K⁺ Channel-SEC11 Binding Exchange Regulates SNARE Assembly for Secretory Traffic

Sakham Waghmare,1,2 Cecile Lefoulon,2 Ben Zhang,2,3 Edita Liliekyte,2 Naomi Donald, and Michael R. Blatt4,5

Laboratory of Plant Physiology and Biophysics, Bower Building, University of Glasgow, Glasgow G12 8QQ, United Kingdom

ORCID IDs: 0000-0003-1918-0673 (S.W.); 0000-0002-1131-3710 (C.L.); 0000-0003-4473-9184 (B.Z.); 0000-0002-0386-7625 (E.L.); 0000-0002-1873-4286 (N.D.); 0000-0003-1361-4645 (M.R.B.).

Cell expansion requires that ion transport and secretory membrane traffic operate in concert. Evidence from Arabidopsis (Arabidopsis thaliana) indicates that such coordination is mediated by physical interactions between subsets of so-called SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptor) proteins, which drive the final stages of vesicle fusion, and K⁺ channels, which facilitate uptake of the cation to maintain cell turgor pressure as the cell expands. However, the sequence of SNARE binding with the K⁺ channels and its interweaving within the events of SNARE complex assembly for exocytosis remains unclear. We have combined protein-protein interaction and electrophysiological analyses to resolve the binding interactions of the hetero-oligomeric associations. We find that the RYxxWE motif, located within the voltage sensor of the K⁺ channels, is a nexus for multiple SNARE interactions. Of these, K⁺ channel binding and its displacement of the regulatory protein SEC11 is critical to prime the Qa-SNARE SYP121. Our results indicate a stabilizing role for the Qbc-SNARE SNAP33 in the Qa-SNARE transition to SNARE complex assembly with the R-SNARE VAMP721. They also suggest that, on its own, the R-SNARE enters an anomalous binding mode with the channels, possibly as a fail-safe measure to ensure a correct binding sequence. Thus, we suggest that SYP121 binding to the K⁺ channels serves the role of a primary trigger to initiate assembly of the secretory machinery for exocytosis.

SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptor) proteins have important roles in membrane traffic. In plants, they contribute to cell homeostasis and responses to biotic and abiotic stress, and they are vital for development and morphogenesis (Shope and Mott, 2006; Campanoni and Blatt, 2007; Kwon et al., 2008; Eisenach et al., 2012). SNAREs facilitate the final steps of vesicle fusion, assembling in complex to bring membrane surfaces together and drive intercalation of the vesicle and target membrane bilayers. SNAREs have been classified as

1This work was funded by the Biotechnology and Biological Sciences Research Council (grants BB/N006909/1 and BB/M001601/1) and by the European Union (grant 678168 to M.R.B.).
2These authors contributed equally to the article.
3Present address: Life Science, Shanxi University, 92 Wucheng Road, Taiyuan City, Shanxi 030000, China.
4Author for contact: michael.blatt@glasgow.ac.uk.
5Senior author.

M.R.B. conceived the studies with S.W.; B.Z. carried out the protein-protein interaction analysis in yeast with N.D.; S.W. and E.L. generated the biochemical tools and carried out pulldown assays; S.W. carried out the CD and oligomerization studies; C.L. generated the molecular tools and carried out oocyte expression analyses with N.D.; M.R.B. wrote the manuscript with S.W. and C.L.

The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantphysiol.org) is: Michael R. Blatt (michael.blatt@glasgow.ac.uk).

[CC-BY] Article free via Creative Commons CC-BY 4.0 license. www.plantphysiol.org/cgi/doi/10.1104/pp.19.00919

1096 Plant Physiology®, November 2019, Vol. 181, pp. 1096–1113, www.plantphysiol.org © 2019 The author(s). All Rights Reserved.
to SNARE complex assembly (Burgoyne and Morgan, 2007; Ma et al., 2013; Demircioglu et al., 2014).

In Arabidopsis (Arabidopsis thaliana), most secretory traffic at the plasma membrane is driven by the QA-SNAREs SYR1/PEN1 (SYP121) and SYP122 through their assembly with the near-identical cognate R-SNAREs VAMP721 and VAMP722 and the Qbc-SNARE SNAP33 (Sanderfoot, 2007; Bassham and Blatt, 2008; Kwon et al., 2008; Enami et al., 2009; Karnik et al., 2013b, 2015; Fujiwara et al., 2014; Zhang et al., 2015). The Arabidopsis SM protein SEC11 associates preferentially with SYP121 and, like its mammalian counterparts, will stabilize the secretion. SEC11 associates with SYP121 that is important for H+-ATPase traffic to the plasma membrane; however, PATROL1 has no impact on traffic mediated by these QA-SNAREs (Hashimoto-Sugimoto et al., 2013). Thus, it remains unclear how SEC11 (de)binding with SYP121 is facilitated to promote secretion.

Evidence gathered over the past decade has uncovered several other protein partners of SYP121 that point to an unconventional mechanism for its regulation. Indeed, SYP121 was first identified as a factor essential for regulation by the phytohormone abscisic acid (ABA) of ion channels in stomatal guard cells leading to stomatal closure under water stress (Leyman et al., 1999, 2000; Geelen et al., 2002). We now know that SYP121 has unique roles in responses to drought and ABA (Leyman et al., 1999; Eisenach et al., 2012). Most important, through the FxRF motif near its N terminus, SYP121 binds directly with a subset of K+ channels, including KAT1 and KC1, already present at the plasma membrane to promote channel gating (Honsbein et al., 2009, 2011; Grefen et al., 2010). The K+ channels incorporate a unique RYxxWE motif at the cytosolic surface of their voltage sensor domain (VSD), and binding of the SNARE through this motif effectively commandeers the voltage-dependent conformations of the VSD to enhance secretion in parallel with voltage-driven K+ uptake (Grefen et al., 2015; Lefoulon et al., 2018). Significantly, both binding motifs are unique to land plants and are highly conserved within a subset of plasma membrane K+ channels and QA-SNAREs, suggesting that their functional coordination was an important evolutionary step in the colonization of the dry land environment (Honsbein et al., 2011; Grefen et al., 2015; Karnik et al., 2017).

The cognate R-SNAREs VAMP721 and VAMP722 also bind with these K+ channels, but in contrast with SYP121, R-SNARE binding suppresses channel gating (Zhang et al., 2015). The R-SNAREs incorporate, within their regulatory (so-called longin) domain, a linear binding motif for the channels of GHFNYLVEgxxY centered around Tyr-57 (Zhang et al., 2015, 2017). At present, we do not know the complementary motif on the K+ channels. However, since overall the channel-SNARE interactions promote vesicle traffic with K+ uptake (Grefen et al., 2015), it is likely that a binding exchange with the K+ channels occurs between the SNAREs, possibly during SNARE complex assembly. Intriguingly, the SYP121 FxRF channel-binding motif overlaps with one of the two sites for SEC11 binding at the QA-SNARE N terminus (Grefen et al., 2010). This SEC11 binding site is highly conserved and is thought to be important for SYP121 transit from the closed to the open conformation (Karnik et al., 2013b, 2015). Thus, a more complex sequence of binding exchanges may be engaged with SEC11, the QA-SNAREs, and R-SNAREs, and centered on the K+ channels.

Here, we have analyzed the coordinate binding interactions of SYP121 and its cognate partners with SEC11 and the K+ channels KC1 and KAT1. We show that the cognate SNAREs each recognize a common binding motif on the K+ channels that underpins sets of competitive interactions, several with consequences for channel gating. Using gating as a proxy for function, we uncover differential effects of SEC11 binding that are consistent with a subset of binding exchanges essential for SNARE complex assembly. These and additional data lead us to propose that the K+ channels fill a functional role analogous to that of MUNC13, but in a manner that engages membrane voltage to trigger the final protein binding events that drive vesicle fusion.

RESULTS

VAMP721, SNAP33, and SEC11 bind the K+ Channel VSD

We used the mating-based split-ubiquitin system (mbSUS) assay, previously employed successfully to identify K+ channel binding motifs (Honsbein et al., 2009; Grefen et al., 2010; Karnik et al., 2015; Zhang et al., 2015, 2017; Horaruang and Zhang, 2017), to assess channel binding with the cognate SNAREs and identify the relevant binding motifs. The mbSUS assay takes advantage of protein fusions with the N- and C-terminal halves (Nub-X and Y-Cub) of ubiquitin. Reassembly of ubiquitin leads to transactivator cleavage from the Y-Cub fusion, its transit to the nucleus, reporter gene activation, and yeast growth on selective media. The assay offers a number of advantages over other yeast-based screens for protein interactions, including bait suppression in the presence of Met as a test for interaction specificity and the possibility of using full-length, integral membrane proteins.

