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COMMENTARY

Structure and function of longin SNAREs

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ABSTRACT

Soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) proteins constitute the core membrane fusion machinery of intracellular transport and intercellular communication. A little more than ten years ago, it was proposed that the long N-terminal domain of a subset of SNAREs, henceforth called the longin domain, could be a crucial regulator with multiple functions in membrane trafficking. Structural, biochemical and cell biology studies have now produced a large set of data that support this hypothesis and indicate a role for the longin domain in regulating the sorting and activity of SNAREs. Here, we review the first decade of structure–function data on the three prototypical longin SNAREs: Ykt6, VAMP7 and Sec22b. We will, in particular, highlight the conserved molecular mechanisms that allow longin domains to fold back onto the fusion-inducing SNARE coiled-coil domain, thereby inhibiting membrane fusion, and describe the interactions of longin SNAREs with proteins that regulate their intracellular sorting. This dual function of the longin domain in regulating both the membrane localization and membrane fusion activity of SNAREs points to its role as a key regulatory module of intracellular trafficking.

KEY WORDS: Lipid transfer, Longin, Membrane proteins, SNARE, Vesicular trafficking

Introduction

Eukaryotic cells possess an endomembrane system that comprises membrane-bound organelles – including the nucleus, endoplasmic reticulum (ER), Golgi complex, endosomes, lysosomes, vesicles and plasma membrane – that carry out important cellular functions such as the synthesis and transport of biological molecules within the cell. These organelles communicate with each other through cargo-containing transport vesicles that bud off from a donor membrane and fuse with a target membrane to deliver their membranous and aqueous contents (Söllner and Rothman, 1996). This process is orchestrated by complex protein–lipid and protein–protein interactions that involve several specialized protein families, including vesicle coats, cargo adaptors, Rabs and soluble N-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs) (Pfeffer, 2007; Robinson, 2004). Comparative analyses of the protein domain architectures within the endomembrane system have highlighted four functional entities that are common to the different subcellular protein machineries involved in membrane traffic: the coiled-coil, coatomer, small GTPase and longin domains (De Franceschi et al., 2014). The longin domain (Box 1) was first identified as a regulatory module of membrane fusion proteins belonging to the SNARE family (Rossi et al., 2004), but recent studies have revealed the presence of this domain in several other important trafficking-protein families (De Franceschi et al., 2014). Longin-domain-containing proteins are now classified into seven homologous superfamilies: the longin SNAREs, adaptins, sedlins, SANDs, targetins, differentially expressed in normal and neoplastic cells (DENNs) and APL2 VPS1 synthetic lethal proteins (AVLs); their function is not limited to membrane fusion reactions but it is also important for vesicle budding and tethering events, as well as for the regulation of Rab GTPases (Box 2). These findings highlight the importance of the longin domain in membrane traffic and indicate that it is one of the primordial functional domains of the eukaryotic trafficking machinery. In this Commentary, we present recent structural and functional data on longin-SNARE proteins, in isolation or associated with their binding partner, and emphasize common structural features as well as variable regulatory principles, which allow them to have multiple fundamental functions in cellular processes, such as autophagosome biogenesis or plasma membrane expansion, through vesicular and non-vesicular lipid trafficking.

Longin-SNARE proteins

SNARE proteins constitute the best-described molecular factors that mediate intracellular membrane fusion, and their mode of action and regulators in neurotransmission are now widely used as paradigms for general SNARE function and regulation (Jahn and Scheller, 2006; Sudhof and Rothman, 2009). During neurotransmission, the vesicular (v-)SNARE vesicle-associated membrane protein 2 (VAMP2) – which contains one coiled-coil domain and no longin domain, and resides on synaptic vesicles – forms a membrane-bridging complex (the so-called trans-SNARE complex) with its two target (t-)SNAREs – syntaxin1A, containing one coiled-coil domain, and SNAP25, comprising two coiled-coil domains connected by a flexible linker – which are located at the plasma membrane (Fig. 1). The energy released during this assembly (a zipper-like assembly from the membrane-distal regions towards the membrane-proximal regions of the SNARE coiled-coil domains) is used to bring the membranes into close proximity and to induce their fusion (Li et al., 2007; Söllner et al., 1993; Sutton et al., 1998; Weber et al., 1998). Members of the syntaxin family also possess an N-terminal regulatory domain, called H_{abc}, that can inhibit their activity by folding back onto their coiled-coil domain, thereby preventing formation of the trans-SNARE complex (Dietrich et al., 2003). SNARE proteins also interact with various regulatory proteins that can manipulate assembly of the trans-SNARE complex to allow fusion to be inhibited and/or triggered where and when necessary (Jahn and Fasshauer, 2012; Sudhof and Rothman, 2009; Südhof, 2013).

Like syntaxins, VAMP proteins of the longin-SNARE family possess, in addition to the fusion-inducing coiled-coil domain, an N-terminal longin domain, that can also regulate assembly of the trans-SNARE complex and thus membrane fusion (Filippini et al., 2001; Rossi et al., 2004). The structures of the N-terminal regions of syntaxins and longin SNAREs, however, differ substantially; the

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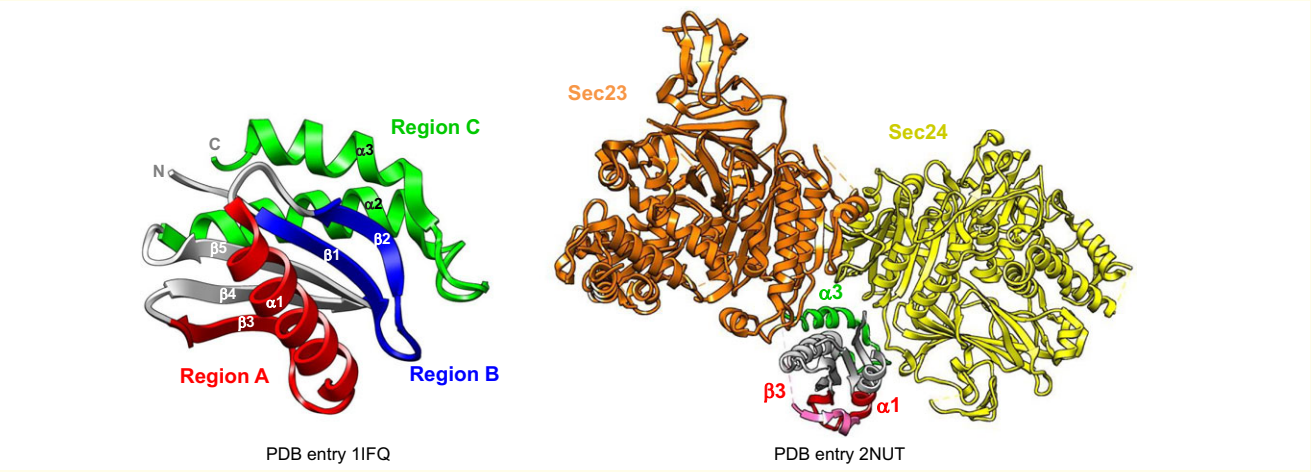
Box 1. Longin domain architecture



