

Evidence that late-endosomal SNARE multimerization complex is promoted by transmembrane segments

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Abstract

Assembly of SNARE proteins into quaternary complexes is a critical step in membrane docking and fusion. Here, we have studied the influence of the transmembrane segments on formation of the late endosomal SNARE complex. The complex was assembled in vitro from full-length recombinant SNAREs and from mutants, where the transmembrane segments were either deleted or replaced by oligo-alanine sequences. We show that endobrevin, syntaxin 7, syntaxin 8, and vti1b readily form a complex. This complex forms a dimer as well as multimeric structures. Interestingly, the natural transmembrane segments accelerate the conversion of the quaternary complex to the dimeric form and are essential for multimerization. These in vitro results suggest that the transmembrane segments are responsible for supramolecular assembly of the endosomal SNARE complex.

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1. Introduction

In eukaryotic cells, an elaborate internal membrane system shapes two highly organized directional routes: one for ingestion of macromolecules from the extracellular milieu to the interior (endocytotic pathway) and the other for the secretion of newly synthesized proteins to the cell exterior (biosynthetic-secretory pathway). Different intracellular compartments are involved in these two pathways and they communicate with one another and the outside of the cell by means of transport vesicles [1,2].

In the endocytotic pathway, the endosomal compartment is a dynamic structure consisting of tubular and vesicular elements, where continuous rounds of fusion and fission take

place. The endocytosed material is first delivered to early endosomes, then to late endosomes and finally to lysosomes, where degradation occurs. Late endosomes have been shown to participate in homotypic fusion reactions, [3,4] which is an important step in morphological remodelling of this compartment and in heterotypic fusion with lysosomes [5,6]. All endosomal fusion reactions are mediated by particular sets of SNARE (N-ethylmaleimide-sensitive factor attachment protein receptor) proteins (reviewed in [7]) that support the specific apposition of cognate membranes and mediate lipid mixing.

The SNAREs responsible for homotypic endosomal fusion comprise the R-SNARE endobrevin (also known as VAMP 8) and the Q-SNAREs syntaxin 7, syntaxin 8, and vti1b. Each of the endosomal SNAREs is composed of a cytosolic domain and a C-terminal transmembrane segment (TMS). Recombinant cytosolic domains assemble to a quaternary complex in a 1:1:1:1 stoichiometry [3]. The structure of the central core of this complex [8] resembles that of the synaptic SNARE core complex [9–11] as it corresponds to a four-helix coiled-coil structure. In contrast to the endosomal SNARE complex, the synaptic complex is formed from two membrane-spanning subunits (synaptobrevin II, syntaxin 1A) and the peripheral SNAP-25 that contributes two helices to the complex.

Abbreviations: BN-PAGE, blue native polyacrylamide gel electrophoresis; CHAPS, 3-[(3-cholamidopropyl)dimethyl-ammonio]-1-propanesulfonate; eb, endobrevin; GST, glutathione S-transferase; HRP, horseradish peroxidase; IPTG, isopropyl-1-thio- β -D-galactopyranoside; SNARE, soluble N-ethylmaleimide-sensitive factor attachment protein receptor; syx7, syntaxin 7; syx8, syntaxin 8; TMS, transmembrane segment

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There is a growing body of evidence suggesting that synaptic SNARE complexes form higher-order multimers at the site of fusion. These multimeric structures appear to be crucial for exocytosis. For example, characterization of SNARE complex assembly in cracked PC12 cells suggested that lipid mixing requires the cooperation of at least three SNARE complexes [12]. Moreover, recombinant [13–16] or native [17–20] synaptic SNAREs form complexes that associate to higher-order multimers *in vitro*. *In vivo*, reduction of the expression level of syntaxin 1A or synaptobrevin II affects the cooperative relationship between calcium concentration and the amount of neurotransmitter released during synaptic vesicle exocytosis. This suggests that the number of synaptic SNARE complexes is critical for synaptic transmission [21]. In the squid giant synapse, SNARE complex multimerization appears to be promoted by the cytosolic protein synaphin/complexin and multimers are required for the fusion of docked vesicles [22].

SNARE complex multimerization may be related to homo- and heterotypic TMS–TMS interactions that were observed for the synaptic SNAREs synaptobrevin II and syntaxin 1A *in vitro* [23–26].

Up to now, the role of TMSs in late-endosomal SNARE interactions has not been studied. Here, we have characterized homo- and heterophilic assembly of recombinant full-length endosomal SNARE proteins and examined the role of the TMSs in these interactions. Our results suggest that some of the TMSs enhance homotypic interactions and promote multimerization of SNARE complexes.

2. Materials and methods

2.1. Materials

Reagents used were from the following companies: enzymes for DNA manipulations from Fermentas (Germany), HisTrap column, GSTrap column, α -GST-HRP conjugate antibody, ECL and ECL Hyperfilm from GE Healthcare (formerly Amersham Bioscience). Endobrevin, syntaxin 7, syntaxin 8 and vti1b antibodies were kindly provided by Drs. C. Schütte and R. Jahn (Göttingen, Germany).

All other reagents were purchased from Sigma, Applichem and Roche. Plasmid manipulations were performed in the *Escherichia coli* strain XL1Blue.

2.2. Methods

2.2.1. Cloning of expression plasmids

The reading frames of rat endobrevin and syntaxin 8, inserted into pGEX-KG, and plasmids encoding rat syntaxin 7 and mouse vti1b were kindly provided by Prof. R. Jahn (Göttingen, Germany). We subcloned syntaxin 7 and vti1b into pGEX-4T-2 (Amersham Bioscience) between *Bam*HI and *Sal*I restriction sites. In all cases, the N-termini of the SNAREs were fused to glutathione S-transferase (GST). The cytoplasmic regions of endobrevin (residues 1–75), syntaxin 7 (1–238), syntaxin 8 (1–212), and vti 1b (1–205) were cloned into pGEX vectors by the same procedure. The TMS alanine mutants were derived from these constructs using appropriate PCR based strategies. All constructs were verified by dideoxy sequencing.