Initially we screened for channel binding with an overlapping series of channel truncations. Growth was recovered with the diploid yeast expressing Nub-VAMP721 with KC1 truncations on selective media, even in the presence of 50 μM Met, provided that residues 65 to 89 were present in each of the Nub-K+ channel fusion constructs (Fig. 1A; Supplemental Fig. S1). Only with yeast expressing the KC1Δ1–89 truncation was little or no growth observed, indicating that the critical motif for binding was localized near the S1 α-helix of the KC1 channel VSD. Assembly of the SNARE complex with SYP121 and VAMP721 draws on the cognate partner
SNAP33 and is subject to SEC11 (de)binding (Karnik et al., 2017). Therefore, we examined channel binding with these proteins. For mbSUS assays with the otherwise soluble baits SNAP33 and SEC11, we incorporated a glycosylphosphatidylinositol (GPI) anchor (Zhang et al., 2018) to express the corresponding protein fusions with NubG-X fusions of the full-length and truncated channel preys. Again, we recovered growth of the diploid yeast with the KC1 truncations, provided that residues 65 to 89 were present in each of the Nub-K+ channel fusions (Fig. 1, B and C; Supplemental Fig. S1). Similar results were obtained in three independent experiments.

We used Ala scanning mutagenesis in the full-length KC1 channel and mbSUS analysis to isolate the corresponding binding motifs. For VAMP721, the analysis showed that residues R85YxxWE at the base of the S1 α-helix were critical for interaction (Fig. 2A; Supplemental Fig. S1). Analysis of KAT1 interactions yielded the same motif, R58YxxWE motif in this K+ channel (Supplemental Figs. S1 and S2). Similarly, we carried out Ala-scanning mutagenesis in the full-length KC1 channel for motifs essential for SNAP33 and SEC11 binding. In this case, we retransformed yeast with the site-mutated constructs for mbSUS analysis with the GPI-anchored SNAP33 and SEC11 baits (Fig. 2, B and C; Supplemental Figs. S1 and S2). With SNAP33 as the bait, yeast growth was suppressed by Ala substitutions within the same R85YxxWE motif of KC1. Parallel analysis with KAT1 indicated a similar dependence on the R58YxxWE motif, although some growth was recovered with the KAT1E63A mutant (Supplemental Fig. S2). With SEC11 as the bait, yeast growth was suppressed by Ala substitutions at positions Y86RxW of the KC1 channel (Fig. 2C), and a similar pattern, albeit weaker, was observed for KAT1 (Supplemental Fig. S2). Again, similar results were obtained in three independent experiments. These findings indicated that within the base of the S1 α-helix of each K+ channel, a linear sequence of residues incorporates overlapping motifs for binding with each of the major components that contribute to SNARE complex assembly with SYP121.

To confirm the mbSUS results, we carried out pull-down studies in vitro after expressing and purifying the N-terminal cytosolic domains of the two K+ channels. These experiments employed the cytosolic domains of SYP121 and VAMP721 used previously for pull-down studies (Karnik et al., 2013b, 2015; Grefen et al., 2015) and with the soluble, full-length SNAP33 and SEC11 proteins. We expressed and purified the

Figure 1. KC1 K+ channel interacts with VAMP721, SNAP33, and SEC11 via a cytosolic N-terminal region of its voltage sensor domain. A, Schematic of Kv channel structure with the VSD comprising α-helices S1 to S4 identified in red and the pore-lining α-helices S5 and S6 in gray (adapted from Grefen et al. [2015]). Segments expressed for the KC1 deletions are indicated below. B to D, Yeast mbSUS assay for interaction of the VAMP721, SNAP33, and SEC11 fusions with KC1 and its deletion constructs (A) as bait with Y-Cub fusions. VAMP721, SNAP33, and SEC11 served as NubG-X prey fusions and SNAP33 and SEC11 were anchored via the GPI signal peptide (Zhang et al., 2018). Positive and negative controls are included in Supplemental Figure S1. Similar results were obtained in three independent experiments. Growth on CSM-LTUM was used to verify the presence of both bait and prey expression. CSM-LTUMAH was used to verify Ade- and His-independent growth of the yeast diploids. The addition of 50 μM Met to CSM-LTUMAH suppressed bait expression as a test for interaction specificity. Yeast was dropped at 1.0 and 0.1 OD600 in each case. Incubation time was 24 h for the CSM-LTUM plate and 72 h for CSM-LTUMAH plates. Immunoblot analysis (5 μg total protein/lane) of the haploid yeast used in mating (right) used the α-HA antibody for the prey fusions and the αVP16 antibody for the bait fusions.
Flag- and His-tagged constructs to carry out pull-down analyses with purified KC167–91-Flag3-StrepII and KAT11–63-Flag5-StrepII immobilized on a Strep-Tactin affinity resin. After incubation with a 3-fold excess of the prey proteins, unbound protein was washed and protein bound to the resin was eluted and analyzed by SDS-PAGE. We included glutathione S-transferase-tagged iLOV protein as a negative control. iLOV is a soluble phototropin from Arabidopsis (Chapman et al., 2008) and is unrelated to either the SNARE or channel proteins. We found (Fig. 3) that the KC167–91 peptide bound individually with each of the interacting partners identified in the mbSUS assays, SYP121ΔC, VAMP721ΔC, SNAP33, and SEC11, as indicated by the presence of bands of their corresponding sizes and their absence in the resin control. By contrast, no binding to the iLOV protein was recovered. Similar results were obtained with the KAT11–63 bait (Supplemental Fig. S3). Thus, we concluded that the N-terminal cytosolic domain of the K+ channels is a prerequisite for binding of all three cognate SNARE partners, as well as the regulatory SM protein SEC11.

The SNARE Complex Can Incorporate the K+ Channel N Terminus

The ability of the K+ channels to interact via overlapping motifs with all three cognate SNAREs raises the possibility that the SNAREs compete for channel binding. To address this question, we carried out pull-down experiments using the Strept-Tactin-immobilized channel peptides in binary combinations with each of the cognate SNAREs while varying the concentration of either SNAP33 or VAMP721ΔC. The results from one experiment with the KC167–91 peptide are shown in Figure 4A. Similar results were obtained in three independent experiments and with the KAT11–63 peptide (Supplemental Fig. S4), and these are summarized together in Figure 4B. We observed no significant change in the band intensity of SYP121ΔC or VAMP721ΔC with increasing SNAP33, suggesting that SNAP33 does not compete for binding with the channel. By contrast, the binary combination of SYP121ΔC and VAMP721ΔC with either KC167–91 or KAT11–63 showed a near-linear exchange in SYP121ΔC binding with that of VAMP721ΔC, indicating a competition between the Qa- and R-SNAREs for channel binding. Indeed, we were not able to recover SNAP33ΔC and VAMP721ΔC in binary complex alone, suggesting that the Qa- and R-SNAREs, either alone or with the channel N termini, fail to assemble together by contrast with pairings incorporating SNAP33.

Figure 2. KC1 K+ channel interacts with VAMP721, SNAP33, and SEC11 via a common N-terminal motif, RYxxWE, at the base of its VSD. A to C, Yeast mating-based split-ubiquitin assay for interaction of the VAMP721, SNAP33, and SEC11 fusions with KC1 and with Ala substitutions of key residues at the cytosolic face of the VSD as bait with Y–Cub fusions. VAMP721, SNAP33, and SEC11 as NubG-X prey fusions and with SNAP33 and SEC11 anchored via the GPI signal peptide (Zhang et al., 2018). Positive and negative controls are included in Supplemental Figure S1. Similar results were obtained in each of three independent experiments. Growth on CSM-LTUM was used to verify the presence of both bait and prey expression. CSM-LTAB was used to verify Ade- and His-independent growth of the yeast diploids. The addition of 50 μM Met to CSM-LTAB suppressed bait expression as a test for interaction specificity. Yeast was dropped at 1.0 and 0.1 OD600 in each case. Incubation time was 24 h for CSM-LTUM plate and 72 h for CSM-LTAB plates. Immunoblot analysis (5 μg total protein/lane) of the haploid yeast used in mating (right), used the αHA antibody for the prey fusions and the αVP16 antibody for the bait fusions.
Figure 3. Pulldown analysis of the SNAREs and SEC11 with the KC167-91 cytosolic domain. Tagged proteins were expressed and purified from *Escherichia coli* using His-affinity and size-exclusion chromatography before incubation in vitro overnight at 4°C. Shown is one of three independent pulldown experiments with Coomassie-stained SDS-PAGE analysis following separation with Strep-Tactin resin-immobilized KC167-91-Flag6-StrepII. Lane sets are the SDS-PAGE results of (left to right) the pulldown, the resin control, and the loading. Incubations were carried out individually with SYP121Ac-Flag-6His, VAMP721Ac-Flag-6His, Flag3-SNAP33-6His, and SEC11-Flag6-6His. The unrelated iLOV-6His protein (Christie et al., 1998) was included as a control. Note the lower-molecular weight fractions that are common for purified SEC11 (Karnik et al., 2013b).

To assess whether all three SNAREs might assemble in complex with the channels, we also performed pulldowns in parallel with these experiments by mixing Strep-Tactin-immobilized KC167-91 together with SYP121Ac, SNAP33, and VAMP721Ac. We recovered all three SNAREs (Fig. 4A). Again, similar results were obtained with KAT1-63 (Supplemental Fig. S4). To confirm whether the channel peptide was associated with the fully assembled SNARE complex, we added the KC167-91 peptide together with the SNAREs at the start of incubation as well as to the preincubated SNAREs. In this case, the mixes were separated by size exclusion chromatography. We recovered SYP121Ac, SNAP33, and VAMP721Ac with the channel peptide when KC167-91 was combined in the mix concurrently with the SNAREs, but when the SNAREs were preassembled in advance to form a SNARE complex, the SNAREs were recovered without inclusion of the KC167-91 peptide (Fig. 4C). A simple interpretation of these results suggests that the SNAREs assemble in complex with the channel N termini when present, but it does not speak to the possible role or mechanics of K+ channel binding.

