 doi:10.20944/preprints202003.0401.v1

Commentary

Roles for the SNAP25 linker domain in the fusion pore and a dynamic plasma membrane SNARE acceptor complex

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Abstract

A recent paper demonstrates the importance of the linker region joining the two SNARE motifs of the neuronal t-SNARE SNAP25 for maintaining rates of secretion with roles for distinct segments in speeding fusion pore expansion (Shaaban et al., 2019, *Elife*. 8). Remarkably, lipid perturbing agents rescue a palmitoylation-deficient phenotype that includes slow fusion pore expansion, suggesting that protein-protein interactions have a role not only in bringing together the granule or vesicle membrane with the plasma membrane but also in orchestrating protein-lipid interactions leading to the fusion reaction. Furthermore, biochemical investigations demonstrate the importance of the C-terminal domain of the linker in the formation of the plasma membrane t-SNARE acceptor complex for synaptobrevin2 (Jiang, et al., 2019, *FASEB J*. 33:7985-7994;Shaaban et al., 2019, *Elife*. 8). This insight, together with biophysical and optical studies from other laboratories (Wang, et al., 2008, *Molecular Biology of the Cell*. 19:3944-3955; Zhao, et al., 2013, *Proc Natl Acad Sci U S A*. 110:14249-14254) suggests that the plasma membrane SNARE acceptor complex between SNAP25 and syntaxin and the resulting trans SNARE complex with the v-SNARE synaptobrevin form just milliseconds before fusion.

Article

Of the four SNARE motifs that comprise the prototypical four helical bundle in neuronal SNARE proteins, two of the motifs (SN1 and SN2) are joined by a palmitoylated linker in the t-SNARE, SNAP25 (**Fig. 1**). Palmitoylation enables binding of the protein to the plasma membrane. Additional functions for the linker were determined several years ago (Wang et al., 2008). First, joining SN1 and SN2 together with a palmitoylated linker facilitates the formation of membrane complexes with syntaxin that bring the N-termini of SN1 and SN2 close together, as reflected by increased FRET in labeled probes. Second, linking SN1 and SN2 enables almost complete rescue of robust secretion in cells expressing the light chain of Botulinum neurotoxin type E (BoNT E cleaves SN2), whereas expressing unlinked SN1and SN2 or SN2 alone resulted in little rescue. In contrast, when secretion rates were slow, expression of SN2 alone completely rescued secretion. (Constructs used in the experiments were resistant to BoNT E.) The results suggested that high rates of exocytosis require linked SNAP25 SNARE domains. Secretion was measured biochemically in cell cultures and had limited time resolution.

The SNAP25 linker and fusion pore expansion. A study by Shaaban et al. (Shaaban et al., 2019) confirms the importance of the linked SNARE domains in exocytosis. Most importantly, it greatly expands our understanding of linker function by investigating single cell responses using techniques with high temporal resolution- patch clamp capacitance recordings and amperometry. SNAP25 analogues differing only in the linker were introduced by viral infection into chromaffin cells from mice with genetic ablation of SNAP25.

Full substitution of the linker domain with a flexible peptide produced a non-functional SNAP25 mutant. A careful structure-function investigation demonstrated important and different functions for the N-terminal and C-terminal domains of the linker. Compared to wild type SNAP25, partial substitution of segments at either end of the linker greatly reduced the amount of primed secretion and slowed the rate of fusion. The N-terminus of the linker immediately adjacent to SN1 has 4 cysteine residues which are sites for palmitoylation (**Fig. 1**). Amperometry demonstrated that cysteine-to-serine mutations greatly prolonged the initial fusion pore expansion as measured by the prespike foot, *without altering the main spike*. This is a beautiful result indicating that palmitoylation not only causes plasma membrane binding, but also specifically speeds opening of the fusion pore. The location of the palmitoylation sites is also critical since fusion pore expansion is greatly slowed in mutants in which the palmitoylation sites were relocated away from the wild type position. The authors postulated that if the membrane interactions of the N-terminal domain of the linker are important for lipid intermediates in fusion, then membrane-active agents might rescue the effects of a N-terminal segment mutant without the palmitoylation sites. Remarkably, intracellular application of the membrane-active reagents methanol and oleic acid reduced or reversed the effects of the mutant protein. The authors suggest that the linker-mediated lipid interaction directly regulates membrane curvature at the initial fusion pore, thereby facilitating fusion pore expansion.