The longin domain belongs to the mixed α-β class of proteins, which encompasses proteins with α-helical and β-sheet secondary structure elements and includes almost half of the proteins described in the class, architecture, topology and homologous (CATH) protein structure classification database (Cuff et al., 2009). This domain displays a globular-fold of 120–140 amino acids, arranged as an α-β-α sandwich architecture, which is one of the most common folds found in nature. This architecture was first linked to that of profilin (Rossi et al., 2004), a protein involved in actin polymerization and actin interaction with the plasma membrane. Recently, a comparative bioinformatics study has revealed that all seven superfamilies of longins share a unique topology (five antiparallel β-strands sandwiched by two α-helices on one side, and one α-helix on the other) that is not found in profilin proteins (De Franceschi et al., 2014). Three binding regions (regions A, B and C) have been identified within longins: region A (red in the figure) is involved in intra- and inter-molecular interactions; region B (blue) is implicated in interactions with small GTPases; and region C (green) is involved in conformational interactions with bulky protein complexes. In the longin-SNARE subfamily, region A is involved in both intramolecular interactions with the SNARE coiled-coil domain (to regulate its activity) and intermolecular binding to other trafficking proteins in order to target longin SNAREs to their site of action (De Franceschi et al., 2014). For example, in the case of the complex between Sec22b and the COPII subunits Sec23–Sec24 (Mancias and Goldberg, 2007) that is illustrated in the right panel of the figure, helix α1 and strand β3 of region A (red) bind to the coiled-coil domain (pink), and helix α3 of region C (green) binds at the interface of Sec23 (orange) and Sec24 (yellow). PDB, Protein Data Bank.

H_{abc} domain comprises an autonomously folded three-helix bundle, whereas the longin domain is arranged in the form of an α-β-α sandwich architecture (Fig. 1). Three subfamilies of longin SNAREs have been identified based on homology to the mammalian Ykt6, VAMP7 and Sec22b proteins. VAMP7 and Sec22b contain a C-terminal transmembrane domain, whereas Ykt6 is targeted to membranes through two lipid anchors and also exists as a soluble cytosolic pool (Figs 1 and 2). These three proteins are enriched in neurons, where they have important functions such as plasma membrane expansion during neuronal development (Rossi et al., 2004; Petkovic et al., 2014). Interestingly, the pollen VAMP7-like protein PiVAMP726 also mediates membrane fusion during pollen tube tip growth in plants (Guo and McCubbin, 2012), suggesting that longin SNAREs perform specialized functions related to membrane growth and remodelling that are conserved across different kingdoms.

Various lines of evidence indicate that longin domains also have a function that goes beyond merely regulating assembly of the trans-SNARE complex. In plants, a genomic comparative analysis has described the Phytolongin subfamily; although proteins of this subfamily still have the longin and transmembrane domains, the SNARE coiled-coil domain is replaced by a previously unidentified domain, the PhyL region, the exact function of which remains to be determined (Vedovato et al., 2009). In rice, there is a Sec22b-like protein that only possesses a longin domain and thus lacks both the coiled-coil and transmembrane domains (Vedovato et al., 2009). In mammals, the Sec22b prototypical protein has two isoforms (Sec22a and Sec22c) that lack a coiled-coil domain but nevertheless might be involved in protein trafficking from the ER to the Golgi (Tang et al., 1998). Finally, a genetic analysis has shown that alternative splicing of the human *VAMP7* gene results in an isoform that lacks a SNARE coiled-coil domain; this isoform has a different tissue expression profile and subcellular localization, but its function is still unknown (Vacca et al., 2011). Longin domains are also found in non-SNARE protein families that are involved in key trafficking steps, such as vesicle budding and tethering (Box 2) (De Franceschi et al., 2014; Vedovato et al., 2009). Taken together, these observations strongly indicate that longin domains play a central role in intracellular trafficking. As discussed below, in the case of the prototypical longin SNAREs Ykt6, VAMP7 and Sec22b, longin domains are important for regulating the localization of SNAREs to their site of action, in addition to acting as an 'on/off' switch for their fusogenic activity.

The three subfamilies of longin SNAREs

Ykt6

Ykt6 was initially identified as a v-SNARE protein that is involved in ER-to-Golgi transport (McNew et al., 1997) but was later also implicated in intra-Golgi (Fukasawa et al., 2004), endosome–Golgi and vacuolar transport steps (Kweon et al., 2003), as well as in membrane fusion reactions that result in expansion and closure of the autophagosome membrane (Nair et al., 2012) (Fig. 2). Interestingly, Ykt6 has been shown to suppress the toxicity of α-synuclein in models of Parkinson's disease (Cooper et al., 2006; Thayanidhi et al., 2010). Parkinson's disease pathogenesis is characterized by the aggregation of α-synuclein, which forms insoluble fibrils that inhibit ER-to-Golgi trafficking and thus induces abnormal protein accumulation at the ER. In yeast, this

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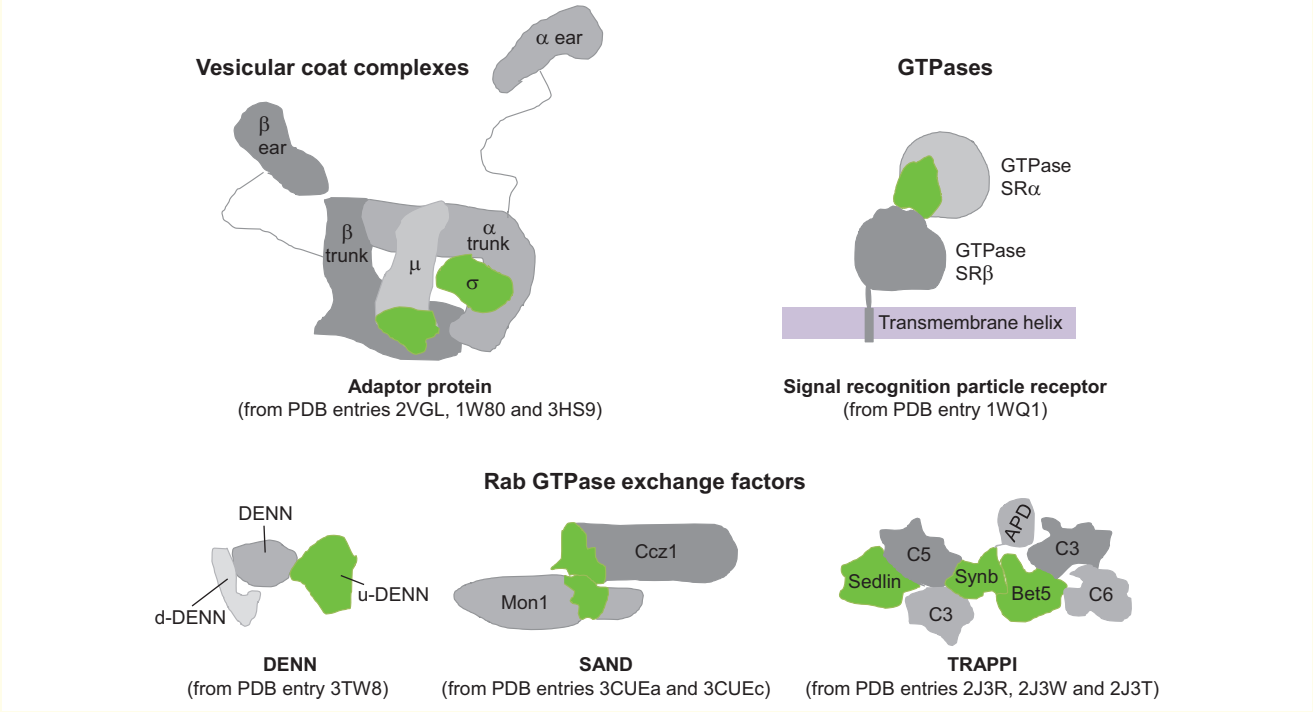
Box 2. Non-SNARE longin proteins that are involved in membrane traffic