SYE21 Coordinates with SNAP33 and VAMP721 in K+ Channel Gating

Voltage-gated K+ (Kv) channel monomers, including those of plant K+ channels, assemble as tetramers to form a functional channel pore (Véry and Sentenac, 2003; Tombola et al., 2006; Dreyer and Blatt, 2009). KAT1 assembles as a homotetramer, but its close homolog, the so-called “silent” KC1 channel monomer, normally functions in vivo as a heterotetramer with the AKT1 K+ channel subunit (Duby et al., 2008; Honsbein et al., 2009). Both channel assemblies show gating that is significantly altered, with opposing effects, by SYP121 and VAMP721 (Honsbein et al., 2009; Grefen et al., 2010, 2015; Zhang et al., 2015, 2017; Lefoulon et al., 2018). However, it is not known whether these characteristics are retained in combination with the cognate Qbc-SNARE SNAP33. To address this question, we assayed channel gating as a proxy to assess the effects of SNARE protein combinations. KAT1 was expressed alone and in combinations with SYP121, VAMP721, and SNAP33 in *Xenopus laevis* oocytes for analysis by two-electrode voltage clamp (Honsbein et al., 2009; Lefoulon et al., 2018). Using KAT1 avoided the technical complications of coexpressing KC1 with AKT1, CIPK23, and CBL1, which are necessary for KC1-AKT1 channel activity (Xu et al., 2006; Honsbein et al., 2009).

Figure 5 shows the mean, steady-state current-voltage curves, and representative current traces from at least five independent experiments with oocytes expressing each combination of proteins. To extract the gating characteristics for the K+ channel in each case, we fitted the steady-state currents to a Boltzmann function of the form

$$I_K = \frac{g_{\text{max}}(V - E_K)}{1 + e^{\delta(V - V_{1/2})/RT}}$$

where $g_{\text{max}}$ is the conductance maximum, $E_K$ is the K+ equilibrium voltage, $V$ is the membrane voltage, and $R$ and $T$ have their usual meanings. Here, the gating characteristic is defined by $V_{1/2}$, the midpoint voltage at which the current reaches half-maximal conductance, and by $\delta$, the apparent gating charge that reflects the sensitivity to a change in voltage. In each case, expression of the proteins was verified by immunoblot analysis (Supplemental Fig. S5). When expressed on its own, KAT1 yielded a large, inward-directed current that activated strongly at voltages negative of ~100 mV, with a $V_{1/2}$ near ~135 mV and $\delta$ of 1.9 (Supplemental Fig. S5), much as has been reported in the past (Hoshi, 1995; Gajdanowicz et al., 2009; Lefoulon et al., 2018). As before (Grefen et al., 2015; Zhang et al., 2015, 2017; Lefoulon et al., 2018), co-expression with SYP121 and with VAMP721 (Fig. 5, A and B) showed opposing effects on KAT1 gating; SYP121 co-expression
Figure 4. The cytosolic domain KC167–91 associates differentially with the cognate SNAREs in binary combination and in complex. A, Tagged proteins were expressed and purified from *E. coli* using His-affinity and size-exclusion chromatography before incubation in vitro overnight at 4°C. Shown is one of three independent pulldown experiments with Coomassie-stained SDS-PAGE analysis following separation with Strep-Tactin resin-immobilized KC167–91-Flag3-StrepII. Lane sets are the SDS-PAGE results of pulldowns with a 3-fold excess of SYP121D and VAMP721D with competing additions of 0, 0.3-, 1-, and 3-fold excess of VAMP721D SNAP33, as indicated (above). Also shown are the pulldown results on incubation of all three SNAREs with the K+ channel peptide (right). SNAREs were incubated at 4°C overnight with Strep-Tactin-immobilized K+ channel peptide-Flag3-StrepII. Fixed concentrations (5 mM) of SYP121D and VAMP721D were used in each case. Similar results for KAT11–63 are shown in Supplemental Figure S4. B, Bound fraction analysis of KC161–97 and KAT11–63 pulldowns from all six independent experiments. Data are means ± SE of the ratios of SNAREs to K+ channel peptide. Lowercase letters indicate statistical differences at *P* < 0.05. C, Comigration of KC167–91 peptide-SNARE complex on gel filtration. The KC167–91 peptide-SNARE complex assembly reactions...
displaced \( V_{1/2} \) to more positive voltages and enhancing \( g_{\text{max}} \), while VAMP721 co-expression displaced \( V_{1/2} \) to more negative voltages and reduced \( g_{\text{max}} \) when compared with oocytes expressing KAT1 alone. In oocytes coexpressing KAT1 with SNAP33 (Fig. 5A), we observed no appreciable change in KAT1 current and values for \( V_{1/2} \) and \( g_{\text{max}} \) were similar to those resolved from measurements from oocytes expressing KAT1 alone. Thus, SNAP33 appeared to have no significant effect on channel activity, even though the protein bound the KAT1 VSD.

We next asked whether SNAP33 might act together with either of its cognate SNAREs to affect KAT1 activity. Again, we recorded \( K^+ \) current under voltage clamp after expressing KAT1 alone, together with binary combinations of the SNAREs SYP121, VAMP721, and SNAP33, and with the tertiary combination of SYP121, SNAP33, and VAMP721, in each case verifying protein expression by immunoblot analysis (Supplemental Fig. S5). Analysis of the results from five independent experiments with each construct combination (Fig. 5, B–D; Supplemental Fig. S5) showed that coexpression of SNAP33 with SYP121 yielded a mean \( V_{1/2} \) similar to that observed on expressing KAT1 with SYP121 on its own. A substantial impact of SNAP33 on KAT1 gating was observed only in binary combination with VAMP721, which displaced \( V_{1/2} \) by \(-25 \) mV compared to KAT1 alone and by \(-6 \) mV compared to KAT1 with VAMP721 (Fig. 5B and Supplemental Fig. S5). We also examined the effect of coexpressing SYP121 with VAMP721; in this case, the result was a displacement of \( V_{1/2} \) similar to that with VAMP721 alone, suggesting a dominance of VAMP721 over SYP121 for channel interaction in binary combination (Fig. 5C). However, when coexpressing KAT1 together with all three cognate SNAREs, we obtained \( K^+ \) currents with \( g_{\text{max}} \) reduced by \( 23 \pm 5 \% \) but with a small positive displacement of \( V_{1/2} \) compared with KAT1 alone (Fig. 5D; Supplemental Fig. S5). The results thus clearly showed a dominance of the Qa- and R-SNAREs in binary SNARE combinations on channel gating and their moderation of the current in tertiary combination. We return to these points later.

**K\(^+\) Channel Facilitates the SYP121 Conformational Changes for Oligomerization**

Previously we hypothesized that \( K^+ \) channel binding with SYP121 facilitates transition of the Qa-SNARE to the open conformation for assembly with its cognate SNARE partners (Karnik et al., 2017). A central question behind this idea is whether channel binding alters SYP121 conformational structure. To address this question, we used circular dichroism (CD) to compare the secondary structural content of equal concentrations...
of SYP121\textsuperscript{C} and KC1\textsuperscript{67–91} alone and in mixture. Equivalent experiments were carried out with the KAT1\textsuperscript{1–63} peptide. The far-UV CD spectrum typically shows maxima and minima between 200 and 230 nm that reflect this structural content. Thus, we anticipated that the spectrum of each mixture would be identical to the arithmetic sum of the spectra for channel peptide and SYP121\textsuperscript{C} alone, provided that neither affected the structure of the other. In fact, the CD spectra of SYP121\textsuperscript{C} with KAT1\textsuperscript{1–63} yielded a 15 ± 4% enhancement in the minima at 208 and 222 nm (Supplemental Fig. S6) and a 28 ± 5% enhancement with the KC1\textsuperscript{67–91} peptide (Fig. 6A), suggesting that interaction with the channel peptides was sufficient to alter the Qa-SNARE and channel peptide conformations. We also added KC1\textsuperscript{67–91} together with SEC11. In this case, we observed no substantive change in the CD spectrum beyond that calculated from the sum of the individual peptides (Fig. 6A), indicating that the SM protein suppressed the effects of the channel on SYP121.

