoxin needed to cleave half of the maximal substrate concentration (EC_{50}) was 1.8 ± 0.3 nM (Fig. 1C), which is significantly higher than that reported for recombinant BoNT E LC [5]. Most surprisingly, analysis of the thermal stability of the enzymatic activity revealed that pre-incubation of 20 nM His-BoNT A LC at 30 °C for 60 min, before adding SNAP25, caused complete inactivation (Fig. 1B, bottom). This result is consistent with the previously reported thermosensitivity of the catalytic activity of the native neurotoxin that was abrogated by pre-incubation at 37 °C [3]. Taken together, these observations suggest that the enzymatic activity of the BoNT A LC is thermally sensitive.

To investigate whether the thermal instability is due to the presence of unfolded protein, the structural characteristics of the His-BoNT A LC were studied by CD in the far-UV (Fig. 1D). The shape of the CD spectrum exhibits a double negative maxima at 207 and 222 nm, and a positive maximum around 190 nm, consistent with the presence of an α-helical secondary structure. Indeed, the apparent secondary structures inferred from the curve-fitting of the CD spectra were 15% α-helix and 25% β-pleated sheets. CD spectra are quite sensitive to structural changes caused by denaturating heat, thus providing an operational assay to study the protein thermostability. Specifically, we studied the variation of the θ_{222} as a function of the temperature (Fig. 1D, inset). The experimental data can be readily described by a sigmoidal curve whose inflexion point corresponds to the denaturating temperature, i.e., 47.3 ± 0.7 °C. This value is remarkably higher than that obtained for the catalytic activity (Fig. 1C), suggesting that the temperature-induced loss of function is not related to profound structural changes in the recombinant protein, but rather may be due to subtle conformational changes in the enzyme active site.

Tyrosine phosphorylation of clostridial BoNT A modulates its catalytic activity and thermal stability [3,4]. Thus, we evaluated whether the recombinant His-BoNT A LC is a substrate of the tyrosine kinase Src. As illustrated in Fig. 2A, incubation of His-BoNT A LC with recombinant Src at 30 °C resulted in a time-dependent phosphorylation with $t_{1/2} \approx 10$ min and saturation at 60 min. No phosphorylation of His-BoNT A LC was observed when the ATP was omitted from the reaction (data not shown). To investigate the effect of the phosphorylation on the catalytic efficacy, the recombinant neurotoxin was phosphorylated with Src at 30 °C for 60 min, and thereafter the enzymatic efficacy of the phosphorylated and unphosphorylated proteins was assessed in vitro using recombinant SNAP25. Unphosphorylated samples refer to His-BoNT A