2.2.2. Protein expression and purification

Transformed *E. coli* BL21 cells (Novagen) were grown overnight at 37 °C in 4 ml of Luria–Bertani (LB) medium containing 100 μ g/ml ampicillin. 400 μ l of the overnight culture were centrifuged, resuspended in same amount of fresh LB medium containing 200 μ g/ml ampicillin and added to 400 ml of LB medium containing 100 μ g/ml ampicillin. Cells were grown at 37 °C to

logarithmic phase ($A_{600} \approx 0.6$), induced with 1 mM isopropyl-1-thio- β -D-galactopyranoside (IPTG) and growth was continued for 3 h. For endobrevin, syntaxin 7, syntaxin 8, and their mutants, the cells were harvested and resuspended in 1/25 of the original culture volume of 25 mM HEPES–KOH, pH 7.4, 0.5 M NaCl, 20% (v/v) glycerol, 2% (v/v) Triton X-100, 2% (w/v) CHAPS containing 100 μ g/ml of lysozyme and incubated on ice for 30 min. Bacteria were lysed by sonication (power level 8, 50% duty cycle) on ice for 3 cycles of 3 min each in a “Cup Horn” sonifier (Branson sonifier 450). Before sonication, 1 mM phenylmethylsulfonyl fluoride (PMSF) was added. After sonication, 2 mg/ml DNase plus 10 mM $MgCl_2$ was added and lysates were incubated on ice for 10 min. The lysates were centrifuged at 19,000 \times g for 30 min. The supernatants were loaded onto a GSTrap FF column and proteins were purified according to the manufacturer’s instructions using as binding buffer phosphate buffered saline plus 0.5 M NaCl, 1% (w/v) CHAPS, 10% (v/v) glycerol and as elution buffer 50 mM Tris–HCl, pH 8, 0.5 M NaCl, 1% (w/v) CHAPS, 20 mM reduced glutathione, 10% (v/v) glycerol. Proteins were also solubilized in Triton X-100 and Thesit, however the yield of purified proteins was lower than in CHAPS.

Due to precipitation or insolubility, the protocol was modified for purification of vti1b and its mutants. These proteins were purified according to a protocol developed for solubilization of insoluble GST-fusion proteins [27]. The supernatants were loaded onto a GSTrap FF column (Amersham Biosciences) and proteins were purified according to the manufacturer’s instructions using as binding buffer phosphate buffered saline plus 1 M NaCl, 2% (w/v) n-octyl- β -glucopyranoside, 10% (v/v) glycerol and as elution buffer 50 mM Tris–HCl, pH 8, 1 M NaCl, 2% (w/v) n-octyl- β -glucopyranoside, 20 mM reduced glutathione, 10% (v/v) glycerol. Vti1b and its mutants were also purified in CHAPS; however, the yield of soluble purified protein was significantly lower than in n-octyl- β -glucopyranoside. To avoid precipitation or aggregation of vti1b and its mutants, 1 M NaCl was required in the purification buffers and for storage at 4 °C. All proteins were analyzed by SDS-PAGE and determined to be at least 90% pure, except for syntaxin 8 that after purification presents a proteolytic degradation product.

2.2.3. Sucrose gradient centrifugation

The individual recombinant SNARE proteins or equimolar mixtures thereof were loaded on top of continuous sucrose gradients (15–60% (w/v) sucrose) in 50 mM Tris–HCl, pH 8, 0.5 M NaCl or 1 M NaCl (for vti1b and its mutants); 1% (w/v) CHAPS or 2% (w/v) n-octyl- β -glucopyranoside (for vti 1b and its mutants) and centrifuged for 15 h at 4 °C at 100,000 \times g in a Beckman Optima LE80K ultracentrifuge (rotor SW60Ti). Fractions were collected from the bottom to the top and the proteins were precipitated by trichloroacetic acid prior to SDS-PAGE and Western blotting. Single protein markers (BSA, 66 kDa; carbonic anhydrase, 150 kDa; β -amylase, 200 kDa) were run in parallel experiments. For control, the 26 kDa GST protein without a fused SNARE was examined in parallel; it localized to fractions 9–10 and thus did not dimerize under these conditions (data not shown). Each sucrose velocity separation was repeated at least three times using different batches of purified proteins.

2.2.4. Electrophoresis and Western blot

Routine SDS-PAGE was done according to Laemmli [28]. For detection of SNARE complexes, “mild SDS-PAGE” was employed, where samples were dissolved in “low SDS sample buffer” (50 mM Tris–HCl, pH 6.8, 1% (w/v) SDS, 10% β -mercaptoethanol, 10% (v/v) glycerol, 0.1% (w/v) bromophenol blue) and separated in gels cast without SDS. Proteins were visualized by Western blotting using anti-GST-HRP conjugate antibody with the enhanced chemiluminescence (ECL) system. Gels were run at 4 °C, limiting voltage to 50 V and current to 10 mA. Samples were not boiled prior to electrophoresis except where stated otherwise. Marker proteins were carbonic anhydrase (29 kDa), egg albumin (45 kDa), bovine albumin (66 kDa), phosphorylase b (97.4 kDa), β -galactosidase (116 kDa), myosin (205 kDa) (Sigma), un-reduced α_2 -macroglobulin (380 kDa).

Blue Native (BN)-PAGE was done as described [29,30] with few modifications. Briefly, individual proteins or complexes, solubilized in the appropriate detergent buffer, were diluted 1:1 with 1 M Tris–HCl, pH 7.0 plus 10% (v/v) glycerol, and one tenth of 10 \times native loading buffer (1.6% (w/v) Coomassie Brilliant Blue G-250, 100 mM Bis-Tris, pH 7.0, 500 mM 6-aminocaproic acid) was added. The cathode buffer (15 mM Bis-Tris, pH

7.0, 50 mM Tricine, 0.02% (w/v) Coomassie Brilliant Blue G-250) was exchanged for the same buffer without the Coomassie dye after the samples had crossed the stacking gel. The anode buffer contained 50 mM Bis-Tris, adjusted to pH 7.0 with HCl. For complex detection, marker proteins were BSA (66 kDa), lactate dehydrogenase (140 kDa), catalase (232 kDa), ferritin (440 kDa), thyroglobulin (669 kDa) (Amersham Bioscience). The highest molecular mass band was corrected to be 880 kDa [31]. For individual protein detection, due to the large amount of detergent bound, markers were re-calibrated according to Heuberger et al. [32]. For Western blot, the blue native gel was soaked in transfer buffer (20 mM Tris, 150 mM glycine, 0.08% (w/v) SDS) for 10 min and proteins were transferred to polyvinylidene fluoride (PVDF) membranes (Millipore) in the same buffer plus 10% (v/v) methanol, using the semi-dry blotting technique. Protein bands were visualized by Western blotting using anti-GST-HRP conjugate antibody with the ECL system. For the second dimension in SDS, a lane was cut out of the first BN-PAGE dimension and incubated for a few minutes in 1% (w/v) SDS. The strip was then placed on top of a SDS-polyacrylamide gel for separation in the second dimension.

2.2.5. Assembly of SNARE complexes

Purified endosomal SNARE proteins and mutants were mixed at equivalent concentrations (15–30 μ M) and incubated at 4 °C for the indicated times prior to analysis of interactions. The reaction buffer contained 0.6 M NaCl, 1% (w/v) CHAPS, 1% (w/v) n-octyl- β -glucopyranoside, 10 mM reduced glutathione, 10% (v/v) glycerol. The assembly was stopped by adding the sample buffer for SDS-PAGE or the native loading buffer for BN-PAGE. Protein concentration was estimated by Bradford assay [33].