Several mammalian and yeast Qa-SNAREs are known to form homo-oligomers in the open conformation when the N-terminal Habc a-helices release the so-called H3 domain, exposing it for SNARE binding (Fasshauer et al., 1998b; Lerman et al., 2000; Misura et al., 2001; Sieber et al., 2006, 2007). SYP121 is no exception to this mode of interaction (Kargul et al., 2001). As an independent test of the Qa-SNARE conformation, we therefore asked whether the channel peptide might promote SYP121\textsuperscript{C} homo-oligomerization. We incubated SYP121\textsuperscript{C} in solution alone and together with KC1\textsuperscript{67–91}, thereafter carrying out size-exclusion chromatography to determine the resulting elution patterns. The results (Fig. 6, B and C) showed a single prominent peak when SYP121\textsuperscript{C} passed through the column on its own. With increasing additions of the KC1\textsuperscript{67–91} peptide, a reduction in the size of this peak was evident with the appearance of shoulders and peaks at higher molecular weight retention volumes, the appearance of SYP121\textsuperscript{C} in the early-eluting fractions (Fig. 6C) along with a peak near 13 kD that corresponded with the channel peptide (Fig. 6, B and C). The lack of obvious coelution of the channel peptide in

Figure 6. The K\textsuperscript{+} channel binding domain alters SYP121 structure to promote the open conformation. A, Far-UV CD spectra of KC1\textsuperscript{67–91} with SYP121\textsuperscript{3C–2PA} mixed and incubated overnight in equimolar ratio shows roughly a 24% increase in spectral peak amplitudes between 200 and 230 nm above the arithmetical sum of individual spectra for the two proteins (top). This shift in peak amplitudes is suppressed on coincubation with SEC11 (bottom). Data are from one of three independent experiments, all of which yielded equivalent results with a mean of 28 ± 5% in peak amplitude above the spectral sum. Similar results were obtained with KAT1\textsuperscript{1–63} (see Supplemental Fig. S6). B, Size-exclusion chromatography of SYP121\textsuperscript{3C–2PA} incubated overnight at 4°C alone and on coincubation with KC1\textsuperscript{67–91}. In the closed conformation, SYP121\textsuperscript{3C–2PA} eluted as a single peak corresponding to a molecular weight around 48 kD. When mixed with increasing amounts of the channel peptide, this peak was reduced in favor of a higher-molecular weight shoulder and peak spanning to near 650 kD, consistent with transition to the Qa-SNARE open conformation and formation of multimers. Molecular weights of standards are indicated above the columns. C, SDS-PAGE analysis of the gel filtration eluate collected in 0.75-mL fractions for fractions 11 to 24 collected after overnight incubations of SYP121\textsuperscript{3C–2PA} alone, with KC1\textsuperscript{67–91}, and with KC1\textsuperscript{67–91} together with SEC11. Note the shift of the SYP121\textsuperscript{3C–2PA} to higher-molecular weight fractions in the presence of the channel peptide and its suppression when SEC11 is included in the incubation mix. Molecular weights of standards are indicated below the columns.
this case is consistent with its lower component fraction in incubation, suggesting a catalytic role in promoting the Qa-SNARE open conformation and exclusion of the channel peptide from the SYP121^{AC} oligomers. This interpretation is also consistent with the effects of SEC11. When the SM protein was included, we observed a loss in Qa-SNARE oligomers but no change in the channel peptide distribution (Fig. 6C). Thus, these findings strongly suggest that the channel is able to catalyze conformational changes in SYP121^{AC} that expose the H3 domain, facilitating homo-oligomerization of the Qa-SNARE, but in the presence of SEC11, these conformational changes are suppressed (Fasshauer et al., 1998a).

SEC11 Coordinates SNARE Binding Differentially with Channel Gating

SEC11 is an important regulator of SYP121-mediated vesicle traffic (Karnik et al., 2013b, 2015). Like other SM proteins, SEC11 binds with SYP121 in both the closed and open conformations via two different domains of the SM protein. Binding of a minor cleft of SEC11 with the N terminus of SYP121 overlaps with the K^{+} channel-binding motif F^{x}RF (Grefen et al., 2010), but SEC11 binding also occurs in association with a separate domain independent of channel binding with the Qa-SNARE and associated with the R^{20R}^{21} motif of SYP121 (Zhang et al., 2019). SEC11 itself also binds the K^{+} channels, the binding motif overlapping with that for channel-SYP121 binding (Figs. 1 and 2). These are characteristics expected of a three-way binding exchange between SYP121, SEC11, and the K^{+} channels. Thus, we wanted to know whether the channel peptide and SEC11 compete for or facilitate SYP121 binding, and whether the SM protein might have complementary effects on binding between the other cognate SNARE partners.

To examine the role of SEC11 in the SNARE channel, we used pulldown experiments as before, employing the Strep-Tactin-immobilized channel peptides and cognate SNAREs without and with SEC11 in molar ratios of 0.3-, 1-, and 3-fold the concentration of SNAREs. Adding SEC11 to the cognate SNAREs individually (Fig. 7, A–C) resulted in a corresponding loss in recovery of the SNAREs, notably with VAMP721 and SNAP33. Adding SEC11 to binary SNARE combinations (Fig. 7, D–F) showed an enhanced recovery of SNAP33 with SYP121^{AC} compared to on its own. By contrast, in binary combination with VAMP721^{AC}, increasing SEC11 showed a reduced recovery of both SNAREs. Finally, combining the cognate SNAREs (Fig. 7G) led to a recovery of all three SNAREs in constant ratio of three independent experiments in each case. Similar results were obtained with the KAT1^{1-43} peptide (Supplemental Fig. S7), and the results of all experiments are summarized in Figure 8.

Figure 7. SEC11 competes differentially with KCI^{67-91} for cognate SNARE binding. Pulldown assays with KCI^{67-91} alone and with 0.3-, 1-, and 3-fold molar excess of SEC11 together with each of the cognate SNAREs, as indicated (top). Binding was tested with the SNAREs singly (A–C), in binary (D–F), and in tertiary (G) combinations, as indicated. Incubations were carried out overnight at 4°C before separation. Shown are Coomassie-stained SDS-PAGE gels of the bound proteins from one
with increasing SEC11, although at high concentration of the SM protein the total recovery was reduced. Similar results were obtained in each of three independent experiments with KAT11–63 (see also Supplemental Fig. S7), and the combined results are summarized in Figure 8. These findings suggest that SEC11 affects differentially the association of the channel with SYP121AC and with VAMP721AC, it favors SNAP33 together with SYP121AC, and it stabilizes the SNARE complex when all three cognate partners are present.

To assess the consequences of SEC11 in combination with the cognate SNARE partners in vivo, again we recorded KAT1 current under voltage clamp on coexpression with SEC11 and the several SNARE combinations in Xenopus oocytes. Figure 9 summarizes the mean steady-state current-voltage curves recorded from at least five independent experiments, in each case with representative current traces for each combination of SNARE components as well as the fitted parameters for gating (Supplemental Fig. S5). Immunoblots confirming protein coexpression are included in Supplemental Fig. S5. Expressing KAT1 with SEC11 alone had no significant effect on the K+ current or its gating characteristics, even though the SM and channel proteins

Figure 8. Bound fraction analysis of KC161–97 and KAT11–63 pulldowns from all six independent experiments alone and with SEC11 additions. Binding was with the SNAREs singly (A–C), in binary (D–F) and in tertiary (G) combinations as indicated and includes the results of experiments shown in Figure 7 and Supplemental Figure S7. Data are means ± SE of the ratios of SNAREs and SEC11 bound to K+ channel peptide. Letters indicate statistical differences at P<0.05.
bound in vitro (Fig. 3; Supplemental Fig. S3). Similarly, expressed in combination with VAMP721 or with SNAP33, we found that SEC11 protein had little or no effect on channel current and gating (Fig. 9, B and C); in each case, the current characteristics and fitted values for \( V_{1/2} \) (Supplemental Fig. S5) were indistinguishable from those with the SNAREs alone. Similarly, coexpression of SEC11 with the SNARE combinations with SNAP33 and SYP121 with VAMP721 had no appreciable effect additional to that of the SNAREs alone (Fig. 9, D and F; Supplemental Fig. S5). Expressing SEC11 with SYP121 and SNAP33 recovered a current similar to that of KAT1 alone, albeit with a small but significant positive shift in \( V_{1/2} \) (Fig. 9D). However, expressed with VAMP721 and SNAP33, SEC11 strongly suppressed the channel current (Fig. 9E), shifting \( V_{1/2} \) to extreme negative voltages (Supplemental Fig. S5).

Of particular interest, we also found that expressing SEC11 with SYP121 alone increased \( g_{\text{max}} \) and yielded a modest negative-going shift in \( V_{1/2} \) when compared with the effect of coexpressing SYP121 alone (Fig. 9A), an effect that was essentially complete at a SEC11:SYP121 molar ratio of unity (Supplemental Fig. S8). In this case, the result was a steady-state current that followed the KAT1 current at more positive voltages and the KAT1+SYP121 current at more negative voltages, indicating a pronounced voltage dependence to the action on gating of SEC11 with the Qa-SNARE, as if the efficacy of the SM protein is suppressed with negative-going voltage. Finally, when coexpressing all three SNAREs with SEC11, we found that the KAT1 current was moderated (Fig. 9G), much as in the absence of SEC11 (Fig. 5D) and with no significant change in \( V_{1/2} \) (Supplemental Fig. S5). These data, together with the biochemical analyses above, indicate a critical role for SYP121 interactions with the K\textsuperscript{+} channels and their coordination with SEC11 in triggering SNARE complex assembly. We return to this point below.