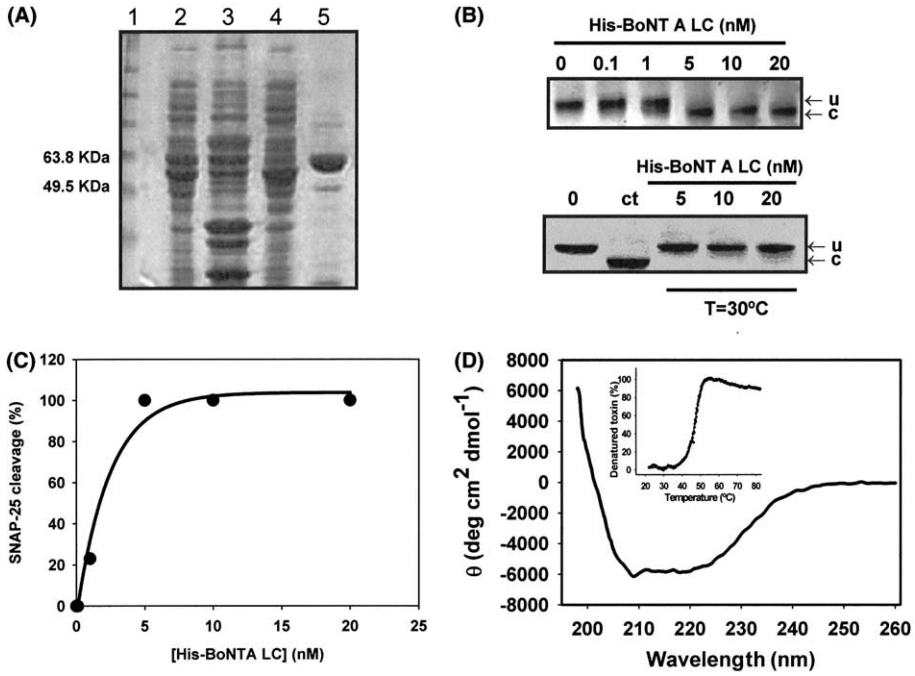

Fig. 1. (A) Purification of BoNT A LC on a Ni^{2+}–NTA affinity chromatography. Protein fractions were separated on 10% Tris–glycine SDS–PAGE gels and stained with Coomassie blue. Lane 1. Molecular weight markers; Lane 2. Induced bacterial culture; Lane 3. Soluble fraction; Lane 4, Flow through Ni–NTA resin; Lane 5, 100 mM imidazole eluted protein. (B) (Top) His-BoNT A LC is an active endoprotease that cleaves SNAP25. (Bottom) Pre-incubation of His-BoNT A LC at 30 °C for 60 min abolishes endopeptidase activity. ct, denotes 5 nM His-BoNT A LC; **u** and **c** correspond to uncleaved and cleaved SNAP-25, respectively. (C) Quantification of the percentage of SNAP25 cleavage as a function of the His-BoNT A LC. Native PAGE gels were air-dried, digitalized and band quantified as described [5]. Solid lines depict the best fit to a Michaelis–Menten binding isotherm. The estimated EC_{50} was 1.8 ± 0.3 nM. Enzymatic reactions were performed with 6 μM SNAP25 at 30 °C for 1 h. (D) CD spectrum of 4 μM His-BoNT A LC at 16 °C (inset). Thermal denaturation of His-BoNT A LC, as determined from analysis of the CD spectra at increasing temperatures [5]. Solid line depicts the best fit to a sigmoidal curve. Denaturating temperature was 47.3 ± 0.7 °C.

LC incubated with the Src kinase at 30 °C for 60 min in the absence of ATP. As expected, unphosphorylated enzyme was not active even at 20 nM, due to thermal inactivation of the catalytic activity after 60 min incubation at 30 °C (Fig. 2B, inset). In marked contrast, phosphorylated His-BoNT A LC virtually cleaved all SNAP25 at concentrations $\geqslant$ 2 nM. A dose–response relationship provided an EC_{50} of 1.3 ± 0.3 nM for the phosphorylated enzyme. Therefore, these data suggest that tyrosine phosphorylation of the recombinant catalytic domain prevents or delays thermal enzymatic inactivation, but it does not affect the catalytic efficacy of the enzyme. Next, we attempted to study the phosphorylation-induced structural changes in the BoNT A LC by CD. However, the phosphorylated neurotoxin aggregated and precipitated out at concentrations $\geqslant$ 1 μM, thus precluding the conformational analysis.

Tyrosine phosphorylation appears conserved among all clostridial neurotoxins, suggesting the presence of a conserved phosphoacceptor site [3,5]. In the BoNT E LC, the phosphorylation site was mapped at Y67 [5], which corresponds to Y71 in BoNT A LC. Indeed, mutation of Y71 to F (Y71F) fully abolished Src-mediated tyrosine phosphorylation of the His-BoNT A LC (Fig. 2C, inset), thus demonstrating that Y71 is the only phosphate acceptor site. The Y71F mutant was stable and exhibited functional and structural properties that were virtually identical to those characteristic of the wild type protein, namely an EC_{50} of 1.8 nM for cleaving SNAP25, a 15% content in α-helical elements and a denaturating temperature of 48 ± 0.2 °C (Fig. 2D and E). Replacement of Y71 by W (Y71W) yielded a protein that showed unaltered catalytic activity (EC_{50} = 1.6 nM) (Fig. 2C) and denaturated at lower temperature (42 ± 0.5 °C) (Fig. 2D and E). Incorporation of a negatively charged amino acid at position Y71 (Y71E) resulted in an unstable protein that aggregated and accumulated in bacterial inclusion bodies, preventing their functional purification and characterization. Taken together, these results imply that the Y71 site is an important determinant of the protease stability, but plays a marginal role in the protein catalytic activity.