2.2.6. Elution of proteins from gels

For recovery of proteins from SDS gels, Coomassie-stained protein bands were cut and completely destained. Acetic acid was completely removed by repeated incubation in water. The gel pieces were soaked in 10-fold volume of 0.05% (w/v) SDS, 20 mM ammonium hydrogen carbonate (pH 9–10) for 4–7 days. The extracted proteins were precipitated by trichloroacetic acid prior to SDS-PAGE and Western blotting.

Extraction of proteins from BN-PAGE was done as described [34]. Briefly, un-stained pieces of gel were incubated overnight in 10-fold volume of 0.05% (w/v) SDS. The protein solution was freeze-dried and redissolved in a minimal volume of 0.5% (v/v) mercaptoethanol. The samples were subjected to SDS-PAGE and Western blot.

3. Results

To investigate the role of the TMSs in interactions of late-endosomal SNARE proteins, we expressed endobrevin, syntaxin 7, syntaxin 8, and vti1b in *Escherichia coli* (*E. coli*). All proteins were expressed at full-length (wt). The proteins were fused to the C-terminus of glutathione S-transferase (GST). The GST was required to enhance solubility in detergent and to facilitate purification and immunodetection. The removal of the GST portion or the use of another shorter tag, as the His-tag, were not sufficient to solubilize in native form our proteins. The GST protein without a fused SNARE was examined in sucrose gradients; it localized to fractions 9–10 and thus did not dimerize under these conditions.

In addition, their predicted TMSs were either deleted (Δ TMS) or replaced by an oligo-alanine sequence (TMS-Ala) (Fig. 1). Previous results have shown that oligo-alanine sequences form stable α -helices [35,36] and stay monomeric [25,37]. Substituting natural TMSs by oligo-alanine stretches, rather than deleting them, is therefore thought to have minimal effects on SNARE secondary structure without introducing artificial interactions. All proteins were solubilized in detergent and purified to homogeneity by GST-affinity chromatography.

| | | TMS | |
|---------------------|--------|--|---------|
| eb-wt | ...NVK | ⁷⁵ MIVII CVVLIIILIIILFA ⁹⁴ | TGTIPT |
| syx7-wt | ...KTL | ²³⁸ LCII ILILVVGIVIIFFIVW ²⁵⁶ | GLKG |
| syx8-wt | ...SAS | ²¹⁴ CGMIM VILLLLVAIVVVAV ²³² | WPTN |
| vti1b-wt | ...ITN | ²⁰⁶ KLLLS VIIILELAILVGLVY ²²⁵ | YKFFRHH |
| eb-TMS-Ala | ...NVK | ⁷⁵ AAAAAAAAAAAAAAAAAAAA ⁹⁴ | TGTIPT |
| syx7-TMS-Ala | ...KTL | ²³⁸ AAAAAAAAAAAAAAAAAAAA ²⁵⁶ | GLKG |
| syx8-TMS-Ala | ...SAS | ²¹⁴ AAAAAAAAAAAAAAAAAAAA ²³² | WPTN |
| vti1b-TMS-Ala | ...ITN | ²⁰⁶ AAAAAAAAAAAAAAAAAAAA ²²⁵ | YKFFRHH |
| eb- Δ TMS | ...NVK | | |
| syx7- Δ TMS | ...KTL | | |
| syx8- Δ TMS | ...SAS | | |
| vti1b- Δ TMS | ...ITN | | |

Fig. 1. Late-endosomal SNARE proteins. Amino acid sequences of predicted endobrevin (eb), syntaxin 7 (syx 7), syntaxin 8 (syx 8), and vti1b transmembrane segments. In TMS-Ala mutants, the TMSs were substituted by alanines, while in Δ TMS mutants they were removed. Predicted TMS residues and the oligo-alanine sequences are shown in bold.

3.1. Homotypic interactions of endosomal SNARE proteins

The potential homotypic interactions of individual SNARE proteins and their mutants were analyzed in non-denaturing detergent. In one set of experiments, purified proteins were subjected to centrifugation on sucrose velocity gradients, whose fractions were analyzed by Western blot (Fig. 2A). Endobrevin wild-type (eb-wt) and mutants (predicted molecular mass 38 kDa) predominates in fractions 6–9. By comparison to molecular mass markers, these predominant fractions correspond to molecular masses ranging from ~40 kDa to ~80 kDa. Accordingly, wt endobrevin and mutants appears to exist as monomeric and homo-dimeric forms. Syntaxin 7 (syx 7-wt 55 kDa) mainly localizes to fractions 7–9, that correspond to molecular masses lower than ~70 kDa and are therefore likely to contain a monomeric form of the protein. However, some protein is also detected in fractions 3–6, revealing a tendency to form homo-oligomeric forms. In contrast, the TMS-Ala and Δ TMS mutants of syntaxin 7 are limited to fractions 6–9 and thus appear to stay monomeric. Vti1b (vti1b-wt, 52 kDa) mainly localizes to fractions 7–9, but could also be detected in fractions 5 and 6, and in bottom fractions (Fig. 2A). Thus, vti1b appears to exist as a monomer, some homo-dimer, and a low amount of homo-oligomers. TMS-Ala and Δ TMS mutants of vti1b mainly localize to fractions 6–9, but not in the lower fractions, suggesting that they stay monomeric (Fig. 2A). Syntaxin 8 (syx8-wt, 52 kDa) and corresponding mutants are mainly found in fractions 7–9, indicative of a monomer.

Velocity gradient centrifugation has a low resolution and therefore a limited capacity to distinguish between monomeric, dimeric, and oligomeric forms of our 50 kDa proteins. As an alternative approach to assess self-assembly, we performed BN-PAGE (Fig. 2B). For endobrevin-wt, as well as its TMS-Ala and Δ TMS mutants, the stained BN-polyacrylamide gel shows two bands, corresponding to monomeric and dimeric forms of this protein. Less intense bands are also detected at higher molecular masses, revealing a tendency to homo-oligomerize under these conditions. Syntaxin 7-wt runs as a monomer, dimer, and some