**DISCUSSION**

SNARE assembly, including that of SYP121, VAMP721, SNAP33, and the SM protein SEC11 (Lipka et al., 2007; regression to the Boltzmann function of Equation 1. Each panel includes representative current traces cross-referenced by symbol. Representative immunoblot analyses and fitting results are included in Supplemental Figure S5. Data for KAT1 alone and with the individual SNAREs are reproduced in selected panels for reference. Water-injected controls yielded currents of <300 nA at all voltages and are omitted for clarity. Scales represent 1 s (horizontal) and 6 \( \mu \text{A} \) (vertical). A, KAT1 alone (○) and coexpressed with SYP121 (●) and SYP121 and SEC11 (♦). B, KAT1 alone (○) and coexpressed with VAMP721 (▼) and VAMP721 and SEC11 (●). C, KAT1 alone (○) and coexpressed with SNAP33 (Δ) and SNAP33 and SEC11 (▲). D, KAT1 alone (○) and coexpressed with SYP121 (●), SYP121 and SNAP33 (Δ), and SYP121, SNAP33, and SEC11 (▲). E, KAT1 alone (○) and coexpressed with VAMP721 (▼), VAMP721 and SNAP33 (●), and VAMP721, SNAP33, and SEC11 (▲). F, KAT1 alone (○) and coexpressed with VAMP721 (▼), VAMP721 and SNAP33 (●), and VAMP721, SNAP33, and SEC11 (▲). G, KAT1 alone (○), and coexpressed with VAMP721, VAMP721, SNAP33, and SEC11 (▲).
Plant Physiol. Vol. 181, 2019 1107

was affected by SNAP33. These with a three-way exchange between the partners that
conformation and channel gating that are consistent
and with SEC11, showed alterations in Qa-SNARE (3) Finally, SYP121 association with the channels, alone
in tertiary combination with all three cognate SNAREs.
was not, either in binary combination with SNAP33 or
VAMP721 was suppressed, but binding with SYP121
SEC11, channel peptide binding with SNAP33 and
pulldown with the channel peptide. (2) Titrated against
in binary combination without SNAP33; with SNAP33
exhibit a strong competition for channel binding, and only
and stabilized by SNAP33. (1) Only SYP121 and VAMP721
bind with the N-terminal cytosolic domain of the K\(^+\) channels KAT1 and
KC1 through the conserved linear motif RYxxWE
situates at the cytosolic base of the first transmembrane
\(\alpha\)-helix of the channel VSD. This motif overlaps also in
binding of the SM protein SEC11, implying a highly
concerted sequence of binding exchanges with the K\(^+\) channels. Three lines of evidence suggest that channel
binding is dominated by the Qa-SNARE SYP121 and is
coordinated through a binding exchange with SEC11
and stabilized by SNAP33. (1) Only SYP121 and VAMP721
exhibit a strong competition for channel binding, and only
in binary combination without SNAP33; with SNAP33
present, all three cognate SNAREs were recovered in
pulldown with the channel peptide. (2) Titrated against
SEC11, channel peptide binding with SNAP33 and
VAMP721 was suppressed, but binding with SYP121
was not, either in binary combination with SNAP33 or
in tertiary combination with all three cognate SNAREs.
(3) Finally, SYP121 association with the channels, alone
and with SEC11, showed alterations in Qa-SNARE
conformation and channel gating that are consistent
with a three-way exchange between the partners that
was affected by SNAP33. These findings are most easily
understood in the context of channel binding that co-
ordinates initially with SEC11 to drive SYP121 from its
closed to open conformation, enhancing channel ac-
tivity for K\(^+\) flux and thereafter tempering K\(^+\) uptake
with tertiary SNARE assembly. They indicate, too, that
SNAP33 is important in moderating channel binding
with SYP121 and VAMP721. Thus, we suggest that the
primary action of the K\(^+\) channels is in a handover
between SEC11 and SYP121 that allows the conforma-
tional transition of the Qa-SNARE for binding with its
cognate SNARE partners. We also suggest that channel
association with the R-SNARE alone and in combina-
tion with SNAP33 may serve as a fail-safe, precluding
interactions that might otherwise disengage ion trans-
port and secretory traffic.

SNAP33 Facilitates K\(^+\) Channel Activity with SNARE Complex Assembly

Most surprising was the finding that K\(^+\) channel
binding for all three cognate SNAREs was isolated to a
short region of the channel N terminus and incorpo-
rated a common motif (Figs. 1 and 2; Supplemental
Figs. S1 and S2). This motif is located at the cytosolic
face of the plasma membrane immediately preceding
the first transmembrane \(\alpha\)-helix of the channel VSD.
The VSD structure itself is highly conserved among
voltage-gated (Kv) K\(^+\) channels across kingdoms and
it confers a voltage dependence to channel opening,
including that of KAT1 (Lefoulon et al., 2014), by cou-
pling movement of the VSD within the membrane to
the channel pore (Palovcak et al., 2014). Significantly,
the RYxxWE is largely conserved among a subset of Kv
channels of land plants, is critical for binding SYP121
that confers a voltage dependence on secretory traf-
icking, with a dominance of VAMP721
VAMP721 and SNAP33 raises obvious questions of
competition for channel binding and connections
to the voltage sensitivity of channel gating. Indeed,
pulldown analysis with the K\(^+\) channel N termini as
bait showed that SYP121 and VAMP721 compete for
binding (Fig. 4; Supplemental Fig. S4). Furthermore,
functional analysis using the KAT1 channel current
as a proxy for SNARE interaction showed opposing
effects on gating, with a dominance of VAMP721
over SYP121 (Fig. 5; Supplemental Fig. S5). The latter
findings extend those of previous electrophysio-
logical studies implicating the apposition of the Qa-
and R-SNAREs on the K\(^+\) channels (Honsbein et al., 2009;
Grefen et al., 2015; Lefoulon et al., 2015; Zhang et al., 2015). No such action
was evident with SNAP33 alone, however, either in
pulldown or electrophysiological experiments. From
these data, we cannot rule out binary binding pop-
ulations of the channel bait with SNAP33 and its
cognate SNAREs or their possible displacements of
SNAP33 from action on the KAT1 channel in vivo.
However, such explanations are difficult to reconcile
with the observations that the channel N termini re-
covered all three cognate SNAREs in roughly a 1:1:1 ratio when added together (Fig. 4; Supplemental
Fig. S4). Furthermore, SNAP33 in binary combination
with VAMP721 enhanced the effect of the R-SNARE in
suppressing the KAT1 current, while its inclusion to-
gether in tripartite combination abolished the apparent
competition of SYP121 and VAMP721 for KAT1 gating
and tempered channel activity in vivo (Figs. 5 and 9).
These findings argue instead that the channels preferentially coordinate with all three SNAREs as they assemble in complex, and they suggest that SNAP33 acts to moderate the functional impact of the Qa- and R-SNAREs, favoring a relaxation of K⁺ flux through the channels on final SNARE complex assembly.

It is worth noting that the actions of SNAP33, like those of SYP121 in vivo (Honsbein et al., 2009; Lefoulon et al., 2018), differ from those of its animal counterpart, SNAP25. Binding and functional studies have suggested that in mammalian cells, SNAP25 may suppress K⁺ and Ca²⁺ channel gating either alone or together with its cognate Qa-SNARE (MacDonald et al., 2002; Leung et al., 2007; Weiss and Zamponi, 2012). These effects are modest, and there remains some controversy as to whether the actions of SNAP25 are associated with its cleavage products (He et al., 2008). Furthermore, assembly of SNAP25 and its cognate SNAREs Syntaxin 1A and VAMP2 abolishes K⁺ channel binding and eliminates SNARE actions on channel activity (Tsuk et al., 2008), raising a basic question about its functional significance in the temporal coordination of membrane excitability.

**SEC11 Complements K⁺ Channel Binding for SYP121**

Conformational Transitions

A further dimension to SNARE-channel coordination is added with our discovery that SEC11 binds the K⁺ channels. Like other SM proteins (Südhof and Rothman, 2009; Archbold et al., 2014), SEC11 binding with the SYP121 N terminus is thought to serve in part as an anchor point for the SM protein during Qa-SNARE transit from the closed to the open state (Karnik et al., 2013b, 2015). SEC11 binding with SYP121 is subject to the highly conserved Phe-9 that situates within the F9xRF motif for the K⁺ channel (Grefen et al., 2010). SEC11 also binds the Qa-SNARE N terminus via a second site centered on a unique R²⁰R³² motif independent of channel binding, an observation that has led to the suggestion of a binding exchange and displacement of the SM by the channel proteins (Zhang et al., 2019). This concept of an exchange cascade now gains further support. We observed that SEC11 interacts with the channels through a motif overlapping with the RYxxWE SNARE-binding motif (Fig. 2; Supplemental Fig. S2) and, furthermore, that it is able to compete for channel binding with each of the cognate SNAREs individually but with reduced efficacy with the binary combination of SYP121 and SNAP33 (Figs. 4, 7, and 8; Supplemental Figs. S4, S7, and S8). Furthermore, SEC11 interaction with KAT1 showed a pronounced voltage dependence when SYP121 was present (Supplemental Fig. S8). We also found that the channel N-termini facilitated SYP121 homo-oligomerization, a common feature of open Qa-SNAREs that is enhanced in the soluble domain and is suppressed by SM protein binding (Dawidowski and Cafiño, 2013), for SYP121 by SEC11 (Fig. 6; Supplemental Fig. S6). These findings indicate that the channels compete with SEC11 to promote transit to the open Qa-SNARE conformation, which, when the Qbc-SNARE SNAP33 is present, may stabilize SYP121 binding with the K⁺ channels.