In addition to its regulatory function in longin SNAREs, the longin domain (green) is a functional entity that is found in six other key protein families, which are involved in membrane traffic, such as vesicular coat complexes and proteins regulating and/or containing GTPase domains (De Franceschi et al., 2014). Longin-domain-containing vesicular coat complexes mediate the budding of vesicles and the selection of cargo proteins in the endocytic and secretory pathways (adaptor proteins, AP1 to AP5), and retrograde trafficking events from the Golgi to the ER (coat protein complex I, COPI). These complexes comprise two core longin-domain-containing subunits – i.e. σ and μ adaptins in adaptor proteins (Collins et al., 2002) and their homologues σ and ζ in COPI (Lee and Goldberg, 2010) – that stabilize their conformation. The longin domain has also a structural role in the signal recognition particle receptor (SR), a heterodimer of the two GTPases SRα and SRβ (also known as SRPR and SRPRB, respectively), which are important for the recruitment of ribosomes at the surface of the endoplasmic reticulum. The crystal structure of the SR complex reveals the presence of a longin domain in the N-terminal targetin region of SRα that interacts with SRβ (Schlenker et al., 2006). Additionally, three families of Rab GTPase exchange factors (GEFs) contain a longin domain. DENNs and DENN-related proteins (AVLs) are important GEFs in higher eukaryotes, in which an N-terminal regulatory longin domain (u-DENN domain) is adjacent to the GTP–GDP exchange factor DENN domain (Zhang et al., 2012). The longin domain is also found in GEF multi-protein complexes that are involved in membrane tethering events. In SAND heterodimeric complexes, the longin domain mediates the assembly of the two subunits, as well as being part of the binding interface with the associated Rab GTPase (Cabrera et al., 2014). In multimeric TRAPPI complexes, the longin domain is present in three subunits [sedlin, synbindin (Synb) and Bet5 (also known as TRAPPC2, TRAPPC4 and TRAPPC1, respectively)] (Brunet and Sacher, 2014). Crystal structures of these subunits reveal a longin domain with strong similarity to that of the longin SNAREs, despite no sequence similarity. In the TRAPPI subunit synbindin, the sequence of the longin domain is even split by the insertion of an atypical PDZ domain (APD), but the resulting longin fold is conserved (Fan et al., 2009). C3, C5 and C6 are known as TRAPPC3, TRAPPC5 and TRAPPC6, respectively. PDB, Protein Data Bank.

trafficking block is relieved by the expression of proteins promoting forward ER-to-Golgi transport, including Ykt6 (Cooper et al., 2006). In addition, Ykt6 can functionally replace Sec22b in ER-to-Golgi trafficking (Liu and Barlowe, 2002) and is a more effective suppressor of α-synuclein toxicity than Sec22b (Thayanidhi et al., 2010). In contrast to having a protective role in Parkinson's disease, Ykt6 is found to be upregulated in metastatic tumours (Kluger et al., 2004; Ooe et al., 2007), and moreover, overexpression of Ykt6 in epithelial cell lines accelerates the cell cycle (Thayanidhi et al., 2012). In addition, other recent findings demonstrate that, in neurons, a fraction of the soluble pool of Ykt6 is redirected to large membrane-associated particle aggregates that do not colocalize with any of the known components of the endomembrane system (Hasegawa et al., 2003; Thayanidhi et al., 2012). There, Ykt6 does not function as a SNARE protein, but instead, its aggregation has been proposed to act as a buffering mechanism to ensure that high levels of the protein are available to mediate its protective role, at the same time preventing its general overexpression, which is toxic to the cell (Thayanidhi et al., 2012). The unique mode of insertion of Ykt6 into membranes is ideally suited to perform such a protective function. As mentioned above, unlike the two other longin SNAREs, Ykt6 does not contain a transmembrane domain that anchors it permanently to membranes, but instead, the protein uses a lipid anchor that allows transient association with the membrane (Fukasawa et al., 2004); thus, Ykt6 can easily insert into potentially affected compartments – for example, ER membranes that have accumulated a toxic amount of proteins in the case of Parkinson's disease – to rescue transport.

The longin domain of Ykt6 has been found to be necessary and sufficient both for targeting Ykt6 to the secretory pathway and for its role in particle formation (Fukasawa et al., 2004; Hasegawa et al., 2004). The molecular mechanisms underlying the aggregation of the soluble pool of Ykt6 are still poorly understood, in contrast to the membrane-anchoring mechanism, which has been fully elucidated and is tightly linked to the conformational transition of Ykt6 from a closed (inactive) to an open (active) state (Fukasawa