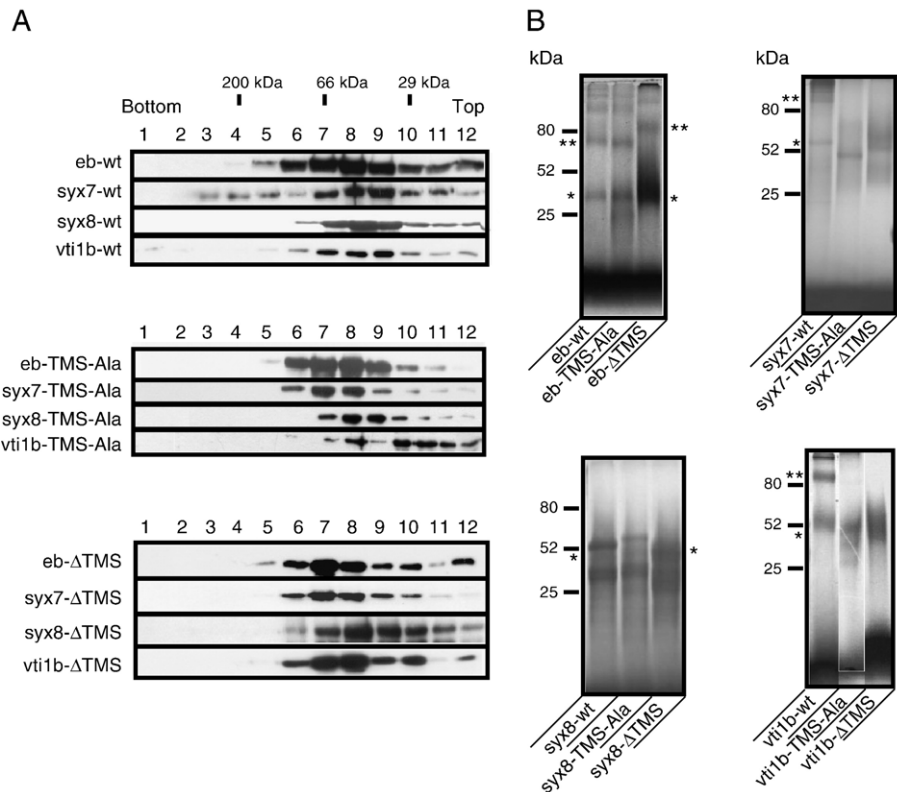


Fig. 2. Homotypic interactions of individual recombinant SNAREs. (A) Analysis of detergent-solubilized proteins ($\sim 30 \mu\text{M}$ of each protein) by sucrose velocity gradient centrifugation. The gradients were fractionated and analyzed for protein content by SDS-PAGE and Western blotting with the anti-GST antibody. The position of marker proteins, as determined in parallel gradients, are shown on the top. The gradients were run at least three times with different batches of purified proteins. (B) Separation of solubilized SNAREs ($\sim 15 \mu\text{M}$) by BN-PAGE. After electrophoresis, the 6–18% gels were stained with Coomassie blue. For vti1b, images of different gels are separated by vertical dividing lanes. Single and double asterisks mark the position of monomers and homodimers, respectively.

higher molecular mass forms $>80 \text{ kDa}$, that are lacking in the mutants. Vti1b-wt exhibits two bands corresponding to monomeric and dimeric forms; further, a very high molecular mass band suggests homo-oligomerization. These high molecular mass bands are not detected in the mutants. Syntaxin 8-wt and its mutants are similar in showing exclusively monomeric species. The principal 52 kDa band is consistently accompanied by a lower molecular mass band that is likely to correspond to a proteolytic degradation product, which is also seen in SDS-PAGE (not shown).

Taken together, these results show that three of our proteins, endobrevin, syntaxin 7, and vti1b, are inclined to self-interaction. Endobrevin, syntaxin 7 and vti1b may exist as a monomer and dimer, with a tendency to form oligomers. On the basis of the comparison between wt and mutants the TMSs of syntaxin 7 and vti1b is affecting homo-oligomerization.

3.2. Heterotypic interactions in binary complexes

In order to examine whether the TMSs support heterotypic SNARE interactions, we studied all the possible binary interactions of wt proteins as well as their TMS-Ala and ΔTMS mutants. Equimolar amounts of proteins were mixed and incubated for 15 h before analysis of assembly by sucrose gradient centrifugation. In contrast to the individual proteins,

that predominated in fractions 6–9 (Fig. 2A), most mixtures of wt proteins resulted in co-localization of both proteins in fractions 2–5 (Fig. 3), i.e. around the position of the 200 kDa marker. No significant co-migration to the high molecular mass fractions was seen for the pairs syntaxin 7/syntaxin 8. The pair syntaxin 8/vti1b co-migrates on SDS-PAGE and can therefore not be distinguished from each other (data not shown). These results show that full length endobrevin is able to interact with syntaxin 7, syntaxin 8, and vti1b. Moreover, syntaxin 7 associates with vti1b. Interestingly, co-localization of the binary pairs to the high molecular mass fractions of the sucrose gradients was abolished when the ΔTMS mutants were examined (Fig. 3), suggesting that these heterotypic interactions depend on the respective TMSs.

3.3. Assembly of the endosomal SNARE complex

In order to assemble the quaternary complex, stoichiometric amounts of all four wt proteins or of the respective mutants were mixed and incubated overnight. Complex formation was first assessed by centrifugation on sucrose gradients. For the wt mixture, the four proteins predominate in fractions 1–3 that correspond to molecular masses $>200 \text{ kDa}$ (Fig. 4A). Complexes formed from the TMS-Ala or ΔTMS mutants exhibit a lesser tendency to form high molecular mass complexes.

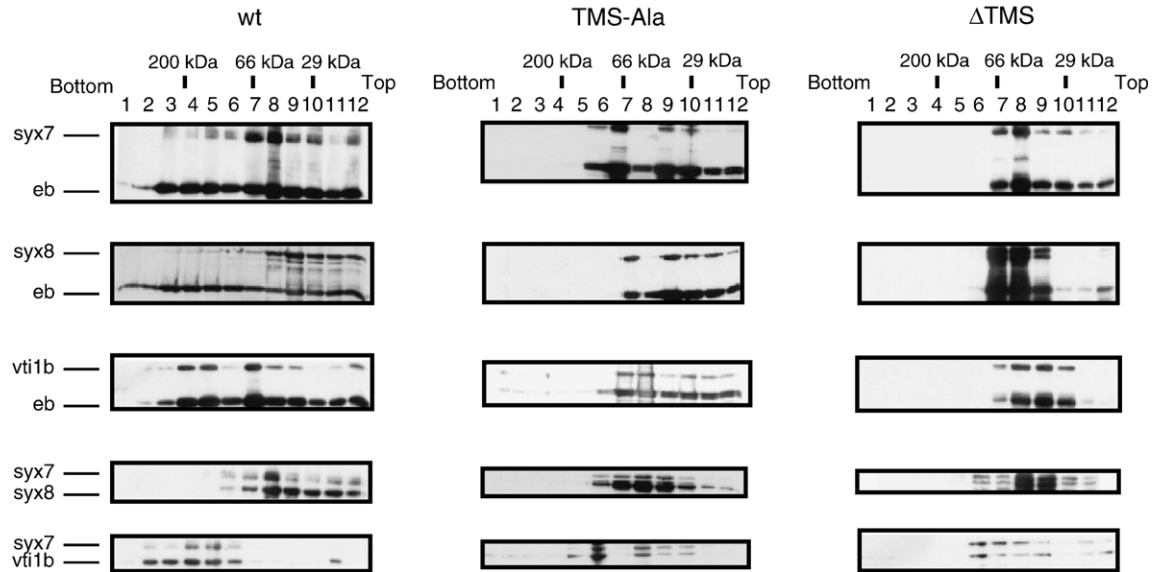


Fig. 3. Binary interactions of SNAREs. (A) Equimolar binary ($\sim 30 \mu\text{M}$ of each protein) mixtures of wt proteins, ΔTMS mutants, or TMS-Ala mutants were incubated for 15 h at 4°C and analyzed by sucrose velocity gradient centrifugation. Gradient fractions were analyzed by SDS-PAGE and Western blotting with the anti-GST antibody. Note that each binary mixture, except syntaxin 7/syntaxin 8 migrates to high molecular mass fractions. Deletion of the TMSs or replacement by oligo-alanine sequences largely abolishes co-migration. The gradients were run at least three times with different batches of purified proteins.

