Synaptotagmins I and II Act as Nerve Cell Receptors for Botulinum Neurotoxin G*

Andreas Rummell‡,†, Tino Karnath‡,†, Tina Henke‡, Hans Bigalke‡, and Thomas Binz‡,†

From the Institutes of ‡Biochemistry and §Toxicology, Medizinische Hochschule Hannover, D-30625 Hannover, Germany

Botulinum neurotoxins (BoNTs) induce muscle paralysis by selectively entering cholinergic motoneurons and subsequent specific cleavage of core components of the vesicular fusion machinery. Complex gangliosides are requisite for efficient binding to neuronal cells, but protein receptors are critical for internalization. Recent work evidenced that synaptotagmins I and II can function as protein receptors for BoNT/B (Dong, M., Richards, D. A., Goodnough, M. C., Tepp, W. H., Johnson, E. A., and Chapman, E. R. (2003) J. Cell Biol. 162, 1293–1303). Here, we report the protein receptor for a second BoNT serotype. Like BoNT/B, BoNT/G employs synaptotagmins I and II to enter phrenic nerve cells. Using pull-down assays we show that only BoNT/G, but neither the five remaining BoNTs nor tetanus neurotoxin, interacts with synaptotagmins I and II. In contrast to BoNT/B, interactions with both isoforms are independent of the presence of gangliosides. Peptides derived from the luminal domain of synaptotagmin I and II are capable of blocking the neurotoxicity of BoNT/G in phrenic nerve preparations. Pull-down and neutralization assays further established the membrane-juxtaposed 10 luminal amino acids of synaptotagmins I and II as the critical segment for neurotoxin binding. In addition, we show that the carboxy-terminal domain of the cell binding fragment of BoNT/B and BoNT/G mediates the interaction with their protein receptor.

Clostridial neurotoxins (CNTs)† are extremely potent bacterial toxins. Among them, the seven serologically distinct botulinum neurotoxins (BoNTs, serotypes A-G) cause botulism, whereas the tetanus neurotoxin (TeNT) provokes the disease tetanus. Each neurotoxin is composed of four domains. Their light chains (LCs) act as zinc-dependent endopeptidases and specifically hydrolyze certain proteins of the vesicular fusion machinery, whereupon the Ca<sup>2+</sup>-triggered fusion of synaptic vesicles with the presynaptic membrane is disrupted (reviewed in Refs. 1–3). The heavy chains (HCs) are tethered to the LCs via a single disulfide bond and encompass the three remaining domains. The HC serves as the vehicle that delivers the LC to the cytosol of neuronal cells. Therefore, the extreme toxicity has to be largely ascribed to the specific binding of the molecule to nerve terminals at the neuromuscular junction. The amino-terminal segment of the HC, the H<sub>C</sub>, domain, is responsible for translocating the LC from the lumen of an acidic intracellular compartment into the cytosol subsequent to cell binding and receptor-mediated endocytosis. The carboxyl-terminal segment of the HC, the so-called H<sub>C</sub>-fragment (H<sub>C</sub>-f), comprises two domains, H<sub>C</sub>N and H<sub>C</sub>C. The latter was shown in the case of TeNT to suffice for cell binding (4) and internalization (5). TeNT travels retrogradely and eventually arrives at inhibitory interneurons in the spinal cord where it provokes spastic paralysis. So far, the corresponding domain has not been identified for BoNTs, which in contrast act locally at motoneurons and cause flaccid paralysis. No function could yet be attributed to the H<sub>C</sub>N domain.