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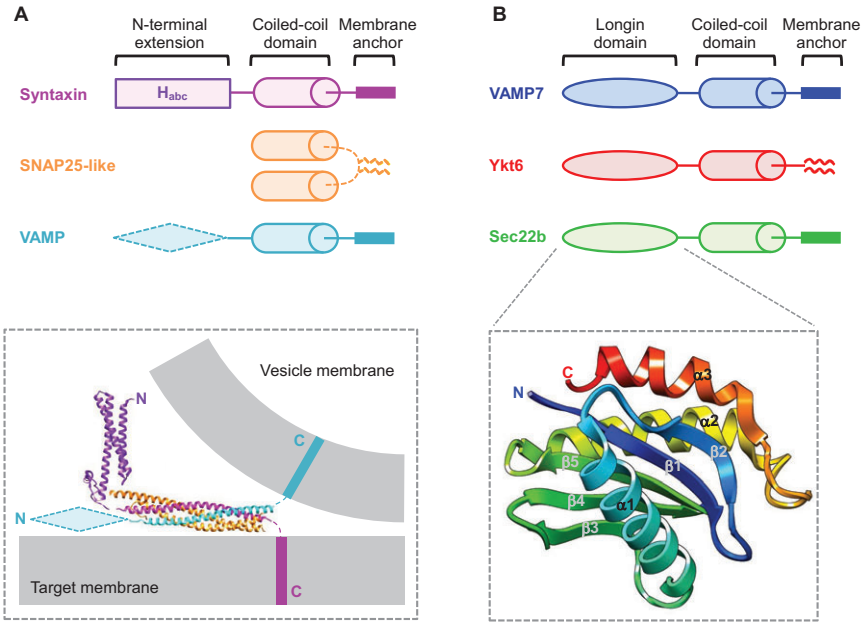

Fig. 1. Domain architecture of SNARE proteins. (A) All SNARE proteins have an α-helical coiled-coil domain (cylinder) that is involved in the formation of a parallel four-helix bundle, which brings the membranes into close apposition (lower panel) and triggers their fusion. Syntaxins (magenta) and vesicle-associated membrane proteins (VAMPs, cyan) each contribute one helix to this bundle, and SNAP25-like proteins (orange) contribute two helices. Syntaxins and VAMPs are usually connected to their respective membranes by a transmembrane domain (see below the exception of the VAMP protein Ykt6), whereas SNAP25-like proteins can have a transmembrane domain, be lipid-anchored or even soluble. All syntaxins possess an N-terminal extension, the H_{abc} domain (purple), comprising three α-helices; the N-terminal region of VAMPs (rhombus) comprises either a short and variable domain (short VAMPs called 'brevins') or a long and conserved longin domain (long VAMPs called 'longins' or longin SNAREs; see panel B). The structure in the lower panel was generated using structural data of the synaptic SNARE complex [Protein Data Bank entries 1SFC and 3C98 (Burkhardt et al., 2008; Sutton et al., 1998)]; the N-terminal domains of Syntaxin and VAMP are positioned arbitrarily. (B) VAMP proteins of the longin-SNARE family exhibit a highly conserved N-terminal extension that is 120–140 residues long – the longin domain. Longin SNAREs are divided into three subfamilies according to the prototype mammalian proteins VAMP7, Ykt6 and Sec22b. VAMP7 and Sec22b have a transmembrane domain, whereas Ykt6 associates with membranes through lipid anchors, and also exists in a soluble cytosolic form (not shown here). The lower panel shows a ribbon depiction of the longin domain of Sec22b [Protein Data Bank entry 1IFQ (Gonzalez et al., 2001)] using a 'rainbow' representation (from blue at the N-terminus to red at the C-terminus). The structure starts with a β-hairpin (β1–β2), followed by a single α-helix (α1), and then leads into three antiparallel β-sheets (β3–β4–β5) where strand β5 interacts in antiparallel with strand β1, and ends with two antiparallel α-helices (α2–α3). The single helix (α1) and the pair of helices (α2–α3) are located on opposite sides of the β-sheet platform. The structure representations in this figure were prepared using Chimera.

et al., 2004; Wen et al., 2010) (Fig. 3). Nuclear magnetic resonance (NMR) data have revealed an auto-inhibitory mechanism, whereby the closed state of Ykt6 (with the longin domain folded back onto the coiled-coiled domain) decreases the kinetics of SNARE-complex assembly (Tochio et al., 2001). The membrane attachment of Ykt6 depends on two lipid molecules, a palmitoyl group and a farnesyl group, which are added onto the first and second cysteine residues of a C-terminal 'CCAIM' motif, respectively. Farnesylation is irreversible and occurs after Ykt6 translation. Surprisingly, this addition does not anchor Ykt6 into membranes, and X-ray crystallography experiments have demonstrated that, following farnesylation, Ykt6 switches from a semi-closed to a predominantly closed and fusion-inactive conformation (Pylypenko et al., 2008; Wen et al., 2010). This traps the lipid molecule inside a hydrophobic pocket that is formed by the interface between the longin domain and the coiled-coil domain, and thus inhibits membrane insertion of Ykt6. In this configuration, the coiled-coil domain folds as five separated domains (four α-helices and one β-sheet) that wrap around the longin domain (without modifying its structure) and interact with it through hydrophobic interactions and hydrogen bonds (Wen et al., 2010) (Fig. 4A). The farnesyl group acts as a molecular stabilizer of this closed conformation and therefore prevents the pairing of Ykt6 with non-cognate t-SNAREs. Unlike farnesylation, palmitoylation of Ykt6 is reversible and results in its membrane anchorage. As a result of membrane association, the partition coefficient of the farnesyl group is shifted towards the membrane, leading to the release of the longin domain and the transition of Ykt6 to an open and active conformation (Fig. 3). This conformational transition from a closed to an open state therefore not only affects the subcellular localization of Ykt6, but also has a role in SNARE-complex formation. Furthermore, the molecular mechanisms underlying this open–closed transition highlight the ability of lipid molecules to actively regulate SNARE protein activity in addition to their ability to function as membrane-structuring and membrane-anchoring elements.

VAMP7

VAMP7 (also called tetanus neurotoxin insensitive-VAMP, TI-VAMP) is involved in various important cellular functions, including phagocytosis, mitosis, cell migration, as well as membrane repair and growth; it fulfils these roles mainly through mediating the fusion of vesicles that are derived from Golgi, late-endosomal and lysosomal compartments with the plasma membrane (Chaineau et al., 2009; Hesketh et al., 2014; Luzio et al., 2009; Rossi et al., 2004) (Fig. 2). VAMP7 has also been implicated in autophagosome biosynthesis (Moreau et al., 2011), the degradation of autophagosomal cargoes through autophagosome–lysosome fusion (Takáts et al., 2013) and in autophagosomal secretion, which is potentially responsible for ATP release into the extracellular space (Fader et al., 2012). VAMP7 was initially identified as a key player in neurite outgrowth during

