Proceedings of the 23rd American Peptide Symposium
Michal Lebl (Editor)
American Peptide Society, 2013

On-Demand Assembly of Macromolecules Used for the Design and Application of Targeted Secretion Inhibitors

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Introduction

Neurological and endocrine pathologies such as acromegalie, Cushing's disease, and neuropathic pain display disregulated exocytosis. Silencing specific cell populations would thus be invaluable to correct these debilitating disorders. To achieve this goal, we re-engineered the Botulinum neurotoxin (BoT), a highly potent pharmaceutical compound capable of inhibiting exocytosis, and fused to it a protein "stapling" domain [1,2]. These peptide motifs, that form an irreversible tetrahelical coiled-coil, are able to link a variety of targeting domains onto the enzyme and thus redirect it towards normally unaffected cells. The conformational diversity of this assembly process greatly supersedes traditional protein expression since multiple targeting domains (homo- and hetero-) can be linked onto one scaffold, larger yields can be produced separately, it permits the combination of solid-phase peptide synthesis with recombinant protein expression, and it can avoid the necessity of an N- to C- translational fusion. With only a few dozen building "blocks" it is possible to construct thousands of different complexes specifically tailored for each purpose as every individual component can be linked onto any other cognate stapling moieties.

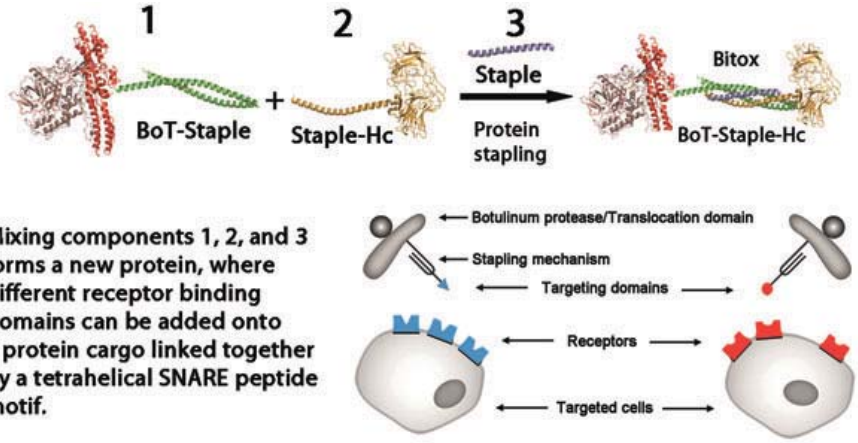

Fig. 1. Schematic representation of SNARE base protein stapling.

We then assayed a range of targeting domains composed of neuropeptides and factors sequences that would exploit each cell's surface receptor expression (see Figure 1).

Results and Discussion

Our results show that Corticotropin Releasing Hormone (CRH), Epidermal growth factor (EGF), Ciliary neurotrophic factor (CNTF), Vasoactive Intestinal peptide (VIP), as well as others, can potentiate the receptor mediated internalization of the BoT enzyme cargo into specific, and pathologically important, cell types [3]. Following this intracellular delivery, those cells displayed significant reductions of hormone and neurotransmitter release.

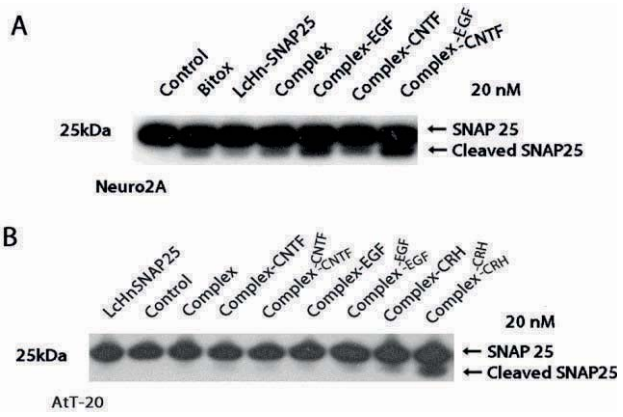

Fig. 2. a) Western immunoblotting of total SNAP25 of neuro2A cells. b) Western immunoblotting of total SNAP25 in AtT-20cells.

Using botulinum neurotoxin's highly sensitive efficacy readout, which is the intracellular cleavage of SNAP25, we have been able to characterize these new cargo amenable targeting domains. This strategy enabled the retargeting of BoNT/A to neuroendocrine cells that are normally unaffected by the native toxin's receptor binding domain. These new toxin complexes can bind to- and be internalized into PC12, Neuro2A, SH-SY5Y, AtT20, and Min6 cells as well as different subpopulations of *ex-vivo* rat cortical cells. Once internalized by receptor-mediated endocytosis, the enzyme cargo cleaves SNAP25 and thus inhibits exocytosis. Furthermore, the facility at which one can recombine the different targeting domains onto the enzymatic cargo permits us to explore novel conformations that protein recombination does not permit. As shown in Figure 2, using single EGF and CNTF targeting domains, domain on Neuro2A cells permits protease entry. However, when they are both included as a hetero bifunctional targeting domains onto a single complex, the efficacy of internalization is increased (Figure 2A). Such approaches could undoubtedly heighten the cell selectivity to the cargo delivery. While testing the corticotroph AtT-20 cells, which are amenable to CRH targeting, we saw that the homodimer targeting domains greatly enhanced the cytosolic entry compared to the monomer domain (Figure 2B). Such approach enables receptor dimer targeting while also elevating the probability of statistical rebinding. The functional targeting domains such as EGF, CNTF, CRH, and VIP, identified in this study, can be complexed with other stapled domains of diverse utilities (e.g. fluorophores, chelators, reactive groups). Combinations of stapled domains enable a "swiss-army knife" configuration to the pharmaceutical molecule. Such as ameliorated efficacy through heightened avidity, a strategy that remains intransigent for single strand protein expression. This targeted secretion inhibitor assembly platform has numerous biotechnological and medical implications [1,3]. Current endeavors are aimed towards testing our compounds in animal models, and subsequently, for clinical trials. Future work will be directed towards monitoring *in vivo* activity of our compounds to treat endocrinopathies and extend this approach to other pharmaceutically useful cargos.

Acknowledgments

We would like to thank the supporting staff at MRC LMB for helping with this work. Supported by MRC grant U10578791.

References

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