Specifically, the four proteins were never observed in fraction 1, but still prevalent in fraction 2–4 and predominated in fractions 5 to 8. It appears, therefore, that SNARE complexes $\geq 200 \text{ kDa}$ are less stable in the absence of the natural TMSs. Since wt

syntaxin 8, wt vti1b, and their TMS-Ala mutants are almost co-migrating on SDS-PAGE, their co-localization in the gradient was verified by reprobing the blots with specific syntaxin 8 and vti1b antibodies (data not shown).

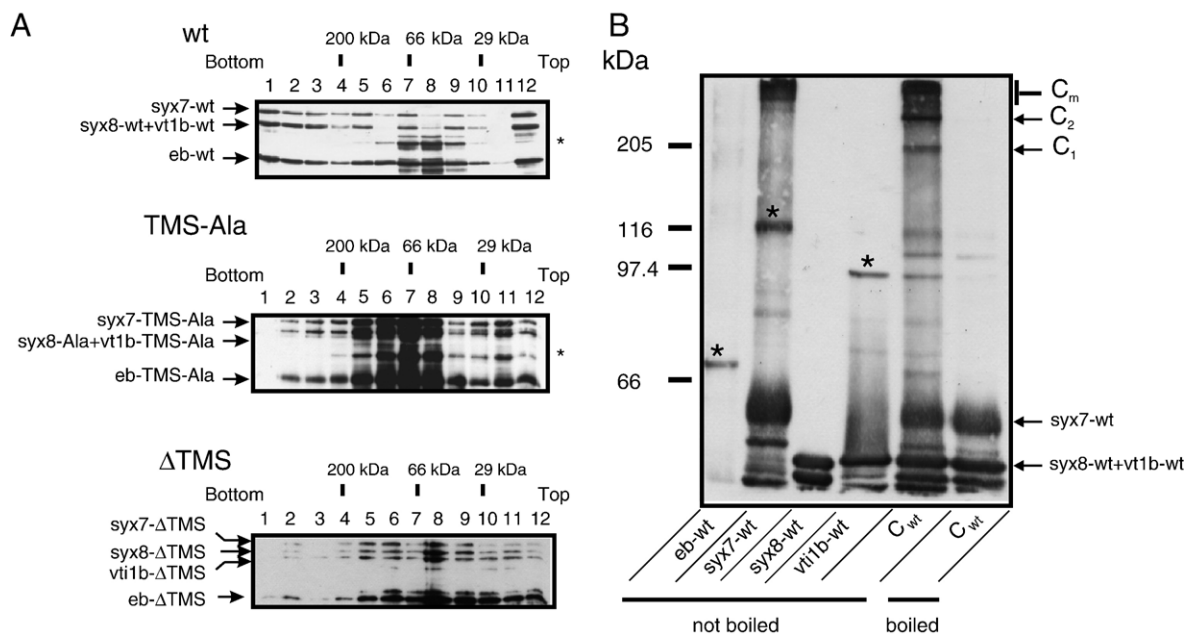


Fig. 4. Assembly of quaternary late-endosomal SNARE complexes. (A) Equimolar quaternary mixtures ($\sim 30 \mu\text{M}$ of each protein) of wild type proteins (wt), ΔTMS mutants, or TMS-Ala mutants were incubated for 15 h at 4°C and analyzed by sucrose velocity gradient centrifugation. Gradient fractions were analyzed by SDS-PAGE and Western blotting with the anti-GST antibody. Note that a significant amount of the wt proteins sediments to high molecular mass fractions. Co-sedimentation was strongly reduced with the TMS-Ala or ΔTMS mutants. The wt and TMS-Ala forms of syntaxin 8 and vti1b co-migrate on SDS-PAGE and are therefore superimposed. The asterisks denote a degradation product of vti1b. The gradient with wt-proteins was repeated six times and the ones with ΔTMS mutants and TMS-Ala mutants three times with similar results. (B) SDS-resistant complexes. Individual full length proteins (eb-wt, syx7-wt, syx8-wt and vti1b-wt) or an equimolar mixture ($\sim 15 \mu\text{M}$ of each protein) thereof (C_{wt} , C_{wt} boiled) were incubated overnight at 4°C and subjected to mild SDS-PAGE on a 7.5% polyacrylamide gel. C_1 , C_2 , and C_m denote monomeric, dimeric, and multimeric forms of the quaternary SNARE complex. Proteins were immunodetected with anti-GST antibody. The asterisks denote homodimers of the individual proteins. The position of SNARE monomers is given, endobrevin has eluted from the gel.

The late-endosomal complex formed from Δ TMS proteins (not fused to GST) has previously been determined to be of ~ 85 kDa by SDS-PAGE, suggesting an equimolar ratio of all four subunits [4]. Here we examined the complex assembled from full length SNAREs by SDS-PAGE upon mixing all four proteins at a 1:1:1:1 stoichiometric ratio (C_{wt}). SDS-PAGE done at mild conditions without sample boiling (see Experimental Procedures), followed by Western Blot, reveals partial assembly of the subunits to distinct high molecular mass complexes that are denoted C_1 , C_2 , and C_m in Fig. 4B. One of these complexes (C_1) runs at an apparent molecular mass of ~ 200 kDa and is thought to correspond to a 1:1:1:1 complex of our GST-fusion proteins (calculated molecular mass = 198.7 kDa). The other complexes migrate at ~ 400 kDa (Figs. 4B and 5A) and above and probably correspond to dimeric (C_2) or multimeric (C_m) assemblies of the quaternary C_1 complex. These different complexes dissociate upon sample boiling, thus demonstrating their non-covalent nature. Neither of these complexes was seen when individual subunits were electrophoresed under the same conditions (Fig. 4B). Rather, individual endobrevin, syntaxin 7 and vti1b, but not syntaxin 8, partially homodimerize (asterisks in Fig. 4B) in agreement with the results obtained by sucrose gradient and BN-PAGE analysis (Fig. 2). Syntaxin 7 also forms

multimers; these are distinct from the C_m complex. No significant amounts of C_1 , C_2 , or C_m complexes were formed when different ternary subunit mixtures (syntaxin 7/syntaxin 8/vti1b, endobrevin/syntaxin 8/vti1b, endobrevin/syntaxin 7/vti1b, endobrevin/syntaxin 7/syntaxin 8) were tested (data not shown). This suggests that the C_1 , C_2 , and C_m complexes are based on specific protein–protein interactions.