Features of the K⁺ currents offer additional insight into possible mechanisms of a binding exchange with the SNAREs. Unlike the pulldown experiments, channel currents recorded under voltage clamp speak to the interactions of the full-length channel protein in the membrane and the impact of membrane voltage. These data (Figs. 5 and 9; Supplemental Fig. S8) show that SEC11 had little impact on KAT1 channel gating with VAMP721 alone. However, when coexpressed with SYP121, SEC11 promoted a KAT1 current that approximated the characteristics of KAT1 alone near and positive of −120 mV and was augmented like that of KAT1 with SYP121 alone at more negative voltages. In other words, SEC11 associated functionally with the KAT1-SYP121 combination such that the functional impact of its binding was suppressed at more negative voltages. At present, it is not possible to resolve the voltage dependence of any of these complex interactions directly in vitro. Nonetheless, the current characteristics suggest that membrane hyperpolarization favors K⁺ channel association with SYP121 binding, overcoming SEC11 action, and that the voltage dependence introduced by SEC11 is moderated when SNAP33 is also present. These findings are entirely consistent with the enhancement of K⁺ uptake and voltage dependence conferred on traffic by the channel VSD (Honsbein et al., 2009; Grefen et al., 2015).

How might these characteristics of SNARE and SM binding and channel gating be understood in the context of SNARE assembly? Secretary traffic mediated by SYP121 shows a substantial dependence on membrane voltage coordinated through the channel VSD with K⁺ flux enhancement (Honsbein et al., 2009; Grefen et al., 2015) and is uniquely subject to SEC11 (Karnik et al., 2013b, 2015). We note, too, that SEC11 binding altered channel voltage sensitivities in association with SYP121, but not in association with VAMP721 alone (Figs. 5 and 9). Thus, it follows that K⁺ channel binding with the R-SNARE “locks out” further transition of the complex to suppress secretory traffic and K⁺ flux. This interpretation accords with previous findings that the vamp721 mutant showed a modest enhancement of growth and that growth was suppressed on overexpressing VAMP721 (Zhang et al., 2015, 2017). We speculate that binary interactions centered around the R-SNARE reflect a fail-safe mechanism that prevents departure from the temporal sequence of binding transitions that lead to vesicle fusion.

By the same token, we note that SYP121, like other Qa-SNAREs (Südhof and Rothman, 2009; Rizzo and Südhof, 2012; Archbold et al., 2014), is thought to be held in closed conformation by SEC11, with the SNARE-binding (H3) domain occluded. Thus, debinding of the SEC11 major cleft and opening of the Qa-SNARE is an early step driving SNARE assembly and vesicle fusion (Karnik et al., 2013b, 2015). Here, channel binding
with SYP121 is important for the transition of SYP121 to the open form, likely displacing SEC11 binding with the Qa-SNARE through a three-way binding exchange that promotes the K⁺ current and culminates with SYP121 in the open conformation, bound with the channel, SNAP33, and SEC11, the latter via its minor cleft (Karnik et al., 2013b, 2015). Our results are most easily explained in the context of such a model (Fig. 10). The data are consistent with a stabilization of this state through its association with SNAP33, thereby priming the Qa- and Qbc-SNAREs for final assembly with VAMP721. The model is also consistent with an enhanced rate of K⁺ uptake that is temporally coordinated with vesicle fusion. Indeed, even a 2- to 3-fold increase in K⁺ flux, as implicated for the current enhancement with SYP121 (Figs. 5 and 9), is likely to be sufficient to support K⁺ with cell expansion if it is maintained for 20% of the cycle period, as illustrated

Figure 10. K⁺ channels KAT1 and KC1 facilitate a binding exchange with SEC11 to promote SNARE assembly for vesicle fusion. The SM protein SEC11 (A) holds SYP121 in the closed conformation through its major cleft and also binds via L₁²⁸ (circled L) with the Qa-SNARE N-terminal motif centered on residue F₉ (circled F; Karnik et al., 2013b, 2015). SYP121 harbors a second SEC11-binding site centered on its R²⁰₀⁻R²¹¹ motif (circled RR) that does not affect channel binding (Zhang et al., 2019). SEC11 interacts with the K⁺ channels through the RYxxWE motif (circled RE) and, with membrane hyperpolarization (+, −), undergoes a three-way binding exchange between SEC11 and SYP121 that culminates with binding through the two motifs, the channel RYxxWE motif (RE) and the Qa-SNARE FxRF motif (F), thereby conferring an apparent voltage dependence on the channel-SM protein interaction between SEC11 and SNAP121 (Supplemental Fig. S8) and enhancing channel gating and activity (Honsbein et al., 2009; Grefen et al., 2010, 2015). The Qbc-SNARE SNAP33 (B) stabilizes the complex of SYP121, SEC11, and the K⁺ channel to moderate the open channel, Qa-SNARE, and SM protein conformations (Fig. 9). Recruiting the R-SNARE VAMP721 (C) facilitates final assembly of the SNARE core complex and transfer of channel binding (D) to SNAP33 (unknown site [circled U]) while relaxing channel gating and conductance (Fig. 9). Finally, disengaging channel binding with the cis SNARE complex (E) is followed by SNARE complex disassembly. Red arrows by each step in the cycle indicate the nominal channel activity for K⁺ uptake and its anticipated enhancement with the open conformation of SYP121 prior to assembly with VAMP721. Note that binding motifs on SEC11 and SNAP33 for the K⁺ channels are yet to be determined. The binding motif on SEC11 associated with the SYP121 R²⁰₀⁻R²¹¹ motif is also unknown. The time-averaged K⁺ flux needed to support cell expansion, for example during stomatal opening, is equivalent to a current of 1 to 3 µA cm⁻² on a cell surface basis and is readily accommodated by KAT1 at voltages near −100 to −120 mV in vivo (Jezek and Blatt, 2017).
In effect, our interpretation implies a role for the K+ channels analogous to that of MUNC13 in orchestrating the binding release of the SM protein Munc18 from Syntaxin 1A for secretion in mammalian neurons (Ma et al., 2013; Wang et al., 2019). It accords, too, with previous studies demonstrating that the K+ channels accelerated secretory traffic dependent on binding with the channel VSD (Grefen et al., 2015), and with the finding that, once bound, the Qa-SNARE interaction with the channel is stable relative to the conformational lifetimes of the channel (Lefoulon et al., 2018).

In conclusion, we propose that binding exchange between SEC11 and the K+ channels with SYP121 represents the critical starting point that regulates the final steps to vesicle fusion while promoting K+ uptake. Such a mechanism ensures the timely engagement of the Qa-SNARE with membrane voltage as a common measure of the transport activities driving solute flux for cell expansion. We suggest, too, that K+ channel association with the cognate SNARE SNAP33 is important to stabilize the nascent Q-SNARE complex at the plasma membrane in preparation for final assembly with the R-SNARE and vesicle fusion. Thus, SEC11 and K+ channel binding unite to define a unique, temporal sequence that juxtaposes ion transport with enhanced secretory vesicle traffic.

MATERIALS AND METHODS

Molecular Biology

Escherichia coli strains of BL21(DE3) were used as a host for protein expression (Invitrogen) and constructs were cloned into pETDuet (Carp et al., 2006) and pRSFDuet (Addgene) expression vectors. We used a classical cloning strategy to generate expression cassettes combining the multiple flag tags (Flag, and Flag3) of the vectors with selections of 6His, 2Protein A (2PA), or Strep-II affinity tags on both upstream and downstream positions. Constructs tagged with 2PA were those described previously (Karnik et al., 2013b). The cassettes were integrated into the backbone of pETDuet and pRSFDuet using NotI and NotI restriction sites. The constructs were obtained by sequenc- ing and named pETDuet-Flag and pRSFDuet-Flag. Open reading frames for SYP121 and VAMP21 were cloned into pDONR207 as described previously (Grefen et al., 2010, 2015; Grefen et al., 2015; Horaruang and Zhang, 2017). Bait constructs were transformed into THY.AP4 and prey constructs were transformed into THY.APS. Ten to 15 yeast colonies were selected and inoculated into selective media (complete supplement mixture [CSM]_LM for THY.AP4 and CSM_AH for THY.APS) for overnight growth at 180 rpm and 28°C. Liquid cultures were harvested and resuspended in yeast peptone dextrose (YPD) medium. Yeast mating was performed in sterile PCR tubes by mixing equal aliquots of yeast containing bait and prey constructs. Aliquots of 5 μL were dropped on YPD plates and incubated at 28°C overnight. Colonies were transferred from YPD plates onto CSM_AH and incubated at 28°C for 2 to 3 d. Diploid colonies were selected and inoculated in liquid CSM_LMTU media and grown at 180 rpm 28°C overnight before harvesting by centrifugation and resuspension in sterile water. Serial dilutions at OD600 1.0 and 0.1 in water were dropped (5 μL per spot) onto CSM_AH+Met plates with added Met. Plates were incubated at 28°C and images were taken after 3 d. Yeast was also dropped on CSM_LMTU control plates to confirm mating efficiency and cell density, and growth was imaged after 24 h at 28°C. To verify expression, yeast was harvested and extracted for protein immunoblot analysis using commercial hemagglutinin (HA) antibody for prey and commercial VP16 antibody (Abcam) for the bait.