It has long been known that polysialogangliosides, i.e. glycosphingolipids that are particularly enriched in the outer leaflet of neuronal cell membranes, are crucial for the binding of CNTs to neurons. TeNT and BoNTs exhibit affinities in the high nM range for isolated polysialogangliosides. The binding of CNTs to neuronal tissue, however, exhibits yet much higher affinities (reviewed in Ref. 6). This discrepancy as well as other findings led to the proposal of the now confirmed two-receptor model (7). This model suggests that the abundant polysialogangliosides trap and accumulate CNTs in the plane of the cell membrane. Here, the neurotoxins wait until achieving contact with their sparsely occurring protein receptor(s), which are assumed to mediate the subsequent specific endocytosis. Synaptotagmin (Syt)-I and Syt-II, two homologous membrane-anchored proteins of synaptic vesicles (8, 9) thought to link vesicular fusion to Ca<sup>2+</sup> entry (10, 11), were demonstrated to interact with BoNT/B in the presence of GT1b (12–14). Recently, it was conclusively shown that their luminal domain, which becomes temporarily exposed at the synaptic cleft upon fusion of synaptic vesicles, interacts with BoNT/B and mediates the entry of BoNT/B into neurons (15).

In the present study, we investigated whether other CNTs than BoNT/B can utilize Syt-I or Syt-II as protein receptors. Our results establish that BoNT/G interacts directly with both Syt-I and Syt-II in a ganglioside-independent fashion. Moreover, neutralization studies employing peptides derived from the luminal domain of Syt-I or Syt-II effectively block the toxicity of BoNT/G in mouse phrenic nerve preparations. Thus, the interaction of BoNT/G with Syt-I or Syt-II is crucial for its entry into motor nerve terminals.

EXPERIMENTAL PROCEDURES

Plasmid Constructions and Recombinant Proteins—Plasmids encoding full-length BoNT/A and BoNT/B as well as the H<sub>C</sub>-fragments of TeNT, BoNT/A, and BoNT/B were described previously (16, 17). Corresponding constructs for the expression of BoNT/C, -D, -E, -F, and -G H<sub>C</sub>-fragments carrying a carboxyl-terminal StrepTag were generated by using PCR with suitable primers and purified bacteriophage DNA (BoNT/C and -D) or total bacterial DNA (BoNT/E, strain NCTC 11219;
BoNT/F, strain NCTC 10281; BoNT/G (Clostridium argentinense) as template DNA. Plasmids for Escherichia coli expression of carboxy-terminal H₄, L and H₃, domain-encoding vector. An expression plasmid (pBoNTG-thro) for full-length BoNT/G (0.2662) was assembled from LC and H₄ domain-encoding DNA pieces obtained from E. coli TG1 purified employing glutathione-Sepharose beads. Fractions containing the desired proteins were pooled and dialyzed against Tris/NaCl buffer (20 mM Tris-HCl, 150 mM NaCl, pH 7.2) or, in the case of neurotoxins, directly frozen in liquid nitrogen and subsequently visualized by Coomassie Blue staining. Asterisks denote breakdown products of GST fusion proteins. Arrowheads point toward unspecifically bound H₄.A and H₃.B.

RESULTS

BoNT/G Directly Interacts with the Luminal Domain of Syt-I and Syt-II—Previous studies demonstrated that Syt-I and Syt-II interact with BoNT/B (12–14) and mediate its internalization (15). In contrast, the entry of BoNT/A and BoNT/E does not depend on the presence of Syt-I and Syt-II (15). To screen whether any of the remaining CNTs utilizes interaction with Syts to enter neuronal cells, we analyzed binding of their H₃-fragments to Syt-I and Syt-II in vitro. We conducted GST pull-down experiments employing fusion proteins in which the entire luminal and transmembrane domains of rat Syt-I and Syt-II were fused to GST. As demonstrated in Fig. 1B, GST-Syt-I-1–82 and GST-Syt-II-1–90 pull down the H₃-fragments of BoNT/B and BoNT/G. The finding with BoNT/B agrees with results of Dong and colleagues (15); the finding with BoNT/G constitutes a novel result. Binding of H₃B and H₃G to GST-Syt-I-1–90 is saturable and reaches a 1:1 stoichiometry at saturation (3-fold molar excess of HC; data not shown). We also detected traces of H₃-fragments of BoNTA and BoNT/E pulled down with GST-Syt-I-1–82 and GST-Syt-II-1–90 (Fig. 1B, arrowheads). This interaction appears to be unspecific, as H₃A is pulled down to a similar extent by GST-Syb2 and GST-Syt-III-1–80 (Fig. 1C), and probably explains data of a previous report about the binding of BoNTA and BoNT/E to Syt-I (19).