In conclusion, full-length endobrevin, syntaxin 7, and vti1b, but not syntaxin 8, tend to form homomers. Further, co-assembly of all four proteins results in high-molecular mass complexes that are proposed to correspond to the quaternary SNARE complex and to multimers thereof. Finally, formation of these multimers appears to depend on one or on all of the respective TMSs.

3.4. Assembly kinetics of the endosomal SNARE complex and its multimers

Here, we determined whether the presence of the TMSs affects the relative abundance of C_1 , C_2 , and C_m complexes, as well as the kinetics of their formation. To this purpose, we examined assembly of all wt and mutant subunits as a function of incubation time upon mixing. The complexes were separated by mild SDS-PAGE and visualized by Western Blot (Fig. 5A–C). Upon mixing full length proteins for only 10 min, mainly dimeric complex (C_2) and significant amounts of multimers (C_m) are seen. After 1 h, the monomeric form (C_1) has disappeared, but C_2 still predominates. After 15 h of incubation, C_m is the dominant complex species (Fig. 5A). Δ TMS mutants assemble rather slowly to C_1 and C_2 forms (Fig. 5C) that are detected only after 4 h of incubation; no multimers are seen even after 4 days. The TMS-Ala mutants form C_1 and C_2 already after 10 min. Again, no multimers are seen even after 4 days (Fig. 5B). We conclude that the presence of the oligo-alanine sequences accelerates C_1 and C_2 formation. However, the natural TMSs are essential for multimerization to C_m .

To ascertain that the oligomers of the wt complex are not artifacts of SDS-treatment, we analyzed them under the non-denaturing condition of BN-PAGE. Here, the monomeric complex is likely to correspond to the weak band at ~ 180 kDa, and it is accompanied by a putative dimer (between 232 kDa and 440 kDa) and by multimers (>440 kDa). In contrast to analysis by mild SDS-PAGE, dimers and multimers predominate already after 5 min of incubation. After 3 h, the dimer starts to disappear in favor of multimers (Fig. 5D). It appears, therefore, that dimerization and multimerization of C_1 is a fast process. The assembly kinetics as detected by SDS-PAGE (Fig. 5A) may be slowed down due to the presence of SDS.

To confirm that the detected complexes contain all SNARE proteins, the proteins of the corresponding bands of the SDS polyacrylamide gels were extracted, dissociated by boiling and analyzed by second dimension SDS-PAGE and Western blotting (Fig. 6). The C_1 and C_2 complexes, assembled from the Δ TMS mutants, as well as the C_2 and C_m complexes, assembled from the full length proteins, contain all four endosomal proteins.

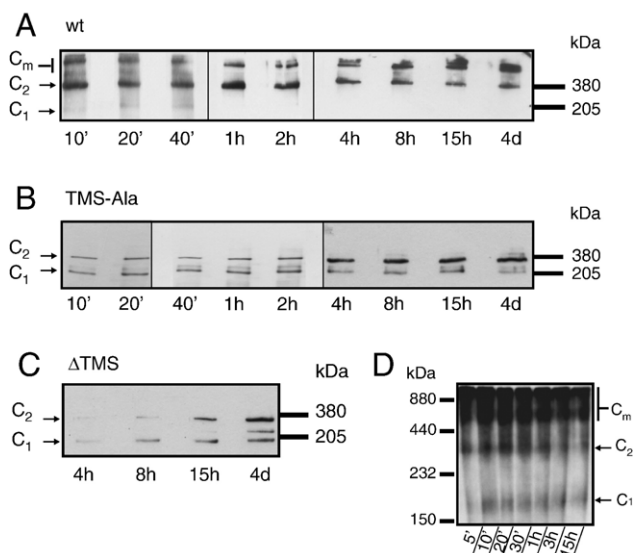


Fig. 5. Kinetics of SNARE complex formation. Equimolar mixtures of proteins (eb, syx 7, syx 8, vti1b; ~ 15 μ M each) expressed as wild type forms (A), TMS-Ala (B) or Δ TMS (C) derivatives were incubated at 4°C for the indicated periods. Proteins were mixed at the different time points and loaded after the indicated incubation periods had passed. For analysis, samples were subjected to mild SDS-PAGE on a 5–12.5% polyacrylamide gel and immunodetected with anti-GST antibody. Only the high molecular mass parts of the gels are shown here; vertical lines divide images from different gels. Note that the natural TMSs strongly accelerate the conversion of C_1 to C_2 and are required for multimer formation (C_m). (D) Time course of formation of the wt SNARE complex as detected by BN-PAGE. Equimolar mixtures of wt-eb, wt-syx 7, wt-syx 8, wt-vti1b (~ 15 μ M each) were incubated at 4°C for the indicated periods. For analysis, samples were subjected to BN-PAGE on 4–12.5% polyacrylamide gels and immunodetected with anti-GST antibody. Here, C_m complexes predominate already after 5 min. Kinetics detected both with SDS-PAGE and BN-PAGE were repeated three times with similar results.