Recombinant Protein Expression and Purification

pETDuet-Flag and pRSFDuet-Flag plasmids containing each construct were chemically transformed into E. coli BL21(DE3) cells. After incubating overnight at 37°C on tryptone glycerol (LB) 1% (w/v) agar plates, single colonies were picked and inoculated into 5 mL LB medium with 100 μg/mL Ampicillin and incubated at 37°C in a shaker at 180 rpm overnight. The 5 mL culture was transferred into 500 mL Terrific Broth (Thermo Fisher Scientific) medium supplemented with 50 μg/mL Ampicillin and incubated at 37°C and 180 rpm to an OD600 of 1.2. Thereafter, the culture was transferred to 18°C and induced with 1 mM isopropylthio-β-D-galactoside overnight before harvesting by centrifugation at 4,000 g for 10 min. Pellets were suspended in 20 mL lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 5% [v/v] glycerol, and 0.1% [v/v] Triton X-100) pH adjusted to 8.0 and centrifuged at 37°C on lysogeny broth (LB) 1% (w/v) agar plates, single colonies were selected and inoculated in liquid CSM-LMTU media and grown at 180 rpm 28°C overnight before harvesting by centrifugation and resuspension in sterile water. Serial dilutions at OD600 1.0 and 0.1 in water were dropped (5 μL per spot) onto CSM_AH+Met plates with added Met. Plates were incubated at 28°C and images were taken after 3 d. Yeast was also dropped on CSM_LMTU control plates to confirm mating efficiency and cell density, and growth was imaged after 24 h at 28°C. To verify expression, yeast was harvested and extracted for protein immunoblot analysis using commercial hemagglutinin (HA) antibody for prey and commercial VP16 antibody (Abcam) for the bait.

Pulldown and Gel Filtration Analysis

Pulldown experiments were performed using KC167, KC167-Flag-StrepII and KAT1-6flag-StrepII as bait immobilized on Strep-Tactin affinity resin. All reactions were carried out by adding prey proteins in the designated molar ratios and incubating the mixtures overnight at 4°C with gentle rotation.
Pulldowns were analyzed after washing with the same buffer by eluting bait and bound proteins from the resin for SDS-PAGE and Coomassie staining.

Gel filtration chromatography was carried out using a Superdex 200 10/300 column (Generon) coupled to an AKTA purifier (GE Healthcare). Samples were loaded to the column equilibrated with 50 mM Tris-HCl and 150 mM NaCl, pH 8.0 buffer. The elution was performed at a flow rate of 0.5 mL/min and monitored at 220 nm, and fractions were collected for analysis by SDS-PAGE. Molecular weights were quantified in parallel chromatographic separations of the established marker proteins bovine thyroglobulin (670 kD), gammaglobulin (158 kD), chicken ovalbumin (44 kD), horse myoglobin (17 kD), and vitamin B12 (1.4 kD). Band intensities on SDS-PAGE were quantified using ImageJ v.1.52 https://imagej.nih.gov/ij/ and standardized against known molar quantities of the same proteins. Bound fractions were calculated from these values relative to the eluted quantities of the relevant bait proteins.

CD Spectroscopy

CD was determined using a Jasco J-810 spectropolarimeter (Jasco) at 20°C. Far-UV CD data were acquired in a quartz cuvette with a 0.02 cm pathlength using a scan rate of 50 nm/min and response time of 0.5 s. Spectra were collected over 185 to 260 nm with a 1-nm bandwidth after correction with blank spectra of the buffer. A final protein concentration of 0.3 mg/mL in 50 mM Tris, 150 mM NaCl, and 20 mM ammonium acetate, pH 8.0, was used in every case. Spectral data were analyzed using Jasco Spectro Analysis software.

Electrophoresis and Immunodetection

The complementary RNA of each construct was transcribed after template linearization using T7 mMessage mMachine (Ambion). Complementary RNA was confirmed by gel electrophoresis and injected into stage VI Xenopus oocytes, as before (Lefoulon et al., 2014, 2018; Grefen et al., 2015) in molar ratios as indicated. Electrical analysis was carried out over a period of 3 to 5 d postinjection. Whole-cell currents were recorded under voltage clamp using an Axoclamp 2B amplifier (Axon Instruments) and data were acquired after filtering with a 1 Hz (f) 8-pole Bessel filter. Measurements were carried out with oocytes under continuous perfusion with osmotically balanced solutions containing 30 mM KCl, 70 mM NaCl, 1 mM CaCl₂, 1.5 mM MgCl₂, and 10 mM HEPES-NaOH, pH 7.4. Recordings were analyzed postacquisition using Henry IV software (Y-Science), as described previously (Honsbein et al., 2009; Grefen et al., 2010, 2015; Lefoulon et al., 2014, 2018).

Oocytes were collected individually after recording for immunoblot analysis. Immunoblots were performed as described before (Lefoulon et al., 2014, 2018; Grefen et al., 2015). Oocytes were homogenized in denaturing buffer (150 mM NaCl, 0.25% [v/v] SDS, 1% [w/v] NP-40, 1 mM EDTA, 1 mM NaF, 1.125 mM PMPS, and 50 mM Tris-HCl, pH 7.4) at 10 μL/oocyte, and centrifuged at 425 g for 5 min. One volume of loading buffer (4 M urea, 10% [w/v] SDS, 40 mM EDTA, 0.2% [v/v] Triton X-100, 0.1% [w/v] bromophenol blue, 20% [v/v] glycerol, 200 mM dithiothreitol, and 100 mM Tris-HCl, pH 6.8) was added to the supernatant, and samples were incubated for 30 min at 37°C before transfer to nitrocellulose and protein detection using the western Blot ECL Advance kit (GE Healthcare) with rabbit α-myc (dilution 1:5000, Abcam) for KAT1, α-HA (dilution of 1:4000, Roche) for VAMP721, and with α-SYP112 antibodies (dilution of 1:4000), α-NAP133P (dilution of 1:20,000), and α-sec11 (dilution of 1:20,000) accordingly (Assaad et al., 2001; Tyrrell et al., 2007; Honsbein et al., 2009; Karnik et al., 2013b; Lefoulon et al., 2018) after binding with secondary horseradish peroxidase-coupled goat, anti-rabbit antibodies (Abcam).

Chemicals and Other Materials

The DNA polymerase KOD-plus-Neo was purchased from Toyobo Life Sciences (Osaka, Japan). Primer synthesis, DNA synthesis, and DNA sequencing were performed by IDT DNA, Genscript, and SourceBioscience, respectively. SDS-PAGE gels NuPAGE and precast M, marker were from Invitrogen. All of the chemicals and reagents were purchased from Sigma-Aldrich unless otherwise indicated. The plasmid mini prep kit was purchased from Qiagen.

Statistical Analysis

Statistical analysis of experiments is reported as means ± s.e as appropriate with significance determined by Student’s t test or ANOVA in SigmaPlot v.11.2 (Systat Software). Joint nonlinear, least-squares fittings were carried out using the Marquardt-Levenberg algorithm of SigmaPlot v.11 (SPSS).

Accession Numbers

Sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL data libraries under the following accession numbers: At5g46240 (KAT1), At4g22650 (KCI), At3G11820 (SYP121), At5G61210 (SNAP33), At1g0750 (VAMP721), and At4G12360 (SEC11).

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. KCI and KAT1 K+ channel interaction controls for experiments with VAMP721, SNAP33 and SEC11.

Supplemental Figure S2. KAT1 K+ channel interacts with VAMP721, SNAP33, and SEC11 via a common N-terminal motif, RyxWE, at the base of its voltage sensor domain.

Supplemental Figure S3. Pulldown analysis of the SNAReS and SEC11 with the KAT11–63 cytosolic domain.

Supplemental Figure S4. The cytosolic domain of KAT11–63 associates differentially with the cognate SNAReS in binary combinations and in complex.

Supplemental Figure S5. KAT1 gating is differentially sensitive to the cognate SNAReS singly and in binary combination, and the effects are moderated in tertiary combination with SEC11.

Supplemental Figure S6. The K+ channel binding domain alters SYP121 structure.

Supplemental Figure S7. SEC11 competes differentially with KAT11–63 for cognate SNARe binding.

Supplemental Figure S8. SEC11 suppresses KAT1 activity stoichiometrically with SYP121 and its action is blocked at more negative voltages.

Supplemental Table S1. Primers and restriction enzymes used in cloning, protein interactions, and protein expression.

ACKNOWLEDGMENTS

We thank George Boswell and Amparo Ruiz-Prado for laboratory support, Sharon Kelly for guidance on CD spectroscopy, and Rucha Karnik for comments on the manuscript throughout its preparation.

Received July 29, 2019; accepted September 4, 2019; published September 23, 2019.