We have previously shown that BoNT A LC is tyrosine-phosphorylated in PC12 cells [3]. Thus, we hypothesized that cellular tyrosine kinase activity may modulate the function of recombinant BoNT A LC. To address this issue, we used the fusion Tat-His-BoNT A LC protein that incorporated the Tat translocation sequence at the N-end of the neurotoxin catalytic domain [11]. Tat-His-BoNT A LC was expressed in bacteria and purified by affinity chromatography with Ni–NTA (Fig. 3A). Fusion of the Tat domain did not affect the in vitro enzymatic activity of the protein, as evidenced by the virtually identical efficacy cleaving SNAP25 of Tat-His-BoNT A LC as that exhibited by the His-BoNT A LC (Fig. 3B). Next, we investigated whether the Tat-His-BoNT A LC translocated through plasma membrane of PC12 cells. For this task, cell cultures were incubated with 300 nM of the protein at 37 °C for 3h. The intracellular localization of the protein was evaluated by immunocytochemistry using an anti-His mAb. As shown in Fig. 3C, PC12 cells incubated

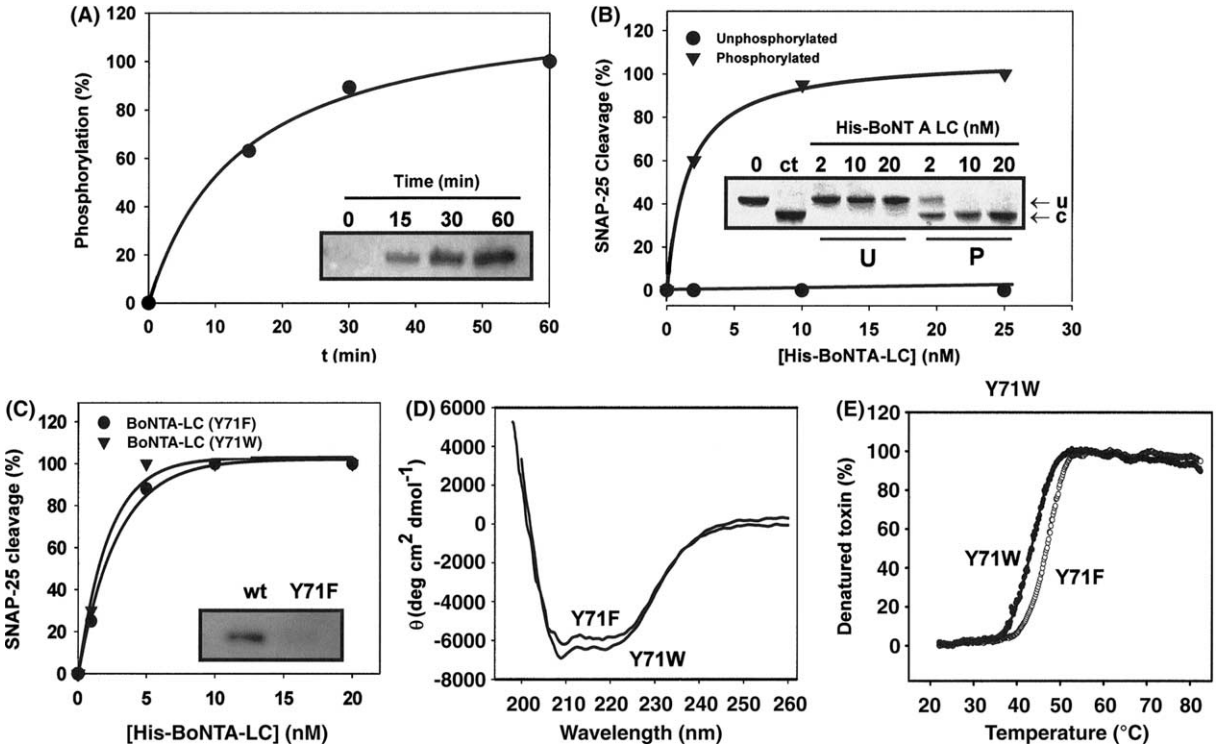

Fig. 2. (A) Src kinases phosphorylate His-BoNT A LC. Tyrosine phosphorylation reaction was carried out using 3 U of Src kinase and 10 nM of BoNTA-LC at 30 °C, and determined by Western inmunoblotting using an anti-phosphotyrosine antibody. Protein bands were visualized with the ECL. Protein immunoblots were digitized and band quantified as described [5,10]. (B) Endopeptidase activity as a function of the concentration of unphosphorylated (U) and tyrosine phosphorylated (P) His-BoNT A LC. The neurotoxin was phosphorylated for 60 min at 30 °C. The catalytic activity was assayed on 6 μM SNAP25 at 30 °C for 60 min, at the indicated enzyme concentrations. The extent of SNAP25 cleavage was analyzed native PAGE electrophoresis as described in the legend of Fig. 1. **u** and **c** correspond to uncleaved and cleaved SNAP-25, respectively. Solid lines depict the best fit to a Michaelis–Menten binding isotherm. The estimated EC_{50} for the phosphorylated His-BoNT A LC was 1.3 ± 0.3 nM. (C) Mutation of Y71 to F abolishes tyrosine phosphorylation of His-BoNT A LC (inset). Catalytic activity of Y71F and Y71W mutants of His-BoNT A LC. The