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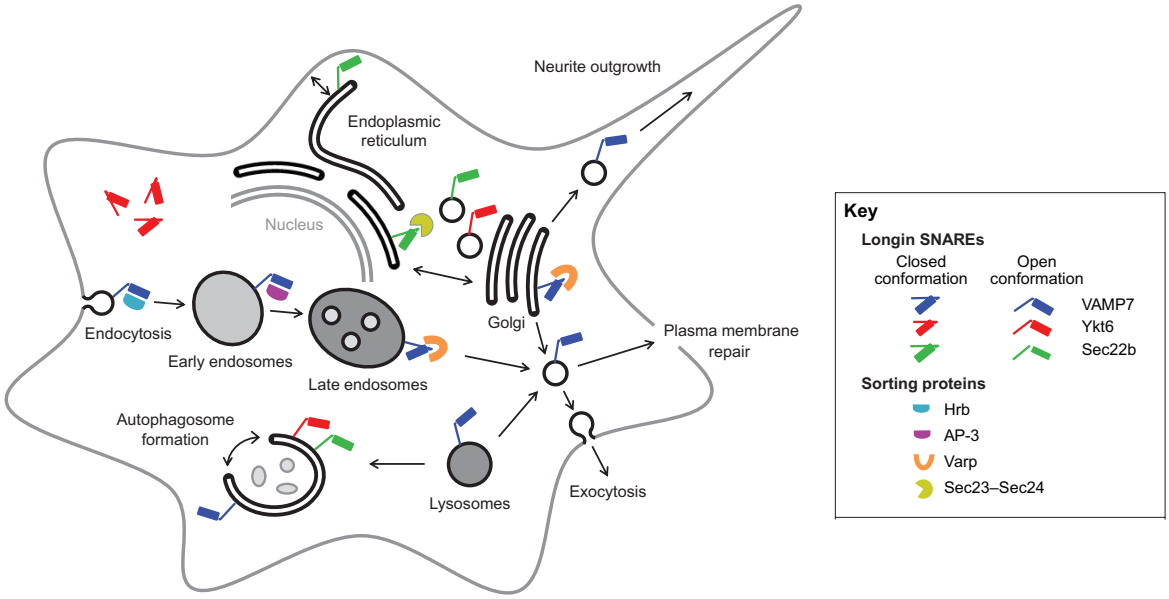

Fig. 2. Overview of the main functions of the three longin SNAREs. Ykt6 (red) and Sec22b (green) are both involved in ER-to-Golgi transport steps and in autophagosome formation. Sec22b also plays a role in the plasma membrane expansion of developing neurons by promoting close contact between the ER and the plasma membrane, potentially facilitating lipid exchanges between these two compartments. VAMP7 (blue) is involved in neurite outgrowth in developing neurons, and in synaptic vesicle exocytosis in mature neurons. VAMP7 is also implicated in plasma membrane repair, in autophagosome formation and in autophagosomal degradation through fusion with lysosomes. Ykt6 exists as a closed cytosolic (inactive) form and as an open membrane-embedded (active) form; the transition between these two states involves lipid modification at the C-terminus of Ykt6 (Fukasawa et al., 2004) (see Fig. 3). Sec22b is transported out of the ER in its closed form, which is stabilized through interaction with the COPII subunits Sec23–Sec24 (Mancias and Goldberg, 2007) (see Fig. 4C and Box 1). VAMP7 is endocytosed in its open conformation, with its longin domain occupied by Hrb (Pryor et al., 2008), and is transported to early endosomes, where its longin domain now binds to AP-3 (Kent et al., 2012), still leaving VAMP7 in an open state. VAMP7 bound to AP-3 is then trafficked to late endosomes. Varp is recruited to late-endosomal (Hesketh et al., 2014) and Golgi membranes (Burgo et al., 2012), where it binds to VAMP7 in its closed conformation. VAMP7-containing vesicles derived from endosomal, lysosomal and Golgi membranes fuse with the plasma membrane during the various VAMP7-mediated processes described above.

neuronal development (Martinez-arca et al., 2000). This process requires the extension of the plasma membrane surface, which is sustained by the VAMP7-mediated fusion of transport vesicles that deliver lipids, adhesion proteins and growth factors. In the PC12 cell model of growing neurites, overexpression of the VAMP7 longin domain inhibits neurite outgrowth, whereas deletion of the longin domain activates SNARE-complex assembly and stimulates neurite outgrowth (Martinez-arca et al., 2000). In fact, these data provided the first evidence for an auto-inhibitory mechanism of longin SNAREs, such as that described above for Ykt6. In mature hippocampal neurons, removal of the longin domain increases spontaneous Ca^{2+}-independent fusion of VAMP7-containing synaptic vesicles with the pre-synaptic plasma membrane (Hua et al., 2011). Interestingly, this effect is still observed after cleavage of VAMP2 with the tetanus neurotoxin, and under this condition, VAMP7 deleted of the longin domain also increases evoked Ca^{2+}-triggered neurotransmitter release, indicating that VAMP7 without its longin domain can mediate both spontaneous and evoked exocytosis of synaptic vesicles. Taken together, these results show that VAMP7 has important functions both in developing and mature neurons, and that the longin domain acts as an 'on/off' switch for its activity.

The inhibitory role of the longin domain of VAMP7 has been confirmed with NMR data, which revealed that the cytoplasmic domain of VAMP7 adopts a predominantly closed conformation in solution (Vivona et al., 2010). Stabilization of this closed structure requires at least residues 126–160 of the SNARE coiled-coil domain; in addition, residues leucine 43 and tyrosine 45 of the longin domain are crucial for its interaction with the coiled-coil domain (Fig. 4B,D) (Vivona et al., 2010). Interestingly, mutation of residue tyrosine 45 to glutamate (which modifies the structure of the protein to mimic as close as possible the effect of phosphorylating tyrosine) increases SNARE-complex formation and VAMP7-mediated vesicle exocytosis *in vivo*, indicating a potential role for tyrosine phosphorylation in the transition of VAMP7 from its closed to open conformation (Burgo et al., 2013). Along these lines, the closed conformation of VAMP7 thus would not require any regulators, either proteins or lipids, in order to be stable, in contrast to Ykt6, which makes use of a stabilizing farnesyl group in order to remain in a closed conformation. Surprisingly, the closed form of VAMP7 does not prevent SNARE-complex formation, but only restrains it as VAMP7 can still interact *in vitro* with its plasma membrane t-SNARE partners syntaxin1A and SNAP25, albeit to a lower extent than VAMP7 without a longin domain (Martinez-Arca et al., 2003; Vivona et al., 2010). The inhibition of SNARE-complex assembly is further enhanced when VAMP7 is bound to Varp, a GDP–GTP exchange factor (GEF) for, and an effector of, the small GTPase Rab21 (Schäfer et al., 2012). The X-ray crystal structure of a complex between the cytoplasmic domain of VAMP7 and a C-terminal fragment of Varp that is rich in ankyrin repeats has shown that Varp binds to the closed conformation of VAMP7 and stabilizes it by interacting simultaneously with the longin and the coiled-coil domains (Schäfer et al., 2012) (Fig. 4E). In this structure, the membrane-distal portion (residues 129–163) of the coiled-coil domain of VAMP7 is ordered, with the isoleucine 139 and isoleucine 144 residues having a key role in the interaction with the longin domain (Fig. 4B,D). Given the importance of Varp in the intracellular trafficking of VAMP7 vesicles (Burgo et al., 2009,