To substantiate the newly discovered BoNT/G-Syt interaction, we studied the binding of the full-length neurotoxin as well. For this, we constructed expression plasmids producing BoNT/G fused to a carboxyl-terminal StrepTag. One variant (scBoNT/G-wt) comprised the native loop region between the two cystein residues that form the interchain disulfide bridge. In a second variant (BoNT/G-thro), a recognition site for thrombin was inserted in this region at the expense of four of the original amino acids (Fig. 2A). Both neurotoxin variants could be isolated from E. coli lysates via their affinity tag and exhib-
conducted under reducing conditions. Or thrombin, (respectively. Electrophoresis was Syt-II-1 to GST – GST/H18528 Syt-II-1 51, suggesting that BoNT/G shares the interaction of the complete interaction site, has no influence on neurotoxicity. Secondly, GST-Syt-I-1–53, as well as the corresponding peptide Syt-I-1–53, does not significantly prolong the paralytic half-time of scBoNT/B because preincubation occurred in the absence of micellar gangliosides (Fig. 5).

BoNT/G proved to be far less active at the mouse phrenic nerve than BoNT/B and BoNT/A, probably because of a lower affinity to synaptosomal membranes as well as to a lower catalytic activity of its LC versus other synaptobrevin-hydrolyzing CNTs (20). A final concentration of 105 nM scBoNT/G–wt resulted in a paralytic half-time of 120 min (data not shown). Bath concentrations of up to 1 mM GST–Syt would have been required for inhibition studies. Attempts were undertaken to acquire nicked, i.e. proteolytically activated, BoNT/G because nicked CNTs are generally far more potent. However, activation of recombinant scBoNT/G–wt with trypsin leads to an inadvertent hydrolysis of peptide bonds within the HC (Fig. 2B). To circumvent this problem, we utilized BoNT/G-thro, which is to a great extent specifically activated by E. coli proteases during the purification procedure. BoNT/G-thro proves to be about 80-fold more active than scBoNT/G–wt. A final bath concentration of 20 nM results in a paralytic half-time of 55.8 ± 3.8 min and was consequently used for neutralization studies. In contrast to BoNT/B, both GST-Syt-I–1–61 and GST-Syt-I–1–53 are able to efficiently diminish the neurotoxicity of BoNT/G-thro on preincubation with a 1000-fold molar excess by 66.2% and 75.1% (according to 74.4 ± 6.5 min, 80.7 ± 2.5 min paralytic half-time), respectively. Syt-II–1–53 applied at 100-fold molar excess decrease the neurotoxicity by 37.9% (63.3 ± 5.9 min) and 66.4% (74.5 ± 4.9 min), respectively, and thus nearly approximate the efficacy of their 10-fold higher concentrated GST-tagged variants. In addition, preincubation of BoNT/G-thro with an equimolar mix-

Fig. 2. Generation of full-length BoNT/G. A, expression plasmids encoding BoNT/G carrying a carboxyl-terminal StrepTag comprised either the native loop region between the LC and HC connecting cystein residues (scBoNT/G-wt) or a thrombin cleavage site that was inserted at the expense of four original amino acids (BoNT/G-thro). The recognition site for thrombin is boxed, the scissile peptide bond indicated by an arrowhead. B, SDS-PAGE analysis of purified recombinant BoNT/G-wt and BoNT/G-thro before and subsequent to protease digestion by trypsin (Tryp) or thrombin (Thro), respectively. Electrophoresis was conducted under reducing conditions. Arrows point at the positions of scBoNT/G, HC, and LC. The asterisk marks an uncharacterized neurotoxin byproduct, the arrowhead carboxyl-terminal breakdown products of BoNT/G.

ited >85% purity (Fig. 2B). BoNT/G-wt was used for in vitro binding studies as single chain protein. Full-length scBoNT/G-wt also interacts with Syt-I and Syt-II, whereas no binding was observed for scBoNT/A, underscoring that the pull down of H$_2$A is indeed unspecific (Fig. 3, middle panel).