calculated EC_{50} were 1.6 ± 0.5 nM and 1.8 ± 0.4 nM for Y71F and Y71W, respectively. (D) CD spectra of Y71F and Y71F mutant proteins. (E) Thermal denaturation of Y71F and Y71F mutant enzymes determined from analysis of the CD spectra at increasing temperatures. Solid lines depict the best fit to a sigmoidal curve. Denaturating temperatures were 480.2 °C for Y71F and 42 ± 0.5 °C for Y71W. Protein concentration was 4 μM.

with the Tat-His-BoNT A LC exhibited a conspicuous intracellular immunostaining, indicating the presence of the protein (Fig. 3C, top, left). Note that, as expected, the Tat-tagged protein accumulated primarily in the nucleus of the cells (Fig. 3C, top, right). In contrast, cells exposed to 300 nM His-BoNT A LC did not show an immunocytochemical labeling (Fig. 3C, Bottom). To further demonstrate the translocation of the Tat-His-BoNT A LC into intact PC12 cells, we evaluated the integrity of cellular SNAP25 by Western immunoblotting using an anti-SNAP25 antibody. Fig. 3D shows that the SNAP25 of PC12 cells exposed to Tat-His-BoNT A LC was $\geqslant$ 50% cleaved, while that of cultures incubated with His-BoNT A LC remained intact. Note that SNAP25 cleavage exerted by Tat-His-BoNT A LC in intact cells was comparable to that elicited by the native neurotoxin [3]. Collectively, these results indicate that the Tat-His-BoNT A LC is a functional enzyme that translocates through the plasma membrane of PC12 cells.

To evaluate the role of tyrosine kinase in the in vivo activity of BoNT A LC, we studied the cleavage activity of the Tat-His-BoNT A LC in cells exposed to PP2 (4-amino-5-[4-chlorophenyl]-7-[t-butyl]pyrazolo[3]pyrimidine), a specific inhibitor of the Src family of tyrosine kinases [12]. Incubation of PC12 cells with 10 nM PP2 notably affected the level of tyrosine phosphorylated proteins, as revealed by Western immunoblot of PC12 whole extracts probed with an anti-phosphotyrosine mAb (data not shown). Noteworthy, the presence of the Src inhibitor reduced the extent of BoNT A-mediated, SNAP25 cleavage by $\geqslant$ 50% (Fig. 3E and F). Therefore, this finding is consistent with the tenet that tyrosine phosphorylation of BoNT A LC modulates its in vivo functionality.