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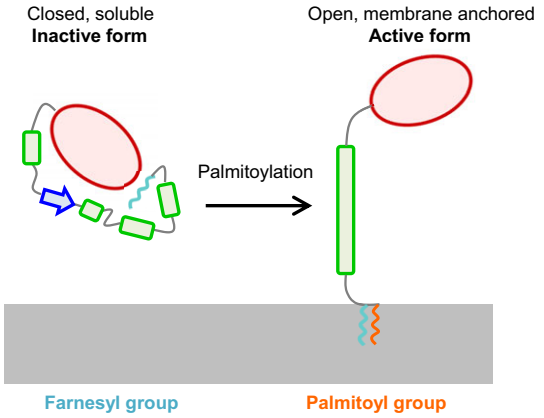

Fig. 3. Molecular mechanisms of the open–closed transition in Ykt6. The soluble inactive conformation of Ykt6 is stabilized by the farnesyl group (cyan) that is sandwiched between the coiled-coil domain (α-helices in green and β-sheet in blue) and the longin domain (in red). Palmitoylation of Ykt6 (orange) drives membrane insertion, farnesyl release and thus opening of Ykt6, which is then able to interact with its t-SNARE partners (not represented here). See Fig. 4A for structural details about the closed conformation of Ykt6.

2012; Hesketh et al., 2014; McGough et al., 2014), these data suggest that VAMP7 travels in a closed conformation (i.e. fusion-incompetent state) within the cell.

Furthermore, the longin domain of VAMP7 is also important for its targeting to its sites of action. The selective sorting of VAMP7 is mediated by direct interactions between its longin domain and the coat components of clathrin-coated vesicles. The longin domain of VAMP7 interacts with the δ-adaptin subunit (also known as AP3D1) of the clathrin adaptor protein 3 (AP-3) complex, which targets VAMP7 to synaptic vesicles (Scheuber et al., 2006), as well as to late-endosomal and lysosomal compartments (Martinez-Arca et al., 2003). Interestingly, Nyv1p, the yeast homologue of VAMP7 that is involved in homotypic vacuole fusion, also interacts with AP-3. This interaction is mediated by an adaptin-dependent sorting signal that is found in the longin domain of Nyv1p and is sufficient to target Nyv1p to the vacuolar membrane (Wen et al., 2006). After fusion of vesicular compartments with the plasma membrane (for example during membrane repair or neurite outgrowth), VAMP7 has to be recycled back to its initial location by endocytosis. Clathrin-mediated endocytosis of VAMP7 is directly mediated by another clathrin adaptor, HIV Rev-binding protein (Hrb; also known as AGFG1), whose interaction with the longin domain is crucial for the efficient retrieval of VAMP7 from the plasma membrane (Chaineau et al., 2008; Pryor et al., 2008). The structures of both the longin–AP-3 and the longin–Hrb complexes have been determined by using X-ray crystallography and have revealed that AP-3 and Hrb both bind to the same region of the longin domain, which is also involved in its interaction with the SNARE coiled-coil domain (Kent et al., 2012; Pryor et al., 2008; Schäfer et al., 2012) (Fig. 4D,E). As a result, AP-3 and Hrb must bind sequentially to the longin domain of VAMP7, and can only do so when VAMP7 is in its open conformation. These successive interactions allow VAMP7 to traffic from the plasma membrane to early endosomes when bound to Hrb, and from there, to late endosomes when bound to AP-3 (Fig. 2). Along this trafficking route, VAMP7 has to be kept in an inactive form to prevent any non-specific fusion events. This could be achieved if VAMP7 is transported as part of a cis-SNARE complex, as previously suggested (Kent et al., 2012), or by an as yet unidentified mechanism, which could, for instance, include VAMP7 dimerization through its longin domain (Vivona et al., 2010) or interaction with other factors. Taken together, the results discussed here show that the longin domain of VAMP7 has at least two important regulatory functions – it controls SNARE-complex assembly through interaction with the coiled-coil domain, and it ensures proper intracellular localization of VAMP7 through interactions with Varp and with the vesicle-coat components AP-3 and Hrb.

Sec22b

Sec22b was originally identified as a SNARE protein that is involved in anterograde and retrograde membrane trafficking between the ER and the Golgi (Burri et al., 2003; Liu and Barlowe, 2002). Like Ykt6 and VAMP7, Sec22b is also involved in fusion events that regulate autophagosome biogenesis (Nair et al., 2012). Interestingly, the trafficking pathway of Sec22b has been shown to be hijacked by intracellular parasites, such as *Leishmania* and *Legionella*; this allows the parasite vacuoles to acquire membrane and cytosolic components of the ER, which are essential for parasite survival and replication (Arasaki and Roy, 2010; Canton and Kima, 2012). Similarly, the vacuoles of dendritic cells that contain the parasite *Toxoplasma gondii* require Sec22b to mature and function properly (Cebrian et al., 2011). We have recently shown that Sec22b also plays an important physiological role in neurons because it is required for plasma membrane expansion during neuronal development (Petkovic et al., 2014). To perform this function, Sec22b interacts with the plasma membrane t-SNARE syntaxin1A to bring the ER and plasma membranes into close proximity, but without inducing their fusion. Recent studies have highlighted the importance of membrane contact sites between the ER and the plasma membrane in facilitating the exchange of lipids between these two compartments, independently of any vesicular lipid trafficking (Schauder et al., 2014; Stefan et al., 2011; Tavassoli et al., 2013; Voelker, 2009). In line with this, the SNARE complex that forms between Sec22b and syntaxin1A does not include any SNAP25-like factors and therefore results in a fusion-incompetent SNARE complex that is unable to mediate vesicle exocytosis in neurons or liposome fusion *in vitro* (Petkovic et al., 2014). Interestingly, in yeast, Sec22 also forms a complex with the plasma membrane t-SNARE Sso1, the yeast homologue of syntaxin1A, and we have shown that both proteins interact with the lipid transfer proteins Osh2 and Osh3. In addition, inhibition of yeast Sec22 compromises the trans-modification of plasma membrane phosphoinositides by an ER enzyme, and introduction of a long rigid linker between the coiled-coil and transmembrane domains of Sec22b, which increases the distance between ER and plasma membranes, impairs axonal and dendritic growth in neurons (Petkovic et al., 2014). Taken together, our results newly identify a conserved non-fusogenic function of the longin SNARE Sec22b in stabilizing close contacts between the ER and the plasma membrane, which contribute to plasma membrane expansion through non-vesicular lipid transfer. Overexpression of the longin domain of Sec22b in developing neurons reduces neurite outgrowth (Petkovic et al., 2014), in agreement with the known inhibitory function of longin domains with regard to SNARE-complex assembly. However, the exact role of the longin domain of Sec22b in this process remains to be determined.