It was recently shown that the interaction of BoNT/B with Syt-I depends on the presence of gangliosides. Therefore, we checked next whether BoNT/G displays the same binding mode. However, BoNT/G-Syt-I complexes already form in the presence of gangliosides (Fig. 3, right panel).

BoNT/G binds to the membrane proximal region of Syt-I and Syt-II—Because the mode of binding differs between BoNT/B and BoNT/G with respect to the requirement of gangliosides, it was interesting to investigate whether BoNT/B and -G associate with the same segment of Syt. Dong et al. (15) showed that the membrane proximal region of Syt-II, i.e. residues 40–60, comprises the binding site for BoNT/B. We therefore mapped the binding site for BoNT/G by truncating Syt-I and Syt-II. Results presented in Fig. 4B show that H$_2$G binds to GST-Syt-II–1–61. No binding is detected, however, to GST-Syt-II–1–51, suggesting that BoNT/G shares the interaction site with BoNT/B. The binding site for BoNT/B in Syt-I could not be determined via carboxyl-terminal-truncated Syt-I, because this interaction only occurs when Syt-I and gangliosides are concomitantly present in micellar structures (Fig. 4A) (15). On the other hand, we were able to establish that the corresponding membrane proximal segment of Syt-I, i.e. residues 43–53, is crucial for the binding of BoNT/G because H$_2$G is pulled down by GST-Syt-I–1–53 but not by GST-Syt-I–1–43. This interaction occurs as with full-length BoNT/G-wt (Fig. 3) whether gangliosides are present or not.

The isolated luminal domain of Syt-I and Syt-II blocks the toxicity of BoNT/G at the mouse phrenic nerve—To provide evidence for the physiological relevance of the BoNT/G-Syt interaction, we investigated whether the neurotoxicity of BoNT/G could be blocked by preincubating the neurotoxin with peptides derived from the luminal domain of Syt-I and Syt-II. As an assay system we chose the established mouse phrenic nerve toxicity test (18). In the first set of experiments, we inspected whether it succeeded in blocking the effect of BoNT/B. scBoNT/B was applied in a 2 nM concentration, which results in a 50% reduction of the hemidiaphragm muscle contractile force within 59.2 ± 3.6 min (paralytic half-time). Preincubation of scBoNT/B with a 1000-fold molar excess of GST-Syt-I–1–61 leads to a 61% inhibition of neurotoxicity (paralytic half-time: 76.0 ± 5.7 min), whereas a 10,000-fold molar excess results in a 95.1% inhibition (paralytic half-time: 132.3 ± 13.2 min; Fig. 5). Removal of the GST portion increases the potency of Syt-I–1–61 10-fold because a peptide concentration of 1.95 μM raises the paralytic half-time to 138.5 ± 10.6 min (corresponding to a 95.9% inhibition). In line with results of the binding experiments, GST-Syt-I–1–51, a variant devoid of the complete interaction site, has no influence on neurotoxicity. Secondly, GST-Syt-I–1–53, as well as the corresponding peptide Syt-I–1–53, does not significantly prolong the paralytic half-time of scBoNT/B because preincubation occurred in the absence of micellar gangliosides (Fig. 5).

A. Rummel and T. Binz, unpublished results.
ture of GST-Syt-II–1–61 and GST-Syt-I–1–53 (−10 µM each) inhibits phrenic nerve paralysis to a similar extent as 19.5-µM concentrations of the individual fusion proteins (63.7%; 73.0 ± 2.8 min; Fig. 5). The shortened constructs GST-Syt-II–1–51 and GST-Syt-I–1–43 do not affect the toxicity of BoNT/G-thro, as indicated by paralytic half-times of 59.5 ± 6.4 and 46.0 ± 4.2 min, respectively, that do not significantly differ from the untreated control. The results with the latter truncated Syt variants also demonstrate that inhibition through GST Syts is specific and does not interfere with any other physiological process in the assay system. As a further control experiment, we assessed whether the toxicity of scBoNT/A is effected by preincu-
vation with GST-Syt, because entry of this neurotoxin has previously been shown to be independent of an interaction with either Syt (15). As presented in Fig. 5, even a 19.5-μM final concentration of GST-Syt-II-1–61 has no detectable effect on the toxicity of 0.22 nM scBoNT/A. This finding further validates the specificity of the assay system. Together, these results prove that Syt-I and Syt-II indeed do mediate the entry of BoNT/G into peripheral nerve cells.