4. Discussion

The salient contribution of this study is that phosphorylation of BoNT A LC notably enhances its thermal stability, akin to the observations reported for the recombinant BoNT E LC [5]. In addition, they corroborate that tyrosine phosphorylation does not modulate the catalytic activity of the recombinant protein. Thus, the proposed enhancement of catalytic efficacy exerted by tyrosine phosphorylation on the native neurotoxin may rather arise from a facilitation of the dissociation of the BoNT A LC form holotoxin and/or stabilization of the separated catalytic domain [3]. As for the holotoxin and the recombinant BoNT E LC, the higher thermostability of the BoNT A LC activity may be due to a

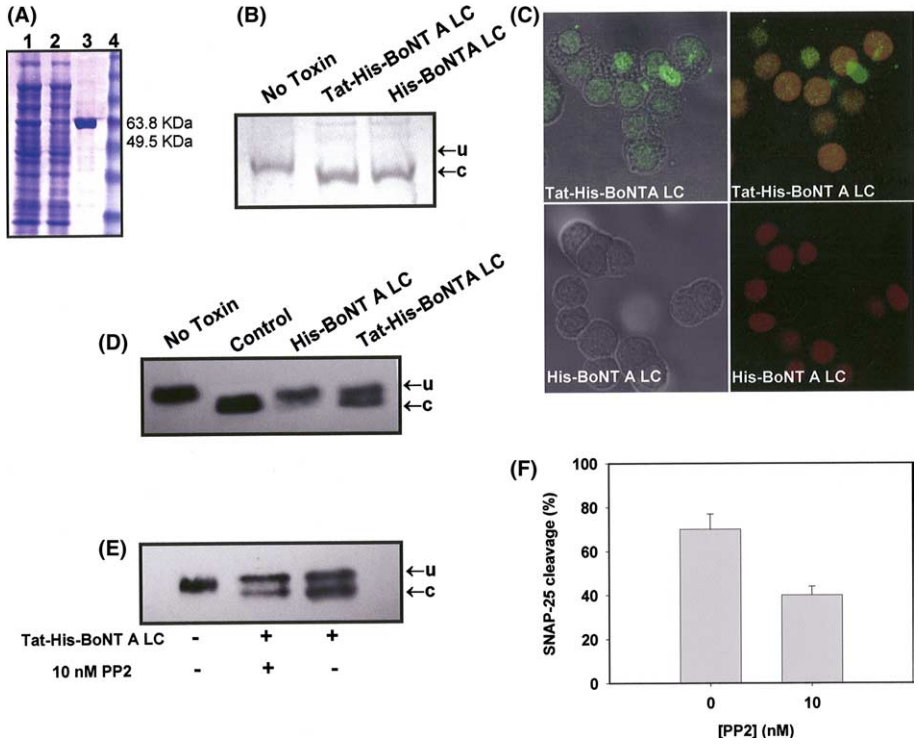

Fig. 3. (A) Tat-His-BoNTA LC was purified by affinity chromatography. Lane 1: soluble fraction; lane 2: flow through Ni–NTA resin; lane 3: protein eluted with 500 mM imidazole; lane 4: molecular weight markers. (B) Tat-His-BoNT A LC is an active endoprotease that cleaves SNAP25 in vitro. Enzymatic reactions were carried out with 6 μM SNAP 25 and 50 nM of endopeptidases at 30 °C for 1 h as described. **u** and **c** correspond to uncleaved and cleaved SNAP-25, respectively. (C) Intracellular localization of Tat-His-BoNT A LC in PC12 cells. Neurotoxin-exposed cell cultures, fixed, detergent-permeabilized and probed with anti-His mAb. Immunocomplexes were visualized with Alexa 488-coupled anti-mouse as secondary antibody (left panels). The cell nucleus was stained with propidium iodide and images overlapped with those of anti-His (right panels). (D) Cleavage of cellular SNAP25 with Tat-His-BoNT A LC in intact PC12 cells. *Control*, denotes the protease activity of 100 nM Tat-His-BoNT A LC in whole cell extracts. SNAP-25 cleavage was determined by immunoblotting using an anti-SNAP25 antibody. (E) Inhibition of Src kinase reduces SNAP 25 cleavage by Tat-His-BoNT A LC. PC-12 cells were exposed to Tat-His-BoNT A LC in the absence or presence of 10 nM PP2. Cell cultures were washed and detergent-permeabilized to determine the extent of SNAP25 cleavage. (F) Quantification of PP2 inhibitory effect of SNAP25 truncation by Tat-His-BoNT A LC. Cell cultures were incubated with 300 nM of Tat-His-BoNTA LC or His-BoNT A LC at 37 °C for 3 h, washed with PBS, before detergent permeabilization.

disorder-to-order transition in the protein structure [4,5]. However, the remarkable tendency of the phosphorylated protein to aggregate at $\geqslant 1$ μM prevented the structural analysis.