Only little is known about Sec22b at the structural level, in particular, whether or not it can adopt a closed conformation. The crystal structure of the longin domain of Sec22b was the first of a non-Syntaxin SNARE N-terminal domain to be determined, but the coiled-coil domain was not included in this structure (Gonzalez et al., 2001) (Fig. 1B). Interestingly, the full-length Sec22b protein

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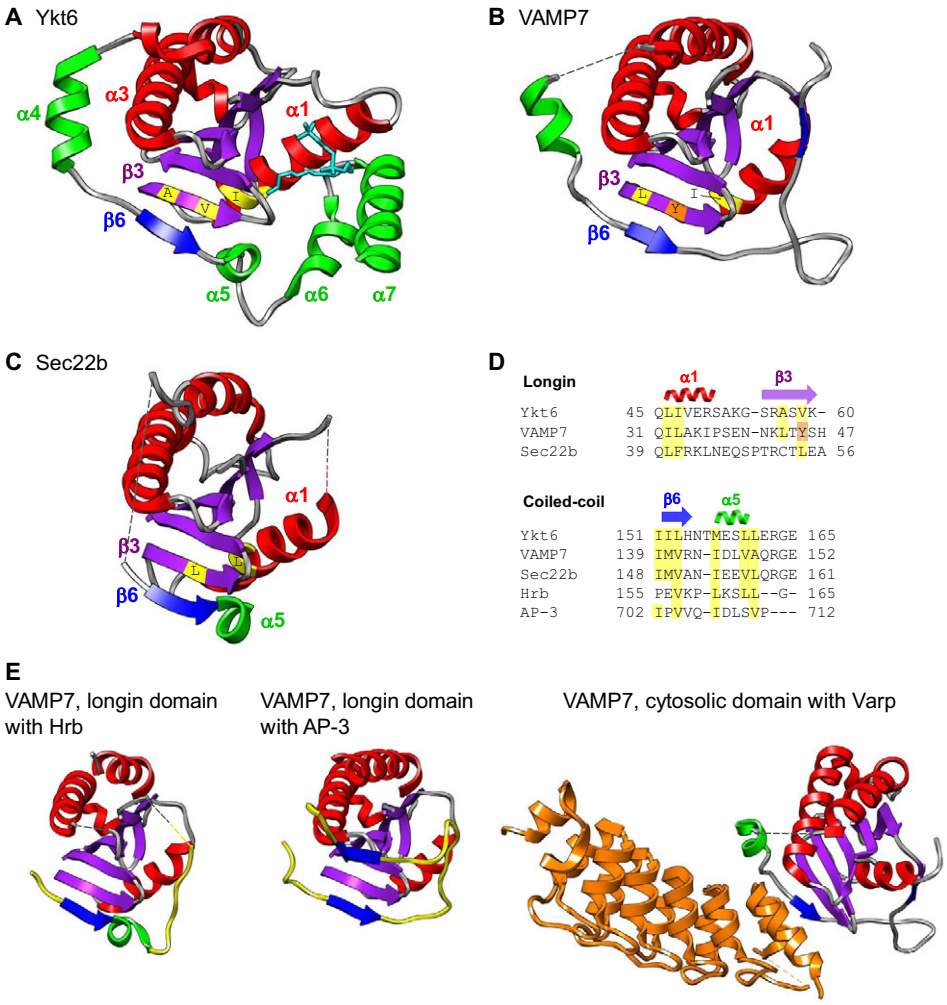

Fig. 4. Comparison of the closed forms of the three longin SNAREs. The longin domain is shown in red (α-helices) and purple (β-sheets), and the coiled-coil domain is depicted in green (α-helices) and blue (β-sheets). The conserved hydrophobic residues of the longin domain that are involved in its interaction with the coiled-coil domain are highlighted in yellow, except for tyrosine residue 45 of VAMP7, which is highlighted in orange. (A) Structure of the closed form of Ykt6 [Protein Data Bank entry 3KYQ (Wen et al., 2010)] bound to a dodecylphosphocholine (DPC) molecule (cyan), which mimics the C-terminal farnesylation of the protein. The entire SNARE coiled-coil domain is visible in the structure. This closed form mainly involves strand β3 and the C-terminus of helix α1 (from the longin domain), which interact with strand β6 and helix α5 (from the coiled-coil domain), and is further stabilized by interactions between the N-terminus of helix α1 (from the longin domain) and the α-helix hairpin α6–α7 (from the coiled-coil domain). The DPC molecule that mimics the natural farnesyl group of Ykt6 binds at the interface between helix α1 and the α-helix hairpin α6–α7, holding the closed conformation in place. (B) Structure of the closed form of VAMP7 bound to Varp [Protein Data Bank entry 4B93 (Schäfer et al., 2012)]; Varp is not shown for clarity (but see also E). The last 25 residues of the coiled-coil domain as well as seven residues connecting the longin domain to the coiled-coil domain are not visible in the structure. Here, the closed form only involves binding of strand β6 (from the coiled-coil domain) to strand β3 and the C-terminus of helix α1 (from the longin domain). (C) Structure of the closed form of Sec22b bound to Sec23–Sec24 [Protein Data Bank entry 2NUT (Mancias and Goldberg, 2007)]; Sec23 and Sec24 are not shown for clarity (but see also Box 2). Only a small portion of the coiled-coil domain (ten residues corresponding to strand β6 and helix α5 of Ykt6) is visible in the structure, and this region interacts with strand β3 and the C-terminus of helix α1 (from the longin domain). (D) Alignment of the regions involved in the interaction between the longin domain and the coiled-coil domain in the three longin-SNARE structures, showing a conserved pattern of hydrophobic residues. Most of the hydrophobic residues in the coiled-coil domain are also found in the regions of Hrb and AP-3 that interact with the longin domain (Kent et al., 2012; Pryor et al., 2008). (E) Structure of VAMP7 bound to sorting partners. Hrb (residues 136–176) and AP3 (residues 680–728) are depicted in yellow (random coils), green (α-helices) and blue (β-sheets); Varp (residues 658–921 corresponding to its C-terminal ankyrin repeats domain) is shown in orange. The structures of Hrb and AP-3 bound to the longin domain of VAMP7 [Protein Data Bank entries 2VX8 (Pryor et al., 2008) and 4AFI (Kent et al., 2012)] are oriented as VAMP7 in B to better show that Hrb and AP-3 both interact with the same region of the longin domain, which is also involved in its interaction with the coiled-coil domain. The structure of Varp bound to the cytosolic domain of VAMP7 [Protein Data Bank entry 4B93 (Schäfer et al., 2012)] has been re-oriented to better show the binding interface between Varp and VAMP7. VAMP7 binds in a closed conformation to the first ankyrin repeat of Varp. The VAMP7–Varp interaction interface involves residues from both the longin and the coiled-coil domains of VAMP7, thereby locking the closed conformation of VAMP7 in place. The figures were prepared using Chimera.