The HCC Domain of BoNT/B and BoNT/G Mediates the Interaction with Syt—It is known that the HCC domain of the TeNT and BoNT HCC-fragments is responsible for ganglioside binding (17, 21, 22). No function could so far be allocated to HCCN. To assign the Syt binding site to either of the two domains, we expressed each as His6-tagged proteins in E. coli and conducted pull-down experiments. HCCB and HCCG served as control peptides in this assay. Fig. 6 clearly demonstrates that the HCCB, but not the HCCG, domain of BoNT/B and -G interacts with GST-Syt-II-1–61. So, in addition to ganglioside binding, the HCCG domain of BoNT/B and BoNT/G mediates the interaction with the protein receptor as well.

**DISCUSSION**

BoNTs, the causative agents of botulism, disrupt the neurotransmission of cholinergic nerves at the neuromuscular junction. More than one decade ago, the underlying molecular basis for the inhibition of neurotransmitter release was deciphered for all BoNTs and TeNT, which turned out to be the prototolysis of one of three intracellular soluble NSF attachment protein receptor proteins by their catalytic domains. On the other hand, receptors that mediate the productive uptake of CNTs into nerve terminals have so far only been unequivocally identified for BoNT/B (12, 15).

In this study, we have identified the cellular receptor for the second of the eight CNTs, BoNT/G, which like BoNT/B is guided into neurons through its specific interaction with Syt-I or Syt-II. Two lines of evidence support this suggestion. First, by means of GST pull-down experiments we were able to show that BoNT/G interacts directly with the luminal domain of Syt-I and Syt-II. Second, peptides derived from the luminal domain of Syt-I and Syt-II are capable of blocking the entry of BoNT/G into motoneurons that innervate the mouse diaphragm. This only occurs when these peptides contain the membrane anchor-juxtaposed luminal 10 amino acids of Syt. The identified segment of Syt-I and Syt-II becomes transiently exposed on the membrane surface only when synaptic vesicles fuse with the presynaptic membrane at synapses. This is in line with the well documented finding that nerve stimulation generally accelerates the uptake of BoNTs and concomitant poisoning of nerve terminals (23). In a similar manner, TeNT was previously also shown to be taken up via recycling of synaptic vesicles (24). Together, these data suggest that CNTs in general enter nerve terminals via this route and may associate with segments of resident synaptic vesicle proteins that are exposed to the luminal side.

In contrast to what was reported for BoNT/B (15), the binding of BoNT/G to Syt-I is apparently independent of the presence of gangliosides. The most obvious explanation for this discrepancy is that a low affinity of BoNT/B for Syt-I does not allow detection by GST pull-down experiments. Here, only the simultaneous interaction with Syt-I and a ganglioside molecule ultimately guarantees high affinity binding. In BoNT/G, sequence variation in its Syt binding fold may account for stronger binding to Syt-I. Alternative explanations like the generation of a high affinity binding site for Syt-I because of ganglioside-induced structural changes is unlikely to occur, because significant structural changes were not observed in several crystals of BoNT/B and its complexes with sialyllactose (21) or doxorubicin (25). Furthermore, gangliosides incorporated in Triton micelles did not support binding of BoNT/B to a truncated Syt-I variant that lacked the membrane anchor domain, indicating strict requirement of both Syt-I and ganglioside within the context of membranes.