Our results substantiate that a single, evolutionary conserved tyrosine residue (Y71 in BoNT A LC) at the N-terminus of BoNTs is the phosphate acceptor site [5,13]. The three dimensional structure of the native and recombinant BoNT A LCs, as well as that of BoNT B LC, shows that the spatial location of this residue is compatible with the impact of its covalent modification on the enzyme properties [14–17]. As depicted in Fig. 4A, the aromatic ring of residue Y71 is located in a small hydrophobic pocket, while its hydroxyl group is solvent-exposed and accessible to the Src kinase. Replacement of Y71 by its phosphorylated counterpart in the BoNT A LC structure shows the occurrence of a strong hydrogen bond interaction of the phosphotyrosine with R374 and with the carbonyl group of L415 at the C-end (Fig. 4B). As a result, the compactness of the proteins appears to increase, a notion consistent with the higher thermostability of the phosphorylated protein [4,5]. Notice that a W at Y71 does not fit into the small cavity that accommodates the residue (Fig. 4C) and that mutation Y71E incorporates a charged residue into this hydrophobic pocket (Fig. 4D). These unfavorable conditions explain the destabilizing effects of these two mutations. Indeed, the estimated variations of free energy of the folded state with respect to wild type endopeptidase ($\Delta\Delta G$) are 2.2 kcal/mol and 3.1 kcal/mol for Y71W and Y71E mutants (calculated with Fold-X). Although these interpretations appear reasonable, the precise structural modulation exerted by phosphorylation will have to be addressed by determining the structure of the phosphorylated enzyme.

We previously demonstrated that BoNT A LC is tyrosine phosphorylated in PC12 cells, implying that this convalent modification may play a role in the biological function of the protein [3]. Here, we have shown that incubation of Tat-BoNT A LC intoxicated cells with a specific, cell-permeable inhibitor of the Src kinase significantly decreased the extent of SNAP25 cleavage, thus suggesting that the phosphorylated enzyme contributes to the in vivo cleavage of SNAP25. Since the BoNT A LC translocates unfolded through the HC [18], it is tempting to hypothesize that phosphorylation-induced conformational changes may contribute to stabilization of the re-folded protein. Collectively, all our findings lend further support to the tenet that phosphorylation may contribute to the in vivo biological behavior of BoNT A. Furthermore, they suggest that acute inhibition of the Src family of tyrosine kinases may be a therapeutic strategy for treating botulinum poisoning. Additional studies are needed to precisely unveil the physiological