and the coiled-coil domain of Sec22b display similar *in vitro* kinetics of assembly with the coiled-coil domains of the t-SNAREs syntaxin1A and SNAP25, suggesting that the longin domain of Sec22b does not fold back onto its coiled-coil domain to inhibit SNARE-complex assembly (Gonzalez et al., 2001). Accordingly, overexpression of a yeast N-terminal deletion mutant of Sec22 does not increase the amount of Sec22-containing SNARE complexes at the ER or Golgi (Liu et al., 2004). In that study, the longin domain was required for the incorporation of Sec22 into coat protein complex II (COPII)-coated vesicles and for its export from the ER to the Golgi, further pointing to an important role of the longin domain in SNARE protein sorting. COPII assembles on the ER from three

subunits (Sar1, Sec23–Sec24 and Sec13–Sec31) and forms vesicles that mediate protein transport from the ER to the Golgi (Kuehn et al., 1998; Sato and Nakano, 2007). Sec22b has been shown to bind to Sec23–Sec24 (Mossessova et al., 2003) and, in the X-ray structure of the complex formed between Sec22b and Sec23–Sec24, a closed conformation of Sec22b binds to the interface between Sec23 and Sec24 (Mancias and Goldberg, 2007) (Box 1). In this closed state, only residues 148–157 of the coiled-coil domain of Sec22b are ordered, and these residues bind to a hydrophobic surface of the longin domain that is formed by helix α1 and strand β3 (Fig. 4C).

The mode of sorting of Sec22b that depends on its closed form is therefore likely to be distinct from that described above for Ykt6 and VAMP7, where membrane targeting of Ykt6 is closely linked to its transition from a closed to an open form, and binding of VAMP7 to the clathrin adaptors AP-3 and Hrb only occurs when VAMP7 is in an open conformation. These results also suggest that the closed form of Sec22b requires additional factors in order to be stable.

Structural comparison of the three longin SNAREs

A close inspection of the X-ray structures of the three longin SNAREs (available in isolation for Ykt6, and bound to molecular partners for VAMP7 and Sec22b) reveals interesting similarities (Fig. 4). In all three structures, the C-terminal region of helix α1 and strand β3 of the longin domain bind to a ∼ten-residue fragment of the coiled-coil domain forming a β-strand (strand β6), which, in the case of Ykt6 and Sec22b, is followed by a short α-helix (helix α5). This binding is mainly mediated by hydrophobic interactions and also involves hydrogen bonds between strand β3 (in the longin domain) and strand β6 (in the coiled-coil domain). Interestingly, the hydrophobic residues that are involved in the longin–coiled-coil-domain interaction are mostly conserved in the three longin SNAREs, and similar hydrophobic interactions are involved in the binding of the VAMP7 longin domain to its molecular coat partners Hrb and AP-3 (Fig. 4D) (Kent et al., 2012; Pryor et al., 2008). In the case of Ykt6, the closed form is further stabilized by hydrophobic interactions between helix α1 of the longin domain and an α-helix hairpin in the C-terminal half of the coiled-coil domain (α6–α7), and is locked in place by the farnesyl group that is sandwiched between these two domains (Figs 3 and 4A). The interaction between strand β3 of the longin domain and strand β6 of the coiled-coil domain is also potentially weaker in VAMP7 and Sec22b, which both display three hydrogen bonds (versus four in Ykt6) and reduced hydrophobic forces – the strong hydrophobic patch Ile-Ile-Leu of strand β6 in Ykt6 is replaced by the less hydrophobic sequence Ile-Met-Val in VAMP7 and Sec22b, and, moreover, Sec22b lacks a hydrophobic residue in strand β3 (Fig. 4C). Finally, in VAMP7, the hydrophobic forces can be further reduced upon phosphorylation of the tyrosine 45 residue. These observations thus point to a very stable closed inactive form in the case of Ykt6, whereas VAMP7 and Sec22b retain some degree of activity, even when they are bound to their molecular partners. In fact, even in the presence of Varp, VAMP7 is still able to engage in SNARE-complex formation *in vitro* (Schäfer et al., 2012). The strong auto-inhibition of Ykt6 fits well with its ubiquitous cellular distribution, including a cytosolic pool, as this will prevent the interaction of cytosolic (closed) Ykt6 with non-cognate t-SNARE proteins (Wen et al., 2010).

Conclusion and perspectives

The specificity of membrane fusion between cargo-containing transport vesicles and their target membrane requires that v-SNARE and t-SNARE proteins are located at the right place, where they need to be activated from an inhibited state in order to be able to interact with each other at the right time. The N-terminal extension of longin SNAREs has an important role in fulfilling both of these fundamental regulatory principles. It controls the intracellular sorting and membrane localization of SNAREs through its interaction with vesicle-coat components (in the case of VAMP7 and Sec22b) or through conformational transition (in the case of Ykt6), and it regulates the fusogenic activity of SNAREs by mediating intramolecular interactions with the coiled-coil domain, as shown for Ykt6 and VAMP7. VAMP7 and Ykt6, by themselves, can adopt a closed and stable conformation that impairs SNARE-complex assembly. The molecular mechanisms leading from the closed inactive state to the open active conformation have been elucidated for Ykt6 and involve a subtle interplay between farnesyl and palmitoyl groups at the C-terminus of the protein. In the case of VAMP7, the molecular triggers leading to the opening of the protein might operate in conjunction with phosphorylation of residue tyrosine 45, which has been shown to increase VAMP7-mediated exocytosis. Proteins like Varp are very likely to participate in the regulation of the open–closed transition of VAMP7, but the dynamics and the precise molecular mechanisms orchestrating this conformational transition are still unknown. At present, there is no clear evidence for an 'on/off' switch for the molecular activity of Sec22b. Therefore, we still do not know whether all three longin SNAREs can transition from an open to a closed conformation, and vice versa. Plants possess an expanded family of longin SNAREs, so it will be important to also use these model organisms to identify conserved fundamental mechanisms that involve longin domains. Interestingly, longin SNAREs appear to participate in cell growth processes in different organisms through membrane fusion or possibly also membrane docking without fusion. Because cell growth needs to be tightly controlled, it is tempting to speculate that the additional layer of regulation provided by the longin domain might be of importance in this process. Outstanding questions mainly relate to the dynamics of the activation of longin SNAREs, particularly in the case of longin SNAREs that have multiple partners, such as VAMP7. What are the molecular determinants of the open–closed equilibrium that would explain the differences between Sec22b on one hand, and Ykt6 and VAMP7 on the other? Where in the cells and when do longin SNAREs become activated to interact with their t-SNARE partners and to mediate membrane fusion? How do sorting partners dissociate in order to allow for the next ones to interact? What is the 'benefit' for a membrane fusion event to involve a longin SNARE versus a VAMP protein without a longin domain? What is the basic function common to all longin domains: auto-inhibition, protein–protein interaction, or both? Answering these questions will help to fully appreciate the exquisite structural and functional flexibility of longin-SNARE proteins.

Competing interests

The authors declare no competing or financial interests.

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