LITERATURE CITED

Archbold JK, Whitten AE, Hu SH, Collins BM, Martin JL (2014) SNARe: the structures of Sec1/Munc18 proteins. Curr Opin Struct Biol 29: 44–51

Assaad FF, Huet Y, Mayer U, Jürgens G (2001) The cytokinesis gene sec11 encodes a Sec1 protein that binds the syntaxin Knolle. J Cell Biol 152: 531–543

Baker RW, Hughson FM (2016) Chaperoning SNARE assembly and disassembly. Nat Rev Mol Cell Biol 17: 465–479

Bassham DC, Blatt MR (2008) SNAReS: Cogs and coordinators in signaling and development. Plant Physiol 147: 1504–1515

Brunner AT, Weniger K, Bowen M, Chu S (2009) Single-molecule studies of the neuronal SNARE fusion machinery. Annu Rev Biochem 78: 903–928

Burgoyne RD, Morgan A (2007) Membrane trafficking: Three steps to fusion. Curr Biol 17: R255–R258

Campanoni P, Blatt MR (2007) Membrane trafficking and polar growth in root hairs and pollen tubes. J Exp Bot 58: 65–74

Carpn LN, Ciufo LF, Shanks SG, Boyd A, Bryant NJ (2006) The Sec1p/Munc18 protein Vps45p binds its cognate SNARe proteins via two distinct modes. J Cell Biol 173: 927–936
Duby G, Hosy E, Fizames C, Alcon C, Costa A, Sentenac H, Thibaud JB (2000) Structural analysis of the neuronal protein iLOV outperforms GFP as a reporter of plant virus infection. Proc Natl Acad Sci USA 97: 2583–2587

Hoshi T (1995) Regulation of voltage dependence of the KAT1 channel by intracellular factors. J Gen Physiol 105: 309–328

Hoshita A, Ichikawa T, Kutsuna N, Hasezawa S, Nakagawa T, Nakano A, Sato MH (2009) Differential expression control and polarized distribution of plasma membrane-resident SYV SNAREs in Arabidopsis thaliana. Plant Cell Physiol 50: 280–289

Hossain A, Blatt MR, et al (2004) A new catch in the SNARE. Trends Plant Sci 9: 187–195

Rizzo J, Südhof TC (2012) The membrane fusion enigma: SNAREs, Sec1/Munc18 proteins, and their accomplishments—Guilty as charged? Annu Rev Cell Dev Biol 28: 279–308

Sanderfoot A, Sanderfoot A, et al (2006) Aminoacylation of the H3 retinoblastoma protein is essential for the formation of syntaxin clusters in the plasma membrane. Plant Cell 18: 2851–2863

Schröder K, Dehmlow C, Stolz W, Siebert C, et al (2007) Munc13-like proteins in Arabidopsis mediate H+–ATPase translocation that is essential for stomatal responses. Nat Commun 8: 2215

Sharma S, Sharma S, et al (2015) Interactions between Sec1-related SNAREs control Arabidopsis K+ channel activity. Plant J 83: 537–549

Kargul J, Gansel X, Tyrrell M, Sticher L, Blatt MR (2001) Protein-binding partners of the tobacco syntaxin NtSyr1. FEBS Lett 508: 253–258

Karnik A, Karnik R, Grefen C (2013a) SDM-Assist software to design site-directed mutagenesis primers introducing “silent” restriction sites. BMC Bioinformatics 14: 105

Karnik R, Grefen C, Bayne R, Honsbein A, Köhler T, Kioumourtzoglou D, Williams M, Bryant NJ, Blatt MR (2013b) Arabidopsis Sec1/Munc18 protein SEC11 is a competitive and dynamic modulator of SNARE binding and SYP121-dependent vesicle traffic. Plant Cell 25: 1368–1382

Karnik R, Waghmare S, Zhang B, Larson E, Lefoulon C, Gonzalez W, Blatt MR (2017) Commandeering channel voltage sensors for secretion, cell turgor, and volume control. Trends Plant Sci 22: 81–95

Karnik R, Zhang B, Waghmare S, Aderhold C, Grefen C, Blatt MR (2015) Binding of Sec11 indicates its role in SNARE recycling after vesicle fusion and identifies two pathways for vesicular traffic to the plasma membrane. Plant Cell 27: 675–694

Kwon C, Neu C, Pajonk S, Yun HS, Lipka U, Humphry M, Bau S, Straus M, Kwaaitaal M, Rampelt H, et al (2008) Co-option of a default secretory pathway for plant immune responses. Nature 451: 835–840

Lefoulon C, Karnik R, Honsbein A, Gutla PV, Grefen C, Riedelsberger J, Blatt MR (2004) Structural analysis of the neuronal protein iLOV outperforms GFP as a reporter of plant virus infection. Proc Natl Acad Sci USA 97: 2583–2587

Leyman B, Geelen D, Quintero FJ, Blatt MR (2000) Localization and control of exocytotic proteins. Endocr Rev 21: 390–409

Leyman B, Geelen D, Quintero FJ, Blatt MR (1999) A tobacco syntaxin with a role in hormonal control of guard cell ion channels. Science 283: 537–540

Lipka V, Kwon C, Panstruga R (2007) SNARE-ware: The role of SNARE-domain proteins in plant biology. Annu Rev Cell Dev Biol 23: 147–174

Ma C, Su, L, Seven AB, Xu Y, Rizzo J (2013) Reconstitution of the vital functions of Munc18 and Munc13 in a neurotransmitter release. Science 339: 421–423

MacDonald PE, Wang G, Tsuk S, Dodo C, Kang Y, Tang L, Wheeler MB, Cattel MS, Lakey JRT, Salapatek AMF, et al (2002) Synaptosome-associated protein of 25 kilodaltons modulates Kv2.1 voltage-dependent K+ channels in neuroendocrine islet β-cells through an interaction with the N terminus. Mol Endocrinol 16: 2452–2461

Misura KMS, Scheller RH, Weis WI (2001) Self-association of the H3 region of Syntaxin 1A. Implications for intermediates in SNARE complex assembly. J Biol Chem 276: 13273–13282

Pavlovic E, Delemotte L, Klein ML, Carnevale V (2014) Evolutionary imprint of activation: The design principles of VSDs. J Gen Physiol 143: 145–156

Pratelli R, Sutter JU, Blatt MR (2004) A new catch in the SNARE. Trends Plant Sci 9: 187–195

Rizzo J, Südhof TC (2012) The membrane fusion enigma: SNAREs, Sec1/Munc18 proteins, and their accomplishments—Guilty as charged? Annu Rev Cell Dev Biol 28: 279–308

Sanderfoot A (2007) Increases in the number of SNARE genes parallels the rise of multicellularity among the green plants. Plant Physiol 144: 6–17

Shope JC, Mott KA (2006) Membrane trafficking and osmotically induced volume changes in guard cells. J Exp Bot 57: 4123–4131

Sieber JJ, Willig KJ, Heinzmann R, Hell SW, Lang T (2006) The SNARE motif is essential for the formation of syntaxin clusters in the plasma membrane. Biophys J 90: 2843–2851

Sieber JJ, Willig KJ, Kutzner C, Gerdling-Reimers C, Harke B, Donnert G, Rammberner K, Egeling C, Hell SW, Grubmuller H, et al (2007) Anatomy and dynamics of a supramolecular membrane protein cluster. Science 317: 1072–1076

Südhof TC, Rothman JE (2009) Membrane fusion: Grappling with SNARE and SM proteins. Science 323: 474–477

Tombola F, Pathak MM, Isacoff EY (2006) How does voltage open an ion channel? Annu Rev Cell Dev Biol 22: 23–52

Waghmare et al.
Tsuk S, Lvov A, Michaellevski I, Chikvashvili D, Lotan I (2008) Formation of the full SNARE complex eliminates interactions of its individual protein components with the Kv2.1 channel. Biochemistry 47:8342-8349

Tyrrell M, Campanoni P, Sutter JU, Pratelli R, Paneque M, Sokolovski S, Blatt MR (2007) Selective targeting of plasma membrane and tonoplast traffic by inhibitory (dominant-negative) SNARE fragments. Plant J 51: 1099–1115

Véry AA, Sentenac H (2003) Molecular mechanisms and regulation of K+ transport in higher plants. Annu Rev Plant Biol 54: 575–603

Wang S, Li Y, Gong J, Ye S, Yang X, Zhang R, Ma C (2019) Munc18 and Munc13 serve as a functional template to orchestrate neuronal SNARE complex assembly. Nat Commun 10: 69

Weber T, Zemelman BV, McNew JA, Westermann B, Gmachl M, Parlati F, Söllner TH, Rothman JE (1998) SNAREpins: Minimal machinery for membrane fusion. Cell 92: 759–772

Weiss N, Zamponi GW (2012) Regulation of voltage-gated calcium channels by synaptic proteins. In MS Islam, ed, Calcium Signaling, Advances in Experimental Medicine and Biology, Vol 740. Springer, New York, pp 759–775

Xu J, Li HD, Chen LQ, Wang Y, Liu L, He L, Wu WH (2006) A protein kinase, interacting with two calcineurin B-like proteins, regulates K+ transporter AKT1 in Arabidopsis. Cell 125: 1347–1360

Zhang B, Karnik R, Alvim J, Donald N, Blatt MR (2019) Dual sites for SEC11 on the SNARE SYP121 implicate a binding exchange during secretory traffic. Plant Physiol 180: 228–239

Zhang B, Karnik R, Donald N, Blatt MR (2018) A GPI signal peptide-anchored split-ubiquitin (GPS) system for detecting soluble bait protein interactions at the membrane. Plant Physiol 178: 13–17

Zhang B, Karnik R, Waghmare S, Donald N, Blatt MR (2017) VAMP721 conformations unmask an extended motif for K+ channel binding and gating control. Plant Physiol 175: 536–551.

Zhang B, Karnik R, Wang Y, Wallmeroth N, Blatt MR, Grefen C (2015) The Arabidopsis R-SNARE VAMP721 interacts with KAT1 and KC1 K+ channels to moderate K+ current at the plasma membrane. Plant Cell 27: 1697–1717