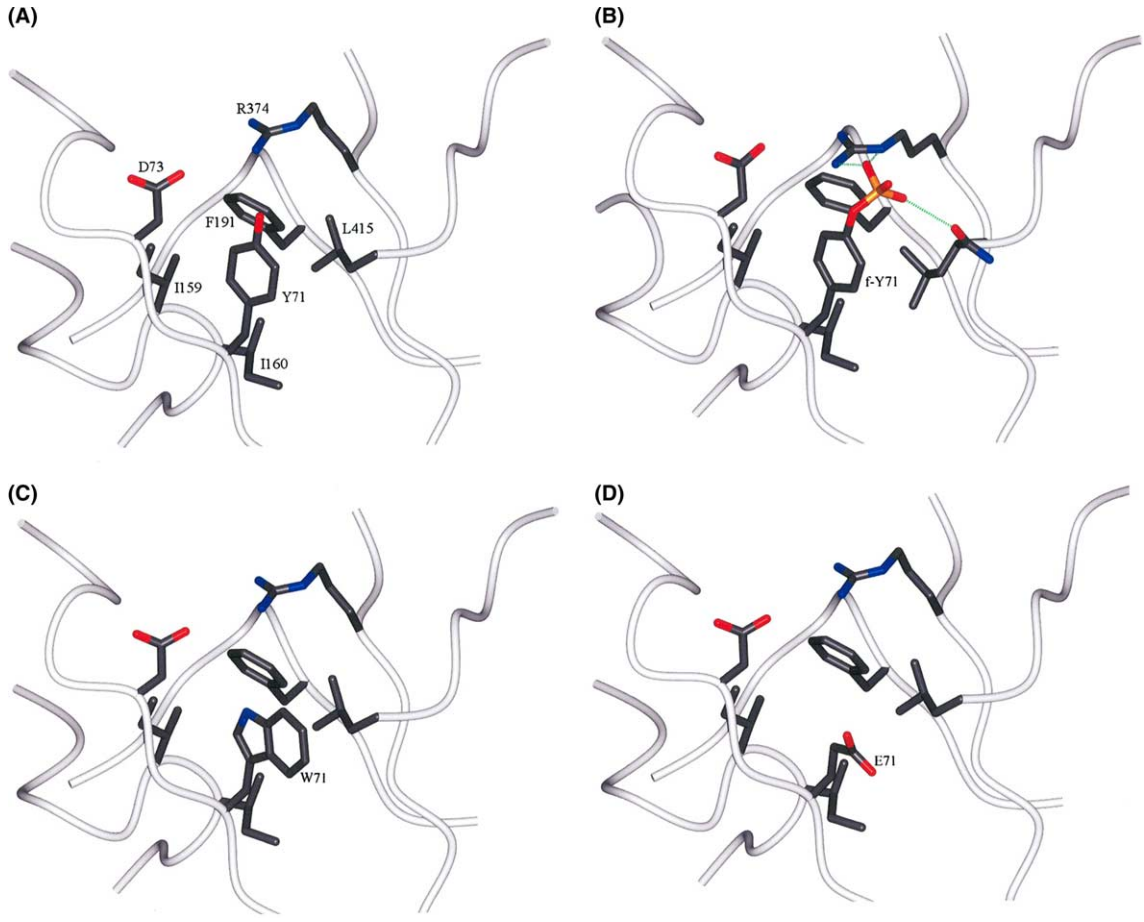

Fig. 4. (A) Residue Y71 is located in a small hydrophobic cavity, but it has its hydroxyl group exposed and accessible to the Src kinase. (B) Phosphorylation of Y71 strengthens the interaction with R474 and L415, inducing a conformation change that increases the compactness of the protein. (C) A tryptophan does not fit into the cavity, thus distorting the structure. (D) A glutamic acid would lie into a hydrophobic pocket, thus destabilizing the folded protein. The structure of the BoNT A LC as used as a scaffold (PDB 1E1H) [14]. The free energy of the folded state of wild type and mutants was calculated with Fold-X (http://fold-x.embl.de). Oxygens are showed in red, nitrogens in blue, and phosphate in yellow.

role of the in vivo phosphorylation of BoNT A LC. It did not escape from our attention that the Tat-BoNT A LC fusion protein or more stable species could be enzymes with improved clinical utility. Indeed, at variance with the high molecular complexity of the current clinical preparations [19], the recombinant catalytic domain might provide a rather homogeneous therapeutic formulation.