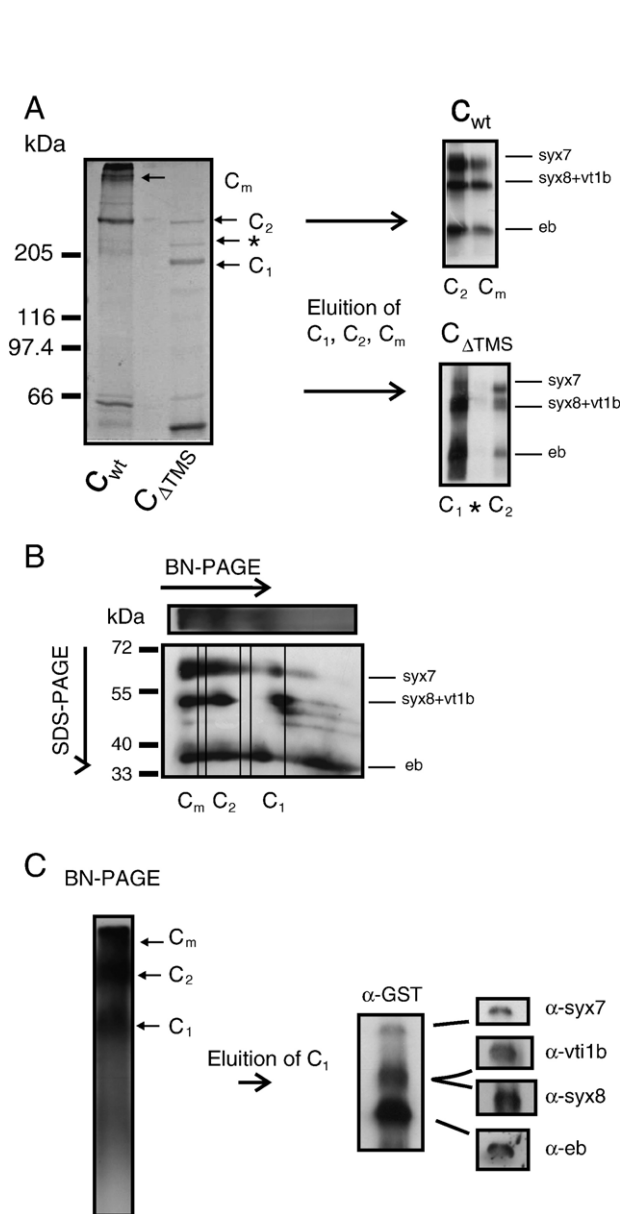


Fig. 6. Compositional analysis of C_1 , C_2 and C_m complexes. Equimolar mixtures of proteins (eb, syx 7, syx 8, vt1b; $\sim 15 \mu\text{M}$ each) expressed as wild type forms or ΔTMS derivatives were assembled at 4°C for approximately 15 h. A) The mixtures were subjected to mild SDS-PAGE on a 5–10% polyacrylamide gel (left panel). The gel was stained with Coomassie R-250. Coomassie-stained protein bands were cut, completely destained and the proteins were extracted as described in Materials and methods. The extracted proteins were precipitated by trichloroacetic acid prior to their analysis by regular SDS-PAGE and Western blotting. Wild-type C_2 and C_m complexes contained all four endosomal SNARE proteins (upper right panel) while C_1 was too faint for compositional analysis. Further, the C_1 and C_2 complexes of ΔTMS proteins contained all SNARE subtypes (right lower panel). The identity of an intermediate band (asterisk in the left panel) is not clear since its compositional analysis gave no result (lower right panel). B) The wt protein mixture was subjected to a first dimension BN-PAGE on a 4–12.5% polyacrylamide gel. The lane was cut and subjected to an SDS-PAGE in a second dimension. The individual proteins forming the complexes C_1 , C_2 , and C_m were visualized by immunoblotting with anti-GST antibody. C) The wt protein mixture was subjected to BN-PAGE on 4–12.5% polyacrylamide gel (left panel). The protein band corresponding to C_1 complex was cut and the proteins were extracted as described in Materials and methods. Extracted proteins were subjected to SDS-PAGE and Western blot with the anti-GST-antibody and with the specific antibody for each protein (right panel).

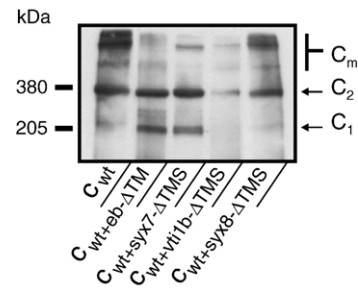


Fig. 7. Role of individual TMSs in complex multimerization. Full length (wt) proteins were mixed, incubated for 6 h at 4°C , and analyzed by low SDS-PAGE on a 5–12.5% polyacrylamide gel and Western blotting. The resulting complex (C_{wt}) is compared to the complexes that form when individual full length subunits are replaced by the respective ΔTMS mutants as indicated. Note that the ratio of C_1 to C_m is increased in the absence of the endobrevin or syntaxin 7 TMSs. Deleting the vt1b TMS reduces the abundance of all complexes, while the syntaxin 8 TMS has no apparent effect. Only the high molecular mass part of the gel is shown.

3.5. Role of individual TMSs in late-endosomal SNARE complex formation

Having shown that the kinetics of formation and supramolecular assembly of the SNARE complex depends on TMSs, we addressed the contribution of the individual TMSs. Accordingly, we assembled “mixed” endosomal SNARE complexes in which individual full-length proteins were substituted by the corresponding ΔTMS mutant. The mixtures were incubated for 6 h (in order to detect the monomeric complex as well) and then subjected to mild SDS-PAGE. Fig. 7 shows that deletion of the endobrevin TMS reduces multimerization and results in C_1 complex. A similar picture is seen upon deletion of the syntaxin 7 TMS. Deletion of the vt1b TMS consistently results in partial disappearance of all complexes, whereas deletion of syntaxin 8 TMS has no discernible effect. This suggests that the TMSs of endobrevin and syntaxin 7 are critical for multimerization of the SNARE complex. For an unknown reason, the TMS of vt1b decreases the efficiency of C_1 complex and multimer formation.

4. Discussion

Our in vitro results suggest that late endosomal SNAREs co-assemble to a putative monomeric complex (C_1), as well as to putative dimeric (C_2) and multimeric (C_m) forms thereof. Interestingly, multimers are not seen in the absence of the natural TMSs implying that multimerization rests on TMS–TMS interactions. C_1 and C_2 complexes also assemble from cytosolic domains, albeit with greatly reduced velocity. Fusing oligo-alanine sequences to the cytosolic domains accelerates C_1 and C_2 formation. The α -helical oligo-alanine tails [35] may stabilize the secondary structures of the individual cytoplasmic domains and thereby support their assembly to the C_1 and C_2 structures. The cytosolic fragments of endosomal SNAREs have indeed been shown to be mainly unstructured in isolation [38].

The assembly kinetics reveal that the ratios of C_2/C_1 and C_m/C_2 , as seen with TMS-Ala mutants and full-length proteins,

respectively, increase with time. This suggests that C_2 is an intermediate in the transition from C_1 to C_m . Moreover, the formation of C_m , but not of C_2 , is only compromised upon deletion of the endobrevin or syntaxin 7 TMSs. C_2 and C_m formation seems to be mediated by the contribution of the oligomerization properties of the individual SNAREs, where the TMSs play a crucial role. In particular, regarding homotypic interactions, we found that endobrevin, syntaxin 7, and vti1b tend to dimerize. Moreover syntaxin 7 and vti1b homodimerization depends on the respective TMSs. In heterotypic binary mixtures, all pairwise interactions detected (endobrevin/syntaxin 7; endobrevin/syntaxin 8; endobrevin/vti1b and syntaxin 7/ vti1b) depend on the TMSs of the interacting partners. C_2 and C_m complexes may arise by different combinations of homo and hetero-interactions, where multiple TMS interfaces are involved, as hypothesized for synaptobrevin II and syntaxin 1A [39].