The SNAP25 linker and the formation of the plasma membrane pre-fusion acceptor complex, a possible late step that helps initiate fusion. There is also compelling biochemical and structural data for a role in fusion of the C-terminus of the linker adjacent to SN2. The C-terminal linker segment (**Fig. 1,** yellow line) strongly facilitates the *in vitro* binding of SN2 to the readily formed complexes of SN1 and syntaxin (Shaaban et al., 2019). It was independently found that the linker-membrane interaction increases linker helical content, which enables the C-terminal linker region to interact with syntaxin (Jiang et al., 2019). These are important findings. They explain the ability of membrane-bound syntaxin to bring the N-termini of linked, but not separated SN1 and SN2 into close proximity, as detected with an intramolecular FRET construct of SNAP25 (Wang et al., 2008). This conformation of the t-SNARE acceptor complex likely initiates interaction with the v-SNARE, synaptobrevin2. Indeed, in an experimental *tour de force,* it was found that the formation of this acceptor complex (again detected with an intramolecular FRET construct) increases milliseconds before fusion at fusion sites in chromaffin cells (Zhao et al., 2013). *This array of experiments suggests that the acceptor complex is dynamic and its formation is an important late step required to initiate fusion.* This concept is consistent with the findings that granules continue to jitter as much as a 100 nm (much greater than the ~10 nm length of the tetrahelical SNARE complex) within 100 ms of fusion (Allersma et al., 2006; Degtyar et al., 2007) and suggests that trans SNARE complexes form just milliseconds before fusion (Degtyar et al., 2007; Jahn and Fasshauer, 2012).

Protein-lipid interactions in exocytosis. The specific, position-dependent effect on fusion pore expansion of palmitoyl groups in the linker domain in SNAP25 highlights the importance of protein-lipid as well as protein-protein interactions in the late steps of exocytosis. Numerous other protein-lipid interactions have been elucidated among components of the fusion complex. The first evidence for a specific role of lipids in Ca^{2+}-dependent exocytosis was the discovery of the requirement for the highly negatively charge plasma membrane phospholipid PIP_2 (Eberhard et al., 1990; Hay et al., 1995; Hay and Martin, 1993; Holz et al., 2000; Milosevic et al., 2005). Synaptotagmin, the Ca^{2+} sensor for exocytosis on the secretory granule membrane, interacts through electrostatic binding to plasma membrane PIP_2. This interaction is thought to bring the granule and plasma membrane into close apposition (Bai et al., 2004) allowing subsequent interaction with plasma membrane phosphatidyl serine to trigger fusion (Zhang et al., 2010). A lipid-binding domain in syntaxin1A that binds negatively charged lipids together with cellular

phospholipase D (produces the negatively charged phosphatidic acid) are both necessary for normal fusion pore dynamics (Lam et al., 2008). Flexibility in the transmembrane domain of synaptobrevin enhances the fusion reaction and, importantly, also speeds fusion pore expansion (Dhara et al., 2016). Although there has been considerable progress in understanding the structural basis of protein-protein interactions leading to formation of the core fusion complex, we have little understanding how these protein-protein interactions orchestrate the above protein-lipid interactions necessary for normal exocytosis.

Summary. The domain in SNAP25 that links the two SNARE motifs can no longer be considered a minor player in exocytosis as had been suggested by earlier work (Chen et al., 1999). The linker participates in the formation of the t-SNARE receptor complex with syntaxin (Jiang et al., 2019; Shaaban et al., 2019), is required for robust secretion (Shaaban et al., 2019; Wang et al., 2008), and functions to quicken fusion pore expansion and to control secretion rates (Shaaban et al., 2019). Normal function requires precise localization of the palmitoylated residues in the linker. Furthermore, biochemical investigations demonstrate the importance of the C-terminal domain of the linker in the formation of the plasma membrane t-SNARE acceptor for synaptobrevin2 (Jiang, et al., 2019, *FASEB J*. 33:7985-7994;Shaaban et al., 2019, *Elife*. 8). This insight, together with *in vitro* and *in situ* FRET measurements of fluorescent SNAP25 (Wang et al., 2008; Zhao et al., 2013), strongly suggests that the plasma membrane SNARE acceptor complex forms just milliseconds before fusion, thereby enabling engagement with granule membrane synaptobrevin2 and other granule membrane proteins to catalyze fusion.

Acknowlegement: This work was supported by NIH Grant R01-170553 to RWH

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Acknowledgement
This work was supported by NIH Grant R01-170553 to RWH.

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 27 March 2020 doi:10.20944/preprints202003.0401.v1

Figure 1. Schematic of the SNAP25. SNAP25 contributes two of the four SNARE motifs that comprise the prototypical four helical bundle in neuronal SNARE proteins. The two motifs, SN1 and SN2, are joined by a linker sequence (light blue and yellow line) with four N-terminal cysteines that are sites for palmitoylation. The linker region is divided into N-terminal (blue) and C-terminal (yellow) domains according to the constructs in Shaaban et al. (Shaaban et al., 2019). SN2 contains the cleavage site for botulinum neurotoxin type E (BoNT E). Botulinum neurotoxin type E (BoNT E) was used in an earlier study to inactivate endogenous SNAP25 (Wang et al., 2008). Shaaban et al. (Shaaban et al., 2019) demonstrate that the N- and C-terminals domains have different roles in enabling the function of SNAP25 in exocytosis. The horizontal distances are to scale.