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References

[1] Simpson, L.L. (2004) Identification of the major steps in botulinum toxin action. Annu. Rev. Pharmacol. Toxicol. 44, 167–193.
[2] Rosetto, O., Seveso, M., Caccin, P. and Montecucco, C. (2002) Botulinum neurotoxins are metalloproteases specific for SNARE proteins involved in neuroexocytosis. Curr. Probl. Dermatol. 30, 117–125.
[3] Ferrer-Montiel, A.V., Canaves, J.M., Dasgupta, B.R., Wilson, M.C. and Montal, M. (1996) Tyrosine phosphorylation modulates the activity of clostridial neurotoxins. J. Biol. Chem. 271, 18322–18325.
[4] Encinar, J.A., Fernádez, A., Ferragut, J.A., González-Ros, J.M., DasGupta, B.R., Montal, M. and Ferrer-Montiel, A. (1998) Structural stabilization of botulinum neurotoxins by tyrosine phosphorylation. FEBS Lett. 429, 78–82.
[5] Blanes-Mira, C., Ibañez, C., Fernández-Ballester, G., Planells-Cases, R., Pérez-Payá, E. and Ferrer-Montiel, A. (2001) Thermal stabilization of the catalytic domain of botulinum neurotoxin E by phosphorylation of a single tyrosine residue. Biochemistry 40, 2234–2242.
[6] Vaidyanathan, V.V., Yoshino, K.-I., Jahnz, M., Dörries, C., Bade, S., Nauenburg, S., Niemann, H. and Binz, T. (1999) Proteolysis of SNAP-25 isoforms by botulinum neurotoxin types A, C, and E: domains and amino acid residues controlling the formation of enzyme- substrate complexes and cleavage. J. Neurochem. 72, 327–337.
[7] Aguado, F., Gombau, L., Majo, G., Marsal, J., Blanco, J. and Blasi, J. (1997) Regulated secretion is impaired in AtT-20 endocrine cells stably transfected with botulinum neurotoxin type A light chain. J. Biol. Chem. 272, 26005–26008.
[8] Blanes-Mira, C., Merino, J.M., Valera, E., Fernandez-Ballester, G., Gutierrez, L.M., Viniegra, S., Perez-Paya, E. and Ferrer-Montiel, A. (2004) Small peptides patterned after the N-terminus domain of SNAP25 inhibit SNARE complex assembly and regulated exocytosis. J. Neurochem. 88, 124–135.

[9] Garcia-Sanz, N., Fernandez-Carvajal, A., Morenilla-Palao, C., Planells-Cases, R., Fajardo-Sanchez, E., Fernandez-Ballester, G. and Ferrer-Montiel, A. (2004) Identification of a tetramerization domain in the C terminus of the vanilloid receptor. J. Neurosci. 24, 5307–5314.
[10] Morenilla-Palao, C., Planells-Cases, R., Garcia-Sanz, N. and Ferrer-Montiel, A. (2004) Regulated exocytosis contributes to protein kinase C potentiation of vanilloid receptor activity. J. Biol. Chem. 27, 25665–256672.
[11] Becker-Hapak, M., McAllister, S.S. and Dowdy, S.F. (2001) TAT-mediated protein transduction into mammalian cells. Methods 24, 247–256.
[12] Gimsa, U., Mitchson, A. and Allen, R. (1999) Inhibitors of Src-family tyrosine kinases favour Th2 differentiation. Cytokine 11, 208–215.
[13] Lacy, B.D. and Stevens, R.C. (1999) Sequence homology and structural analysis of the clostridial neurotoxins. J. Mol. Biol. 291, 1091–1104.
[14] Lacy, B.D., Tepp, W., Cohen, A.C., DasGupta, B.R. and Stevens, R.C. (1998) Crystal structure of botulinum neurotoxin type A and implications for toxicity. Nat. Struct. Biol. 5, 698–902.
[15] Segelke, B., Knaupp, M., Kadkhodayan, S., Balhom, R. and Rupp, B. (2004) Crystal structure of *Clostridium botulinum* neurotoxin protease in a product-bound state: evidence for noncanonical zinc protease activity. Proc. Natl. Acad. Sci. USA 101, 6888–6893.
[16] Hanson, M.A. and Stevens, R.C. (2000) Cocrystal structure of synaptobrevin-II bound to botulinum neurotoxin type B at 2.0 Å resolution. Nat. Struct. Biol. 7, 687–691.
[17] Swaminathan, S. and Eswaramoorthy, S. (2000) Structural analysis of the catalytic and binding sites of *Clostridium botulinum* neurotoxin B. Nat. Struct. Biol. 7, 693–699.
[18] Koriazova, L.L. and Montal, M. (2003) Translocation of botulinum neurotoxin light chain protease through the heavy chain channel. Nat. Struct. Biol. 10, 13–18.
[19] Parish, J.L. (2003) Commercial preparations and handling of botulinum toxin type A and type B. Clin. Dermatol. 21, 481–484.