To our knowledge this is the first time that multimerization has been shown for endosomal SNARE complexes. Although our data were obtained in detergent solutions, they are in agreement with *in vitro* and *in vivo* results obtained for synaptic SNAREs. There is evidence from functional studies that the cooperative action of synaptic SNARE complexes is required for exocytosis [40]. The exact number of complexes that are required for exocytosis is currently not known as different experimental approaches arrive at different conclusions. At least three SNARE complexes have been calculated to cooperate in exocytosis from cracked PC12 cells [12]. Recent studies on norepinephrine release from PC12 cells suggest that five to eight syntaxin 1A molecules are required for fusion pore formation. [41] Other estimates are based on inhibition of synaptic SNARE function by botulinum neurotoxins and suggested that 10–15 complexes are required for neurotrans-

mitter release [42,43]. Despite the uncertainty of these numbers, these results suggest that the cooperative action of non-covalently linked SNARE complexes is crucial for membrane fusion. It has been proposed that higher-order multimers could be required for tightly regulated fast exocytosis from synaptic vesicles, whereas lower-order multimers might suffice for slow exocytosis from secretory granules [40]. Conceivably, these multimers form a ring-like structure around the site of membrane fusion. Synaptic SNARE complex multimers have also been observed by electron microscopy. Purified SNARE complexes from brain form star-shaped multimeric structures [14] that contain three to four individual complexes [20]. These observations agree with numerous observations of high-molecular mass neuronal SNARE complexes detected by SDS-PAGE [15,18,19,22,44,45].

Our results indicate that multimerization is a conserved feature of endosomal and synaptic SNARE proteins. On the other hand, recombinant yeast vacuolar SNAREs form a quaternary complex that does not multimerize under the conditions used in the present study [46]. Although we cannot rule out that the yeast SNARE multimers are disrupted upon detergent solubilization, these finding suggests that multimerization of SNARE complexes may not be required for each type of intracellular fusion.

Different mechanisms may be responsible for multimerization of SNARE complexes in different cell types and organelles. Synaptotagmin and complexin/synaphin were proposed to mediate interaction of synaptic SNARE complexes [22,47]. However, multimers of recombinant or native synaptic SNAREs were indistinguishable, which argues against a critical role of accessory proteins [20]. Kweon et al. suggested that multimerization of synaptic SNARE complexes could proceed via domain swapping, where one of the two SNAP-25 helices

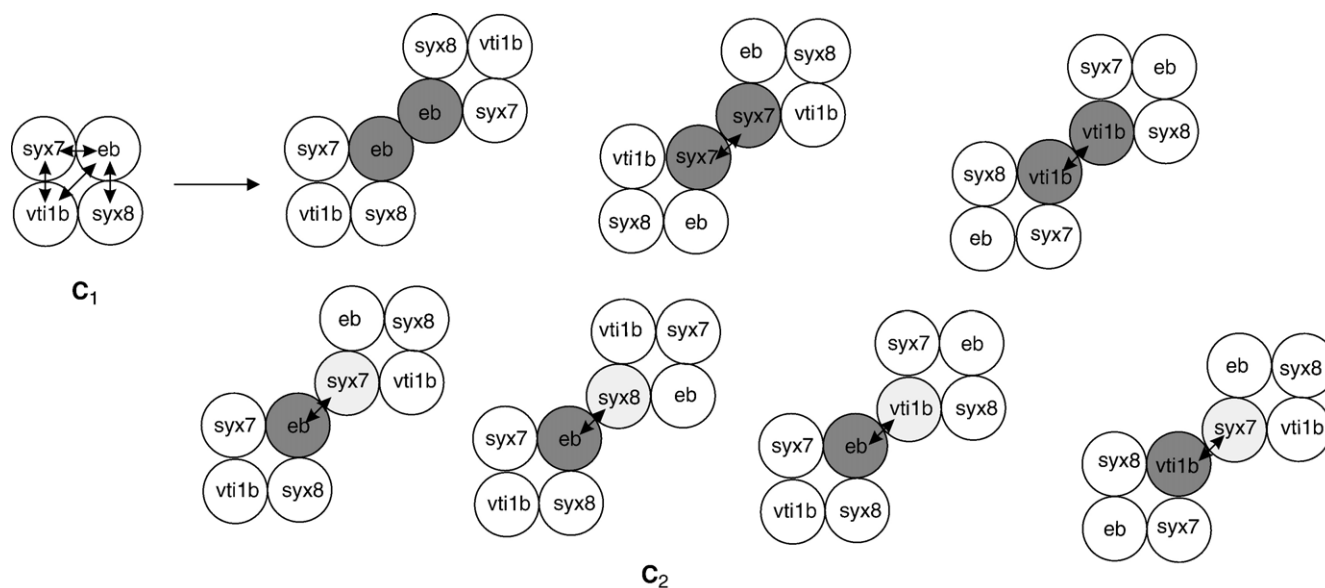


Fig. 8. A hypothetical model of a late-endosomal SNARE complex multimer. The spatial arrangement of the TMSs (circles) in the quaternary complex (C_1) is based upon the order of the C-termini in the previously crystallized soluble version of the complex [8]. TMS–TMS interactions are indicated by double arrows. Assembly of the quaternary complex to the dimeric form (C_2) shown here is thought to involve homo- (dark grey) and hetero-interactions (dark and light grey) between endosomal SNARE proteins.

would be substituted by the equivalent helix from the neighbouring complex [48]. Further, multimerization has been suggested to be mediated by interactions between the TMSs of synaptobrevin II and syntaxin 1A [24,25] since TMSs promoted the stability of the multimers in SDS-PAGE [15,24]. With endosomal SNARE complexes, TMS–TMS interactions appear to be one of the major forces driving multimerization for the following reasons. First, accessory proteins like synaptotagmin or complexin/synaphin are not present in endosomes and are not required for multimer formation of our purified proteins. Second, two distinct Q-SNARE subunits, syntaxin 8 and vtilb, represent the synaptic SNAP-25 in the endosomal complex and thus rule out swapping of covalently linked cytoplasmic helices between adjacent complexes. Third, formation of the multimers depends on the TMSs of endobrevin and syntaxin 7 and it is influenced also by the TMS of vtilb. Strikingly, the TMSs of syntaxin 7 and vtilb support the homodimerization of both proteins.

According to our *in vitro* results a hypothetical model of late-endosomal SNARE complex multimer formation is proposed in Fig. 8, where dimerization of the full complex is mediated by homo- and heterotypic interactions. In homotypic interactions, syntaxin 7 and vtilb, with the respective TMS, and endobrevin, with the cytosolic domain, are involved in multimerization. Endobrevin heterotypic interactions with syntaxin 7, syntaxin 8, and vtilb may be mediated by the TMSs. The TMS-mediated heterotypic interaction may also occur between syntaxin 7 and vtilb. Noticeably, endobrevin may contribute to complex dimerization both with the cytosolic domain (in homo-interaction) and with the transmembrane domain (in hetero-interactions), thus playing a key role in the multimerization. Due to the complexity of the natural lipid bilayer, we would not rule out that lipids may influence the interaction patterns here presented.

In membranes, multimerization is thought to concentrate multiple SNARE complexes prior to actual lipid mixing. SNARE TMSs may fulfill temporally distinct functions in the course of a fusion reaction. At an early step, during membrane docking and trans complex formation, they may support multimerization of SNARE complexes to allow for cooperative SNARE function. At a late step, the TMSs may actively support actual lipid mixing.

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