Our study illustrates that the HCC domains of BoNT/B and BoNT/G are responsible for the binding to their protein receptor. This interaction is requisite for productive uptake into neurons. Indirect evidence for an employment of HCC in this process has recently been suggested for TeNT as well (5). Thus, the HCC domains likely function as a closed cell entry module in all CNTs, whereas the function of the HCCN domain still awaits elucidation.

The novel finding of the present study that BoNT/G, just like BoNT/B, utilizes Syt-I and Syt-II for its entry into nerve terminals is consistent with their degree of sequence conservation. The similarity score for aligning their HCC-fragments is 42.4% and is only exceeded by the score of 58.0% for HCE (strain NCTC11219)/HCCF (Clostridium barati) (ClustaW software). Almost all other pairwise alignments result in less than 30% similarity. We have further shown that the HCC domain of BoNT/B and -G mediates the binding to Syt-I and Syt-II. With respect to sequence alignments of HCC, HCCB/HCCG actually reaches by far the highest similarity score (39.5%), whereas those for many other pairs drop down to less than 20%. This agrees with the notion that none of the remaining six CNTs binds to Syt-I or Syt-II and the observation that BoNT/B and -G are incapable of competing binding of BoNT/A and E to synaptic membranes. These data add further support to the premise that all other CNTs associate with different protein receptors to become endocytosed. Interestingly, TeNT, which is sorted into the retrograde axonal transport route upon endocytosis, was demonstrated to bind simultaneously to two separate carbohydrate structures (16, 26), one of which could be part of the recently discovered glycosylated 15-kDa protein receptor (27). Therefore, the use of a separate protein receptor that ensures exit from the lysosomal transport route of BoNTs is plausible explicitly for TeNT.

**Acknowledgments**—We thank Drs. Thomas C. Sudhof (Dallas) for plasmids encoding rat Syt-I and Syt-II, Massanari Mizuta and Susumu Seino (Chiba) for a Syt-III-specific clone, and William S. Trimble (Toronto) for the rat synaptobrevin 2 cDNA. We thank Beate Laske and Christina Knorr for excellent technical assistance and Dr. Harold V. Taylor for critically reading the manuscript.

**REFERENCES**

1. Niemann, H., Blasi, J., and Jahn, R. (1994) Trends Cell Biol. 4, 179–185
2. Bigalke, H., and Shoer, L. F. (2000) in Handbook of Experimental Pharmacology (Aktories, K., and Just, I., eds) Vol. 145, pp. 407–443, Springer Verlag, Berlin, Heidelberg
3. Schiavo, G., Matteoli, M., and Monteuccio, C. (2000) Physiological Rev. 80, 777–786
4. Halpern, J. L., and LoTurco, A. (1993) J. Biol. Chem. 268, 11188–11192
5. Herreros, J., Lalli, G., and Schiavo, G. (2000) Biochem. J. 347, 199–204
6. Halpern, J. L., and Neale, E. A. (1995) Curr. Top. Microbiol. Immunol. 195,
Synaptotagmins Act as Botulinum Neurotoxin G Receptor

21. Swaminathan, S., and Eswaramoorthy, S. (2000) Nature Struct. Biol. 7, 693–699
22. Sutton, J. M., Chow-Worn, O., Spaven, L., Silman, N. J., Hallis, B., and Stone, C. C. (2001) FEBS Lett. 493, 45–49
23. Black, J. D., and Dolly, J. O. (1986) J. Cell Biol. 103, 535–544
24. Matteoli, M., Verderio, C., Rossetto, O., Iezzi, N., Coco, S., Schiavo, G., and Montecucco, C. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 13310–13315
25. Eswaramoorthy, S., Kumaran, D., and Swaminathan, S. (2001) Acta Crystallogr. D Biol. Crystallogr. 57, 1743–1746
26. Feng, C., Emsley, P., Black, I., Ando, H., Ishida, H., Kiso, M., Sinha, K. A., Fairweather, N. F., and Isaac, N. W. (2001) J. Biol. Chem. 276, 32274–32281
27. Herreros, J., Ng, T., and Schiavo, G. (2001) Mol. Biol. Cell 12, 2947–2960
